CHARACTERIZATION OF A TOSPOVIRUS CAUSING NECROSIS DISEASE OF COWPEA [*Vigna unguiculata* (L). Walp.]

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DECLARATION

I hereby declare that this thesis entitled **"Characterization of a tospovirus causing necrosis disease of 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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Introduction

1. INTRODUCTION

Cowpea [*Vigna unguiculata* (L.) Walp.] is an important high protein food crop widely cultivated in many tropical and subtropical countries, where it contribute an important source of dietary protein. Among legumes, cowpea is one of the vegetable crops grown for its tender pods and as pulse when dried. It is a good cover crop and rich supplier of nitrogen. In India, cowpea is cultivated in West Bengal, Tamil Nadu, Andhra Pradesh, Kerala and Maharashtra. In Kerala, cowpea is cultivated mainly as a vegetable and grain crop.

Diseases and pests are major constraints in increasing the production of the crop. Among the pathogens, viruses are threat to cultivators. Viruses are known to occur and infect cowpea at all stages of the plant growth and its effects can be devastating and are a major constraint to increase production. More than twenty viruses are reported in cowpea from different parts of the world (Thottappilly and Rossel, 1992). Among the viruses infecting cowpea, Blackeye cowpea mosaic virus (BICMV) is the most wide spread in Kerala (Radhika and Umamaheswaran, 2000). Natural infection of tospovirus was recorded at the Instructional Farms of College of Agriculture, Vellayani, College of Horticulture, Vellanikkara and found to cause severe damage to cowpea in many farmers fields. During the last decade, tospoviruses belonging to the family *Bunyaviridae* (Van Regenmortel *et al.*, 2000) are causing substantial losses world wide to crops such as groundnut, potato, tobacco, vegetables and ornamental plants (Moyer, 1999)

The tospoviruses are a highly cosmopolitan group . Many viruses included in this genus have very wide host ranges and can cause problems in an extensive range of food and ornamental crop species. The ability of the tospoviruses to cause severe losses on a broad range of crops, places them amongst the most economically important plant pathogens in the world at present. The symptoms induced by the tospoviruses are highly varied. On non – systemic hosts symptoms are generally restricted to local lesions, with chlorosis and necrosis in some instances. With systemic hosts symptoms include ring spots, line patterns,

wilting-stunting, silvering, mottling, bronzing, chlorosis, necrosis and a range of leaf and stem lesions. The morphology of the tospoviruses is typical of members of Buniyaviridae . It measures about 80 -110 nm in diameter ;consist of an outer membrane envelope and quasi -spherical in shape. Tospovirus genus is classified into four serogroups. Three distinct tospovirus species , Groundnut bud necrosis (GBNV), Groundnut yellow spot virus(GYSV) from groundnut and Watermelon bud necrosis (WBNV) from watermelon have been reported from India. One of the main factors contributing to the global status of the tospoviruses as pathogens is the relation of their vectors, thrips.

Informations on tospovirus disease of cowpea, its symptomatology, host range, transmission and physical properties in Kerala are scanty. These details along with serological detection and host-pathogen interactions are required for formulating effective control measures. Therefore the study was undertaken to investigate the symptomatology, biological and physical charactristics of the virus, host pathogen interaction and virus-vector interaction and for serological characterization of the virus causing necrosis disease of cowpea.

Review of Literature

2. REVIEW OF LITERATURE

2.1. SYMPTOMATOLOGY

The disease known as "Spotted wilt" was first described in Australia by Brittlebank, (1919) and shown to have a viral etiology by Samuel *et al.* in 1930. Ghanekar *et al.* (1979) reported chlorotic ring or chlorotic leaf specking, terminal bud necrosis, axillary shoot proliferation and severe stunting in groundnut (*Arachis hypogaea*) was caused by tomato spotted wilt viruses (TSWV). Francki and Hatta (1981) observed that tospoviruses cause necrosis on several plant parts, chlorosis, ring patterns, mottling, silvering, stunting and local lesions .The symptoms vary depending on the virus strain, host plant, time of year and environment. Iwaki *et al.* (1984) reported that TSWV causes severe chlorotic, necrotic and malformed symptoms on host plants in tropical, sub-tropical and temperate zones. Singh and Krishnareddy (1996) found that symptoms such as leaf crinkling, mottling, yellowing and necrotic streaks on vines, shortened internodes, upright branches and dieback of buds were produced by watermelon strain of tomato spotted wilt virus (TSWV-W). Bhat *et al.* (2001) observed that symptoms of the virus on field infected cowpea plant included necrosis of the growing point of the plant leading to severe stunting and profuse growth of short axillary shoots giving a bushy appearance. Severe infection lead to the death of the plant in all cases. Jain *et al.* (2002) reported that the natural infection of tospovirus on mung bean (*Vigna radiata*) and cowpea (*Vigna unguiculata)* and tomato (*Lycopersicon esculentum.*) was characterized by severe necrosis of leaves, stem, growing buds, pods and fruits at the experimental farms of Indian Agricultural Research Institute, New Delhi and Kerala Agricultural University, Vellayani. Tospovirus infecting soybean induced necrosis of veins, midrib, petiole, stem and bud, other symptoms were yellowing, rugosity, cupping and rolling of leaves (Bhat *et al.*, 2002). Umamaheswaran *et al.* (2003) recorded localized as well as systemic infections characterized by chlorotic and necrotic lesions, veinal and systemic necrosis.

2.2 HOST RANGE OF THE VIRUS

Amin *et al.* (1979) identified that the leaf curl disease in mung and urd beans was caused by TSWV. Ghanekar *et al.* (1979) reported TSWV in groundnut (*Arachis hypogaea*). Host range studies were also conducted and proved that virus induced chlorotic or necrotic local lesions in *Cajanus cajan, Chenopodium amaranticolor, Chenopodium quinoa, Gomphrena globosa, Nicotiana rustica, Nicotiana tabacum, Petunia hybrida* and chlorotic or necrotic spots followed by systemic infection in *Datura stramonium, Glycine max, Vigna unguiculata* cv- C-152, *Lycopersicon esculentum, Vigna radiata, Vigna mungo, Vinca rosea* etc.

The virus causing top necrosis in pea was identified as tomato spotted wilt virus, on the basis of serology, thrip transmission, reactions on cowpea cv. C-152 and *in vitro* properties (Prasada Rao *et al.*, 1985). Tsuda *et al.* (1991) confirmed a virus isolated from tobacco plants as TSWV and reported that virus infection showed severe symptoms on several plants including tobacco, tomato and *Nicotiana benthamiana.* Singh and Krishna Reddy (1996) identified watermelon strain of tomato spotted wilt virus causing an unusual disease of watermelon in India. They also reported that the virus infected systemically many species of Cucurbitaceae and Solanaceae. Moyer (1999) reported that tospoviruses belonging to family *Bunyaviridae* are causing substantial losses world wide to crops such as groundnut, potato, tobacco, vegetables and ornamental plants. Bhat *et al.* (2001) identified tospovirus isolates associated with blackgram, green gram, cowpea and soybean based on bioassay, serology and nucleic acid hybridization. Golnaraghi *et al.* (2001b) observed severe stunting, mosaic, bud necrosis and chlorotic symptoms on groundnut infected with tospovirus. Golnaraghi *et al.* (2001a) reported tomato spotted wilt virus on soybeans in Iran. Umamaheswaran *et al.* (2003) ascertained the experimental host range of tomato tospovirus. The virus could infect fifteen of twenty six different hosts belonging to 4 families. None of the Cucurbitaceous species tested were infected. The virus caused only chlorotic and necrotic lesions on plant species belonging to Amaranthaceae and

Chenopodiaceae families. Both localized as well as systemic infections were observed on Fabaceae and Solanaceae species. The virus caused chlorotic or necrotic lesions followed by veinal necrosis, stem necrosis, leaf deformation and bud necrosis in *Arachis hypogaea*, *V.radiata*, *V. unguiculata*, *Macrotyloma uniflorus* and *Physalis floridana*. Jain *et al.* (2002) recorded natural infection of tospovirus on mung bean (*V. radiata*), cowpea (*V. unguiculata*) and tomato (*Lycopersicon esculentum*) characterized by severe necrosis of leaves, stem, growing point, buds, pods, and fruits at experimental farms of Indian Agricultural Research Institute, New Delhi and Kerala Agricultural University, Vellayani . Mandal *et al.* (2002) observed differential response of selected peanut (*Arachis hypogaea*) genotypes to mechanical inoculation by tospovirus. Martinez-ochoa *et al.* (2003) reported mixed infections of impatiens necrotic spot virus and TSWV in tobacco grown in Georgia.

2.3 TRANSMISSION

2.3.1 Mechanical Transmission

Ghanekar *et al.* (1979) reported mechanical transmission of tomato spotted wilt virus using 0.05M Potassium phosphate buffer (pH-7.0) containing 0.02M 2-mercaptoethanol. Tsuda *et al.* (1991) isolated TSWV from tobacco and mechanically inoculated to several differential host plants using 0.1M Phosphate buffer (pH 7.0) with 0.01M Na_2So_3. Sheng-zhipang *et al.*(1992) inoculated transgenic plants with TSWV isolates using a phosphate buffer (0.033M KH_2PO_4, 0.067M K_2HPO_4 and 0.01M Na_2So_3). The inoculum extracts were immediately rubbed on the carborrundum dusted leaves of transgenic plants and the inoculated leaves were subsequently rinsed with water. de Avila *et al.* (1993) maintained tospovirus isolates in liquid nitrogen and was recovered by mechanical inoculation on *Nicotiana benthamiana* Domin or *Nicotiana rustica* L. using 0.01M phosphate buffer, pH7.0, containing 0.01M $Na_2 So_3$. Singh and Reddy (1996) isolated tospovirus from leaves of watermelon showing bud necrosis using 0.1M phosphate buffer pH7.0 containing 0.02M 2.mercaptoethanol. Extract from

infected plants were inoculated on cowpea (*V.unguiculata* cv. C-152). Cortes *et al.* (1998) reported that iris yellow spot virus, a tospovirus species was isolated in 1992 and maintained in *Nicotiana benthamiana* Domin by mechanical inoculation. Hoffmann *et al.* (1998) reported mechanical inoculation of tomato spotted wilt virus in peanut plants dually infected with peanut mottle virus using 0.01M Tris pH7.8 containing 0.01M Na_2So_3 and 0.1 per cent cysteine hydrochloride. Bhat *et al.* (2001) reported that tospovirus infecting black gram, cowpea, green gram and soybean could be mechanically transmitted. Mechanical inoculation was carried out using 0.05M potassium phosphate buffer pH 7.0 containing 0.01M sodium sulfite as described by Ghanekar *et al* (1979). Bhat *et al.* (2002) maintained and propagated tospovirus causing soybean bud blight on cowpea (*Vigna unguiculata*, cv. Pusa Komal) in glass house through mechanical inoculation using 0.1M phosphate buffer, pH7.2 containing 0.1% 2-mercaptoethanol. Umamaheswaran *et al.* (2003) observed mechanical transmission of tospovirus infecting tomato. The virus was rub inoculated on to cowpea (*Vigna unguiculata* cv. Pusa Komal) under glass house at primary leaf stage using 0.01M potassium phosphate buffer (pH7.2) containing 0.1% 2-mercaoptoethanol. Mandal *et al.* (2001) studied the factor affecting mechanical transmission of tomato spotted wilt virus to peanut. It was reported that use of two anti-oxidants (sodium sulfite and mercaptoethanol) and two abrasives (celite and carborrundum) and inoculation was done by rubbing with a cotton swab dipped in inoculum as well as pricking with a sterlized needle resulted in significantly high rate of transmission. Chaisuekul *et al.* (2003) investigated thrips and mechanical transmission of TSWV to tomato plants of different ages.

2.3.2 Insect Transmission

Tomato spotted wilt virus transmitted by several species of thrips. Ghanekar *et al.* (1979) reported transmission of TSWV by thrips (*Scirtothrips dorsalis*). Sidda ramaiah *et al.* (1980) studied the spread of bud blight disease of groundnut and found that disease was transmitted by *Thrips tabaci.* Palmer *et al.* (1990) used several species of thrips in ICRISAT to test the presence of TSWV

using dot immuno binding assay and found *Thrips palmi* positive for the virus. Gofflot and Verhoyen (1990) reported that TSWV occurrence in Belgium was related to significant increase in *Frankliniella occidentalis* population. Other possible vectors were *Thrips tabaci, F. schultzei* and *F. fusca*. Marchoux *et al.* (1991) detected tomato spotted wilt virus in individual thrips (*F. occidentalis*) and related the spread of TSWV in vegetables and ornamental plants to this vector. Singh and Krishnareddy (1995) investigated the potential of *Thrips flavus* as a vector of the tospovirus infecting watermelon in India. According to their studies adults collected from naturally infected plants showed 28.5% transmission and nymphs were able to transmit the virus to healthy plants following acquisition access on infected plants. Westering *et al.* (1996) reported that TSWV was efficiently transmitted by second instar larvae and adults of *F. occidentalis* in a propagative manner. Ohnishi *et al.* (1998) reported that TSWV was exclusively transmitted by several species of thrips in a unique persistent manner. Nagata *et al.* (1998) studied the development of the infection of TSWV in the midgut of the larvae and adults of *F. occidentalis*. Tospoviruses were biologically transmitted by seven species of the genera Thrips and *Frankliniella* sp. (Chatzivassiliou *et al.*, 1998). Allen *et al.* (2000) suggested that infected thrips, *F.occidentalis* moving into fields were the most significant transmitters of tomato spotted wilt virus to tomatoes in south east Arkansas. Assis Filho *et al.* (2004) studied the acquisition of tomato spotted wilt virus using adults of two thrips species –*Frankliniella fusca* (Tobacco thrips) and *F. occidentalis* (Western flower thrips). They reported that adult thrips feeding on virus infected plants could not transmit the virus. Sakurai (2004) reported that *F. schultzei* originating in Paraguayan tomato fields could transmit TSWV efficiently and is an important vector of the virus.

2.3.3 Seed Transmission

Ghanekar *et al.* (1979) studied seed transmission of TSWV causing bud necrosis of groundnut. Of nearly 6000 seeds collected from the infected plants, 30 per cent produced normal plants, nine per cent produced malformed plants and the reminder failed to germinate. None of the plants developed disease symptoms

and assays on cowpea from the malformed stunted plants gave negative results. The results indicated that the virus was not seed borne. Mali and Pahl (1980) studied the occurrence of tomato spotted wilt virus on groundnut in Maharashtra. They reported that the virus was not transmitted through seeds.

Reddy and Wightman (1988), Goldbach and Peters (1994) and Mumford (1995) suggested that seed transmission does not occur among tospoviruses.

2.3.4 Graft Transmission

Roy and Gupta (1977) reported top necrosis virus disease of pea from India, which was identified as tomato spotted wilt virus, could be transmitted through graft and sap inoculations. Ghanekar *et al.* (1979) established tomato spotted wilt virus causing groundnut bud necrosis in a screen house by graft transmission to healthy groundnut plant. Matsura *et al.* (2004) studied the efficiency of viral transmission from TSWV infected chrysanthemum stock plants to cuttings. The results indicated that transmission efficiency from infected Chrysanthemum stock plants to cuttings was approximately 20-50 per cent.

2.4 PHYSICAL PROPERTIES

Physical properties of the virus include dilution end point (DEP) thermal inactivation point (TIP) and longevity *in vitro* (LIV)

Roy and Gupta (1977) studied physical properties of top necrosis virus disease of pea from India. The physical properties were studied at room temperature (25± 3°C). The DEP was 1 : 10,000, TIP 64° C and longevity *in vitro* and *in vivo* was for 5 and 15 days respectively.

Ghanekar *et al.* (1979) found that TSWV remained infective in buffered sap (phosphate buffer, pH 7.0) of groundnut at a dilution of 10^{-2}- 10^{-3}. The infectivity was retained after storage for four hours at room temperature (30°C) and for ten minutes at 40°C but not at 45°C.

Mali and Pahl (1980) found that tomato spotted wilt virus (TSWV) remained infective in buffered sap of groundnut at a dilution of 10^{-3} but not at 10^{-4}.

The TIP was between 45-50°C and LIV was found to be 1-2h at room temperature.

Silveira Junior *et al.* (1985) identified the pathogen isolated from *Sechium edule* as tomato spotted wilt virus from its host range and properties *in vitro*. The TIP was found between 45-50°C. DEP was 10^{-3} to 10^{-4} and longevity at room temperature was less than 2 h.

Roggero and Pennazio (1997) found that the thermal inactivation point of TSWV in tobacco was 44°C. The virus was infective in *in vivo* for one hour at 50°C. However, the virus was inactivated in plants held for 24 hours at 45°C.

Buzancic and Juretic (1978) studied properties of tomato spotted wilt virus isolate from tobacco in Yugoslavia. They found that the thermal inactivation of the virus was 44-46°C, DEP 10^{-3}-10^{-4} and LIV at room temperature 3-4h.

2.5 IMMUNODETECTION AND CHARACTERIZATION

2.5.1 Enzyme Linked Immunosorbent Assay (ELISA)

Avila *et al.* (1990) conducted serological differentiation of 20 isolates of tomato spotted with virus. The results indicated that ELISA employing polyclonal and monoclonal antisera could be used to differentiate TWSV isolates and to detect defective forms. Resende *et al.* (1991) found that the virus could efficiently be detected in high dilutions of sap from infected plants and at low concentration of purified virus and nucleocapsid protein preparations in cocktail ELISA and DAS ELISA. Porter *et al.* (1991) reported tomato spotted wilt virus in peanuts and virus identification was confirmed by standardized ELISA. Gumodzoe (1993) reported the use of immunosorbent assays for the identification of virus disease 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. Bo *et al.* (1997) used ELISA to detect tomato spotted wilt tospovirus in tissue imprints. Cho *et al.* (1986) reported

ELISA as the method of choice for diagnosis and detection of tospoviruses in plants and thrips. Serological differentiation of Iris yellow spot virus (IYSV) from other tospovirus was proved using DAS-ELISA (Cortes *et al.*, 1998).

2.5.2 Dot Immuno Blot Binding Assay (DIBA)

Hsu and Lawson (1991) used direct tissue blotting for the detection of tomato spotted wilt virus in impatiens. They reported that when biotinylated mouse monoclonal antibody were used, dot blot immuno assay (DBIA) was nearly eight times more sensitive than ELISA for the detection of TSWV in extracts from infected *Nicotiana benthamiana* leaves. Bananej *et al.* (1998) used DIBA for identification of tomato spotted wilt virus from tomato fields in Varamin area in Iran. Bhat *et al.* (2001) reported successful Dot –blot hybridization of healthy and necrosis infected black gram, cowpea, greengram and soybean with nucleocapsid protein (NP) gene probe of GBNV. Whitfield *et al.* (2003) used Tissue blot immuno assay for detection of tomato spotted wilt virus in *Ranunculus asiaticus* and other ornamentals.

2.6 BIOCHEMICAL CHANGES OF HOST PATHOGEN INTERACTION

2.6.1 Carbohydrate

Khatri and Chenulu (1969) reported that reducing sugar content was not appreciably affected by cowpea mosaic virus in resistant and susceptible cowpea cultivars. Ramiah (1978) found that there was decrease 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 10^{th} day after inoculation. Singh and Singh (1979) studied the changes in carbohydrate fractions of mung bean due to mung bean severe mosaic infection. They observed decrease in reducing and non-reducing sugars, total sugar and starch in infected leaves, stems and roots at every stage of infection. Rajendrasingh and Kumarsingh (1980) reported decrease in total sugar and starch in leaf tissues of sunhemp infected with common bean mosaic virus. Singh and

Singh (1984) observed that the virus infection decreased total sugar and starch in cowpea cultivars infected with southern bean mosaic virus and cowpea mosaic virus. 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 sugar and total sugar in bhindi (Sarma *et al.*, 1995).

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 suceptible varieties of cowpea when inoculated with cowpea aphid borne mosaic virus (CABMV).

Mali *et al.* (2000) reported that infection of yellow mosaic virus in moth bean resulted in reduction of total soluble carbohydrate in suceptible when compared to resistant genotype. 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 bhindi 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. Manickam *et al.* (2000) reported that the increase in non-reducing sugars in TSWV inoculated plants was less compared to healthy cowpea plants. Sutha *et al.* (1998a) studied the changes in concentrations of chemical constituents in tomato caused by tomato spotted wilt virus infection and revealed that there was accumulation of carbohydrate in infected plants. Total, reducing and non-reducing sugars decreased in infected plants, however, the reduction was more in the initial stages of infection compared to later stages. In contrast to sugar concentration,

the starch increased in infected plants at all stages of analysis. Sutha *et al.* (1998b) reported that TSWV infection reduced the concentration of total, non-reducing and reducing sugars of tomato fruits.

2.6.2 Chlorophyll

There was significant reduction in chlorophyll content in virus infected susceptible variety Co-2 (Ramiah, 1978). Rajendrasingh and Kumarsingh (1980) reported that inoculation of *Crotalaria juncea* with bean common mosaic virus decreased the net and gross production of dry matter and total chlorophyll in leaves and enhanced respiration. 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 remained unchanged. The total chlorophyll, chlorophyll a and b increased up to 60 days after inoculation and then declined (Rao and Shukla, 1989). They suggested that the yield loss due to virus infection was mainly 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. Sarma *et al* (1995) reported the reduction of total chlorophyll and chlorophyll b in case of yellow vein mosaic virus infecting bhindi. Dantre *et al.* (1998) studied biochemical changes induced by yellow vein mosaic virus in leaves of soybean cultivars. They 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 Uma maheswaran (2000) reported higher chlorophyll content in resistant variety when compared to susceptible variety in case of cowpea infected with BCMV. Sutha *et al* (1998a) investigated the biochemical changes caused in tomato following infection with TSWV and reported that chlorophyll, xanthophyll and carotene contents were reduced in infected plants. It was suggested that this could directly affect the yield potential of infected tomatoes. Sutha *et al.* (1998b) reported that TSWV infection reduced the concentration of carotene of tomato fruits. In contrast lycopene content was increased.

2.6.3 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 investigating the effect of yellow vein mosaic virus 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 protein and soluble proteins were found high in virus free resistant varieties. Mali *et al.* (2000) reported that free aminoacids and soluble protein content increased with increasing levels of yellow mosaic virus infection in susceptible variety of moth bean. Manickam *et al.* (2000) studied the impact of application of a foliar spray of AVPs from *Cocos nucifera, Sorghum vulgare, Sorghum bicolor* and *Croton sparsiflorus* leaves and inoculation of TSWV on the non-reducing sugar and total soluble protein contents of cowpea plants. It was found that the increase in total soluble protein was higher in TSWV - inoculated plants. They also studied the

effect of foliar spray of AVPs and inoculation of TSWV on the RNA content of *Vigna unguiculata* and *Vigna radiata* plants. TSWV inoculation showed a significant increase in the RNA content of both the species tested, while there was no change in RNA content in AVPs treated cowpea plants inoculated with TSWV. Sindhu (2001) studied total soluble protein content in the susceptible cowpea plants inoculated with BICMV. The total soluble protein was found higher in the case of inoculated susceptible plants. Kovalenko and Shepelevitch (2003) found that severe virus infection decreased general content of proteins in leaves but induced at least three pathogenesis associated proteins.

2.6.4 Phenol

Ramiah (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 cowpea aphid-borne mosaic virus. 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 muskmelon and showed an increasing trend of the enzyme activity and phenol component 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 no difference between varieties with respect to total phenol content. Kato *et al.* (1993) extracted and characterized two phenolic compounds from cowpea leaves infected with cucumber mosaic virus. Sohal and Bajaj (1993) reported an increase in total phenols in both resistant and susceptible varieties of mungbean 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. Sutha *et al.* (1998b) reported that TWSV infection reduced the concentration of

total and *ortho*-dihydroxy phenol contents of tomato fruits.

2.6.5 Defence Related Enzymes

Khatri and Chenulu (1970) studied the changes in the peroxidase enzyme activity in leaves of resistant and susceptible cowpea variety and observed the peroxidase activity increased in both resistant and susceptible variety 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 found an increasing trend of enzyme activity when compared to healthy control.

Rathi *et al.* (1986) assayed peroxidase, polyphenol oxidase and isozyme of peroxidase in pigeon pea 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 suceptible 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 phenylalanine ammonia-lyase in response to infection by carnation etch ring virus. The results suggested the existence of a correlation between the elevated levels of phenolics and phenylalanine ammonia-lyase with disease resistance. Ahmed *et al.* (1992) found that enzyme peroxidase and polyphenol oxidase

showed no significant difference in virus free susceptible and resistant plants while studying biochemical basis of resistance to yellow vein mosaic virus in okra. Sohal and Bajaj (1993) reported an increase in polyphenol oxidase activity in resistant variety of mungbean infected with yellow mosaic virus.

Umamaheswaran (1996) reported that there was progressive increase in peroxidase, polyphenol oxidase and phenylalanine ammonia-lyase activity in inoculated and susceptible varieties of cowpea. Mali *et al* (2000) reported that the activity of catalase, peroxidase and nitrate reductase enzymes was found to reduce with increase in intensity of disease in the case of yellow mosaic disease of moth bean (*Vigna aconitifolia*). Radhika and Umamaheswaran (2000) reported higher activity of peroxidase, polyphenol oxidase and phenylalanine ammonia-lyase in resistant variety when compared to susceptible variety of cowpea infected with BICMV.

Sindhu (2001) investigated on changes in defence related enzymes viz., peroxidase, polyphenol oxidase and phenylalanine ammonialyase and indicated that there was significant increase in activities of these enzymes in inoculated plants.

2.7 ELECTROPHORESIS OF SOLUBLE PROTEINS

Satyanarayana *et al.* (1996) performed polyacrylamide gel electrophoresis of nucleocapsid protein of peanut yellow spot virus (PYSV), a distinct tospovirus species based on protein profiling, serology and nucleic acid hybridization. Nucleocapsid of PYSV, purified from peanut plant tissue contained a protein with a molecular mass of 29 kDa.

Cortes *et al.* (1998) characterized iris yellow spot virus (IYSV), a tospovirus species. They estimated the size of IYSV, N-protein and compared it with those of other tospo viral N-proteins. The results revealed that N-protein of IYSV had an estimated molecular weight of 30kDa and is larger than N-proteins of TSWV (28.8 kDa), tomato chlorotic spot virus (28.7 kDa), groundnut ring spot

virus (28.8 kDa) and impatiens necrotic virus (28.7 kDa) but still smaller than that of watermelon silver mottle virus (30.6 kDa)

de Avila *et al* (1993) analysed the protein composition of a number of tospovirus isolates, collected from different geographical areas and different host plants. They found that the migration rates of different isolates correspond with the molecular mass of 29 kDa and 28 kDa.

2.8 ELECTROPHORETIC ANALYSIS OF ISOZYMES

Novacky and Hampton (1967) separated isozymes from TMV infected tobacco and CMV or tobacco ring spot virus infected cowpea by disc electrophoresis and stained with benzidine HCl - H_2 O_2 . Only quantitative changes were observed in both host plants.

Solymosy *et al.* (1967) reported that the changes in peroxidase enzyme patterns was induced by virus infection. They compared the changes in peroxidase isozyme spectrum in various host virus combinations and indicated that the change was determined mainly by the host tissue and not by virus.

Brown *et al.* (1978) found that the isozyme polymorphism was 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 of cowpea.

Sindhu (2001) conducted electrophoretic analysis of isozyme from CABMV susceptible cowpea varieties. She reported five isoforms of PPO in suceptible varieties, Sharika and Malika, whereas other two genotypes, Co-6 and Pallichal local expressed only four iso-PPOs. The isozymes, polyphenol oxidase in CO-6 were more prominent compared to other genotypes analysed.

Materials and Methods

3. MATERIALS AND METHODS

3.1 SYMPTOMATOLOGY

Seeds of cowpea (*Vigna unguiculata* (L.) Walp.) were obtained and they were sown in pots containing potting mixture of sand, soil and cowdung in the ratio of 1:1:1.. Leaves showing chlorosis and veinal necrosis were collected from field and the culture of virus was maintained by repeated transfers on cowpea variety Pusa Komal in insect proof glass house by mechanical inoculation using 0.01M Phosphate buffer of pH 7.2 containing 0.1% 2-mercaptoethanol at 24-32°C. Inoculated plants were incubated for the development of symptoms.

3.2 HOST RANGE

To determine the host range of cowpea tospovirus, the plants belonging to 24 species of 7 families were inoculated by sap inoculation. Ten seedlings of each species were inoculated. The plants which did not show visible symptoms of infection after four weeks were indexed by back inoculation to healthy cowpea plants to find out whether they were symptomless carriers of the virus.

3.3 TRANSMISSION

3.3.1 Mechanical Transmission

Culture of the virus was collected from field and maintained in the insect proof glass house. Sap transmission was conducted using different buffers for extraction. The buffers used were 0.1 M citrate buffer (pH 6.2), 0.1 M citrate phosphate buffer (pH 7.0) 0.01M phosphate buffer (pH 7.0), 0.1M. tris buffer (pH 7.2) and 0.01M phosphate buffer (pH 7.2)(Appendix I). In all sap inoculation studies celite powder was used as abrasive.

The extract was taken from young leaves showing severe chlorotic and necrotic symptom. One part of the leaf tissue was homogenised with one part of

buffer using a chilled mortar and pestle. The homogenate was maintained in an ice box and immediately used for inoculation. Inoculation was done on primary leaf stage of cowpea plants cv Pusa Komal. Prior to inoculation leaves were uniformly dusted with celite powder. Test plants were inoculated with the pestle moistened with the inoculum by gently rubbing on the upper surface of the fully opened leaves. The surface was rinsed off after 5 minutes with distilled water using a wash bottle. Inoculation was also done on local lesion host, *Chenopodium amaranticolor*. Fourth to eight leaves of the local lesion host at 8-15 leaf stage were chosen for inoculation. Inoculation was done using different buffers mentioned above. The buffer which gave maximum number of local lesions on *Chenopodium amaranticolor* was chosen for extraction of sap to inoculate on cowpea plants for further studies. 0.01 M phosphate buffer (pH 7.2) was used for all transmission studies. The plants were kept for 6-7 days for the development of symptoms.

3.3.2 Insect transmission

Insect transmission studies were conducted using insects associated with cowpea namely thrips and aphids.

Inoculation using aphid vectors

The apterous aphids were transferred to petridishes and starved for a period of one hour (pre-acquisition fasting period). The aphids were feed on young infected cowpea leaves for a period of 30 minutes (acquisition feeding period). After that aphids were released to ten cowpea plants using a moistened camel hair brush and allowed to feed for 24 hours. The aphids were killed using 0.1 per cent quinalphos. Per cent transmission was recorded.

Inoculation using thrips vectors

Transmission studies were carried out as per the procedure coined by Ghanekar *et al* (1979). Healthy thrips colonies were raised from 10 adults

collected from the field and maintained on cowpea plants by weekly transfer of nymphs. First or second instar nymphs were allowed acquisition feeds on detached infected cowpea leaves floated on water in a petridish. After an acquisition access period of 2 – 3 days, 10 – 15 nymphs were transferred to individual cowpea plants at the primary leaf stage. After an inoculation feeding period of 12 days the thrips were killed by spraying with 0.1% quinalphos. The test plants were then kept in the screen house for observation.

3.3.3 Seed transmission

Seed transmission studies were conducted using 125 seeds collected form infected plants. They were sown in pots kept is an insect proof glass house and examined for the development of symptoms in the primary and first trifoliate true leaves.

3.3.4 Graft transmission

Small shoots showing chlorotic symptoms were used for preparing the scion. The base of the scion was trimmed to a wedge shape and inserted into a cleft made on the stem of the healthy cowpea plants kept in insect proof glass house.

Thirty day old healthy plants were used as root stocks. The base of the scion was inserted into the cleft of the stock. The graft was then tied firmly using a para film. These plants were kept under observation for the development of systemic symptoms in the new sprouts of the root stock.

3.4 PHYSICAL PROPERTIES OF THE VIRUS

3.4.1 Dilution End Point (DEP)

Five grams of infected cowpea leaves was homogenized in chilled mortar and pestle by adding 5 ml of 0.01 M phosphate buffer (pH 7.2) . The homogenate was strained through a thin layer of cotton. Serial dilutions of the sap, viz., 10^{-1},

10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} were made as follows. Six test tubes were kept in a row in a test tube rack. Nine ml of buffer was dispensed into each of five test tubes starting from the second test tube by using a clean pipette. The extract prepared was transferred to the second tube with 9 ml buffer to get a dilution of 10^{-1}. It was thoroughly mixed and one ml of 10^{-1} dilution was transferred to the next test tube to obtain a dilution of 10^{-2}. This process was continued till a dilution of 10^{-5} was obtained. The dilutions were inoculated on fully opened leaves of *C. amaranticolor*. Five replications were maintained for each treatment. The inoculated leaves were labelled and kept under insect proof conditions and observed for the development of local lesions. Leaves treated with buffer were kept as control.

3.4.2 Thermal Inactivation Point (TIP)

Sap was extracted from infected leaves of cowpea as mentioned in above experiment. 3 ml of sap was pipetted into a thin walled glass test tube. C was taken not to smear the upper part of the test tube. This was placed in a w bath with thermostat arrangement. The level of water was maintained 3 cm a the level of sap in the tubes. 3 ml aliquots of the sap were treated for ten mi each at 35, 40, 45, 50, 55 and 60° C. After each treatment, the tubes were rem and cooled immediately. Control was kept at room temperature (28 ± 2° C) samples were inoculated on five fully opened leaves of *C. amaranti* Observations on number of local lesions produced were recorded.

3.4.3 Longevity *In vitro* (LIV)

Infected leaves were ground in a mortar and pestle as mentioned and the homogenate was filtered through a thin layer of cotton. One ml ea this sap was pipetted into eleven test tubes and closed with cotton wool. tubes were kept at room temperature (28 ± 2° C) and five in a refrig maintained at 8° C. One tube each containing the sap of each treatment was after specific periods viz., 2, 4, 6, 8 and 24 h and inoculated on the lea *C. amaranticolor*. The plants were observed for the development of local les

3.5 IMMUNO DETECTION

3.5.1 Direct Antigen Coating – Enzyme Linked Immunosorbent Assay (DAC-ELISA)

ELISA for the detection of cowpea viruses was done. 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 the virus was carried out following the procedure described by Hugenot *et al.* (1992).

Procedure

The healthy and infected leaf samples were ground separately in coating buffer (carbonate buffer) in the ratio 1:5 (w/v). The homogenate was centrifuged at 5000 rpm for 10 minutes at 4°C. The treatments were replicated twice. After incubation for 2 h at 37°C the wells were washed with PBS-T, 3 times each for a duration of 3 minutes. The plates were tapped on a blotting paper to remove excess PBS-T. Blocking was done with 100 µl of 1% BSA for 30 minutes at 37°C. After incubation blocking agent was removed, plates were washed with PBS-T as before. Antibodies specific for tospovirus and WSMV was used. The antibodies at 1:10000 dilutions in PBS-TPO were added and incubated over night at 4°C. The plates were washed with PBS-T and treated with 100µl of alkaline phosphatase conjugated anti rabbit immunoglobulin diluted in PBS-T (10^{-4}) and incubated for 2 h at 37°C. Wells were washed with PBS-T as before. The substrate *para*-nitro phenyl phosphate (*P*-NPP) in diethanol amine buffer (1 mg/ml) was added to each well (100µl/well) and incubated for 1 h at 37°C. Reaction was stopped by adding 50 µl of 4 % sodium hydroxide. The absorbance was read at 405 nm in an ELISA reader (ECIL, MS5608) (Appendix II).

3.5.2 Dot Immuno Binding Assay (DIBA) for the Detection of Tospovirus

DIBA was carried out to detect the presence of tospovirus in infected cowpea leaves.

Procedure

- Tissue was extracted in antigen extraction buffer (1:10 w/v) and expressed through cheese cloth
- 0.8 ml of expressed sap was taken in an eppendorf to which 0.4 ml chloroform was added
- The mixture was vortexed and centrifuged at 12,000 g for two minutes
- The clarified sap (upper aqueous layer) was mixed with antigen extraction buffer (1:4 ratio) and vortexed
- Nitrocellulose membrane (NCM) with squares of 1 x 1 cm was floated in Tris Buffer Saline (TBS) and air dried
- 10 µl of the sample was spotted to the centre of each square and allowed it to dry
- Treated NCM was immersed in blocking solution with gentle oscillation for one hour at room temperature
- NCM was rinsed in TBS for 10 minutes and incubated overnight at 4°C in crude antiserum diluted in TBS-spray dried milk (SDM).
- NCM was again rinsed in TBS for 10 minutes and incubated for one hour at room temperature in secondary antibody (antirabbit IgG alkaline phosphatase conjugate diluted in TBS-SDM).
- After rinsing in TBS for 10 minutes NCM was incubated in substrate solution at room temperature in the dark After the colour development NCM was rinsed in fixing solution for 10 minutes and then air dried between Whatman filter paper sheets and stored (Appendix III).

3.6 BIOCHEMICAL CHANGES OF HOST PATHOGEN INTERACTION

Biochemical analysis of healthy and diseased plants were carried out. Cowpea cv Pusa Komal was selected for the study. Seeds were sown 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 ammonia lyase were also done. Protein profile study was done using SDS-PAGE. Isozyme analysis in diseased and healthy plants was analysed in native PAGE.

3.6.1 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 hydrolyzed with 5ml of 2.5 N hydrochloric acid (HCl) in a boiling water bath. The hydrolyzate 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 4 ml anthrone reagent was added and heated for eight minutes in a boiling water bath. This was cooled rapidly and absorbance was measured at 630 nm in a spectrophotometer (Systronics UV-VIS Spectrophotometer 118). Amount of carbohydrate present was calculated from standard graph prepared using glucose and expressed in terms of milligrams of glucose equivalent per gram of leaf tissue on fresh weight basis.

3.6.2 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 in volumetric flask was made up to 100 ml. Absorbance of the solution at 645, and 663 nm was read in a spectrophotometer against the solvent (80 per cent acetone) as blank. The chlorophyll content was calculated using the following equations and expressed as milligrams chlorophyll per gram tissue.

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

$$\text{Chlorophyll a} = 12.7(A663)-2.69(A645) \times \frac{V}{1000 \times w}$$

$$\text{Chlorophyll b} = 22.9(A645)-4.68(A663) \times \frac{V}{1000 \times w}$$

3.6.3 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 g for 15 minutes at 4° C. 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. The absorbance was read at 595 nm in a spectrophotometer 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.

3.6.4 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 10000 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. An aliquot of 0.3 ml was pipetted out and made up to 3 ml with distilled water. Folin - Ciocalteau reagent (0.5 ml) was added and 2 ml of 20 per cent sodium carbonate solution was added to each tube after three minutes. This was mixed thoroughly and kept in boiling water for one minute. This was cooled and 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.

3.6.5 Estimation of Defence Related Enzymes

3.6.5.1 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.

3.6.5.2 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.

3.6.5.3 Estimation of Phenylalanine Ammonia-lyase (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 5 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 3 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 3 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. The absorbance was read at 290 nm in a spectrophotometer (Systronics UV-VIS spectrophotometer 118).

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

3.7 ELECTROPHORETIC ANALYSIS OF PROTEINS

Characterization of proteins by PAGE

Electrophoretic separation of soluble protein of cowpea leaves were carried out as per the procedure described by Laemelli (1970). SDS was not used in this experiment since it degrade the virus. Leaf sample of healthy and diseased plants were taken for analysis.

Five hundred milligram each of healthy and infected leaf samples were homogenized in 200 µl of cold denaturing solution at 4°C. The supernatant was mixed with chilled acetone in the ratio 1:4 and the protein was allowed to precipitate by keeping the mixture at 4°C for 30 minutes. The sample was centrifuged at 5000 rpm for 15 minutes at 4°C. The precipitate was resuspended in 20 µl of denaturing solution and vortexed. The homogenate was centrifuged at 5000 rpm for 15 minutes. The supernatant was mixed with equal volume of sample buffer and kept in a boiling water bath for 3 minutes . These samples were used for PAGE. The protein concentration was adjusted in each sample to 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 approximately 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°C.

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°C.

d) Polymerising agents

Ammonium persulphate (APS) 10 per cent prepared freshly before use.

TEMED – Fresh from the refrigeration.

e) Electrode buffer pH 8.3

Tris base	- 6.0 g
Glycine	- 28.8 g
Double distilled water	- 2 litre

f) Sample buffer

Double distilled water	-2.6 ml
0.5 M Tris HCl pH 6.8	-1.0 ml
2-mecaptoethanol	-0.8 ml
Glycerol	-1.6 ml
0.5 % Bromophenol blue	-0.4 ml

g) Staining solution

Comassie 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 R 250

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

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 Ammonium persulphate (APS)
Freshly prepared - 0.10 ml

Tetra methyl ethylenediamine (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

Acrylamide stock - 1.3 ml

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

APS 10 % - 0.05 ml

TEMED - 0.1 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 in 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 overnight 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).

3.8 ELECTROPHORETIC ANALYSIS OF ISOZYME

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 molecular size.

The present work was undertaken to study the enzyme alterations in healthy as well as tospovirus infected cowpea leaves.

Enzyme extraction and assay

Soluble and ionically bound enzymes were extracted by grinding the sample under chilled condition in 50 mM Tris-Cl (pH 7.6) in the ratio of 1:2 w/v. The homogenate was centrifuged at 15,000 rpm for 10 minutes at 4°C. The resulting

Resolving gel acrylamide solution -10 ml

Distilled water -25 ml

APS

supernatant was used for isozyme analysis.The protein content was adjusted in each sample to a strength of 100 µg of protein following 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) with slight modification. Proteins extracted by 50 mM Tris (pH 7.6) 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 out SDS (native gel).

Reagents

a) Separating gel (7.5 %)

Tris chloride buffer stock solution (pH 8.9)	- 5 ml
Resolving gel acrylamide solution	-10 ml
Distilled water	-25 ml
APS	-300 µl

b) Staking gel (4%)

Tris chloride buffer stock, pH 6.7	-2.5 ml
Resolving gel acrylamide solution	-3.1 ml
Distilled water	-14.1 ml
APS	-300 µl

Following electrophoresis, the gel was immersed in a solution of 10 mM L-3, 4 dihydroxy phenyl alanine (L-DOPA) in 100 mM sodium phosphate (pH 7.0) in a plastic tray kept in a shaker for 30 minutes. Zones of enzyme activity

(Polyphenol oxidase) were observed as grey black bands. The Rm value and relative intensities of the isozyme bands of PPO were also recorded.

For peroxidase enzyme, the gel was incubated in 0.6 M sodium acetate buffer (pH 5.4) containing 0.5 per cent 0 – dianisidine HCl for 30 minutes at room temperature. The gel was transferred to 0.1 M hydrogen peroxide until visible bands were developed.

Results

4. RESULTS

4.1 SYMPTOMATOLOGY

Mechanically inoculated seedlings of cowpea cv. Pusa Komal expressed symptoms of viral infection five days after inoculation. Initial symptoms appeared as chlorotic spots in the inoculated leaves (Plate 1a). Chlorotic spots later turned necrotic. Symptoms were also observed in the newly emerged trifoliate leaves as chlorotic spots. As the infection progressed the leaves become necrotic and showed veinal necrosis (Plate 1b). Severely infected leaves became distorted and reduced in size. Generally the plants were seen stunted and showed bud necrosis in the later stages.

Symptoms on naturally infected plants were characterized by necrosis of the growing point of the plant leading to severe stunting, profused growth of short axillary shoots giving the bushy appearance (Plate 1c). Severe infection led to the death of the plants.

4.2 HOST RANGE

Host range studies were conducted with 24 plant species belonging to 7 families. The result showed that 12 species belonging to 5 families ie., Chenopodiaceae , Amaranthaceae, Leguminosae, Solanaceae and Malvaceae produced symptoms of virus disease (Table 1, Plate 2-13).

4.3 TRANSMISSION

4.3.1 Mechanical Transmission

The virus was transmitted successfully through mechanical inoculation. This was done both on cowpea and *Chenopodium amaranticolor*, the local lesion host of the virus. Infectivity of the virus in different buffers was rated based on the development of local lesions in *C. amaranticolor*. Phospshate buffer (0.01M,pH 7.2) gave maximum number of lesions (65.5) compared to other buffers tested

Symptoms of tospovirus infected cowpea

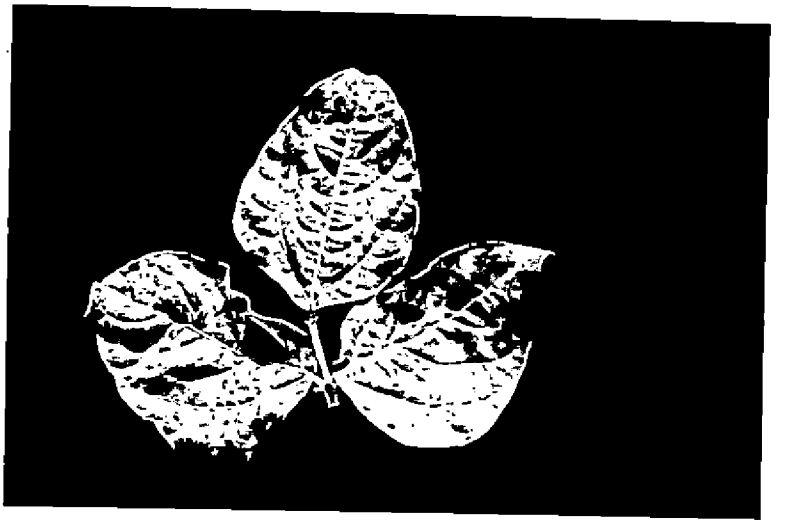


Plate 1a Chlorotic spots

Plate 1b Veinal necrosis

Plate 1c Distortion and reduction in leaf size (Bushy appearance)

Table 1 Host Range studies of tospovirus isolates from cowpea

Host	Symptoms	Percent transmission
I. Amaranthaceae		
Gomphrena globosa	Chs, NE, SI, BN	100
Amaranthus tricolor	NS	-
II. Chenopodiaceae		
Chenopodium amaranticolor	Chs, NLL	100
Chenopodium quinoa	Chs, NLL	90
Chenopodium album	Chs, NLL	50
III. Cucurbitaceae		
Cucumis sativus	NS	-
Cucumis melo	NS	-
Cucurbita moshata	NS	-
Benincasa hispida	NS	-
Trichosanthes anguina	NS	-
Momordica charantia	NS	-

Table 1 Continued

Host	Symptoms	Percent transmission
IV. Leguminosae		
Vigna unguiculata(L.) Walp cv *pusa komal*	Chs,NE,VN,BN,SI	87
Arachis hypogaea	Chl,BN,SI	60
Cajanus cajan	NS	-
Glycine max	Chl	30
Vigna radiata	Chs,VN,BN,SI	90
V. Solanaceae		
Capsicum annum L.	NS	-
Datura stramonium	Chs, NLL, SI	90
Lycopersicon esculentumL.	NL, SI	47
Nicotiana benthamianaL.	NS	-
Solanum melongena L.	Chs	30
VI. Apocynaceae		
Catharanthus roseus	NS	-
VII. Malvaceae		
Abhelmoschus esculentus	NS	-
Petunia hybrida	DV	40

*NS – No Symptoms, Chs – Chlorotic Spots, NLL – Necrotic Local lesions, SI – Systemic Infection, DV – Discoloration along Veins, BN – Bud Necrosis, NE – Necrosis, VN – Veinal Necrosis.

Plate 2. Bushy appearance in cowpea

Plate3. Ground nut bud necrosis

Plate 4. Bud necrosis in green gram

Plate 5. Chlorotic spots on green gram

Plate 6. Necrotic spots in tomato

Plate 7. Stem necrosis of tomato

Plate 8. Chlorotic spots in brinjal

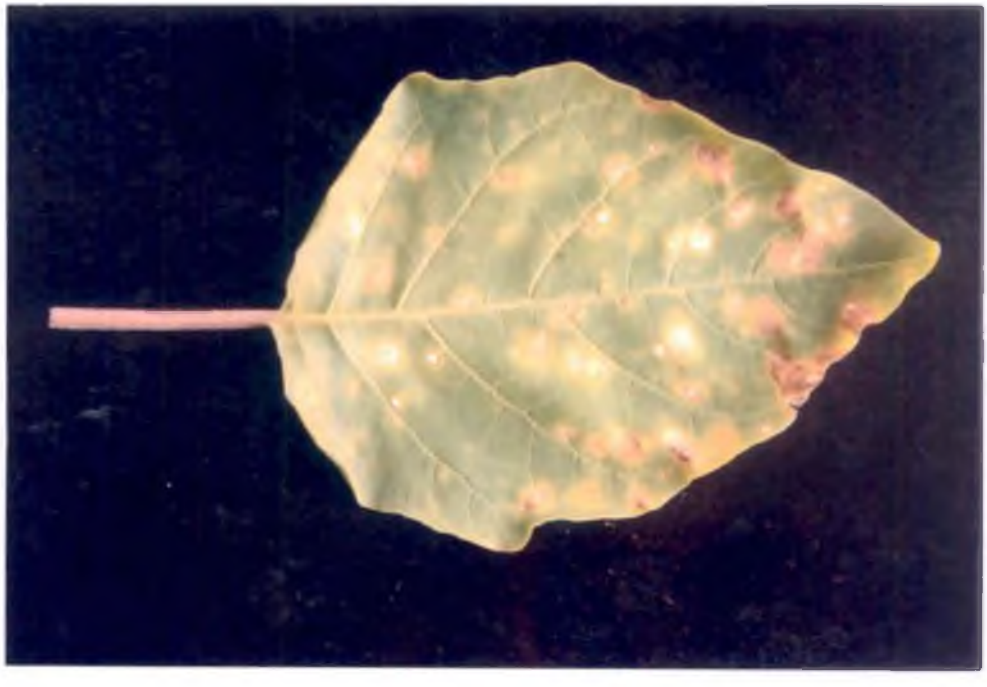


Plate 9. Necrotic spots in datura

Plate 10. Chlorotic spots on chenopodium

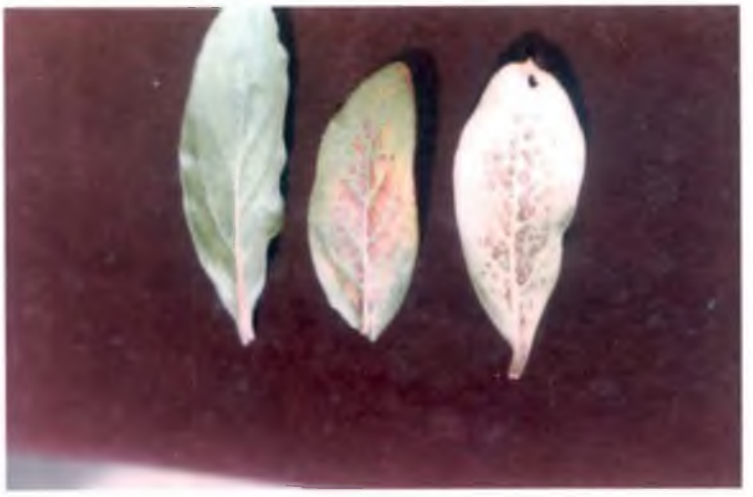


Plate 11. Purple spots on gomphrena

Plate 12. Bud necrosis in gomphrena

Plate 13. Discoloration along veins in petunia

Table 2 Effect of different buffers on infectivity of cowpea tospovirus isolate on *C. amaranticolor*

Sl. No.	Buffer	Molar Concentration	P^H	Mean No. of lesions
1	Citrate	0.1	6.2	56
2	Citrate Phosphate	0.1	7.0	10
3	Phosphate	0.01	7.0	38
4	Tris	0.1	7.2	12
5	Phosphate	0.01	7.2	65.5

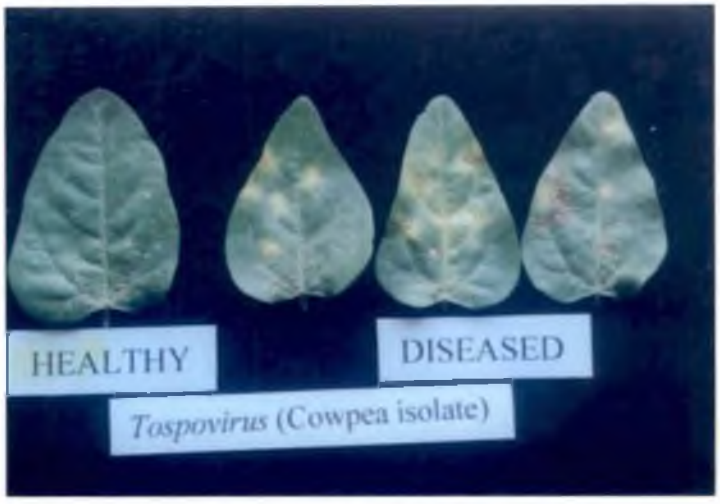


Plate 14. Local lesions in cowpea (*Vigna unguiculata*)

Plate 15. Local lesions in *Chenopodium amaranticolor*

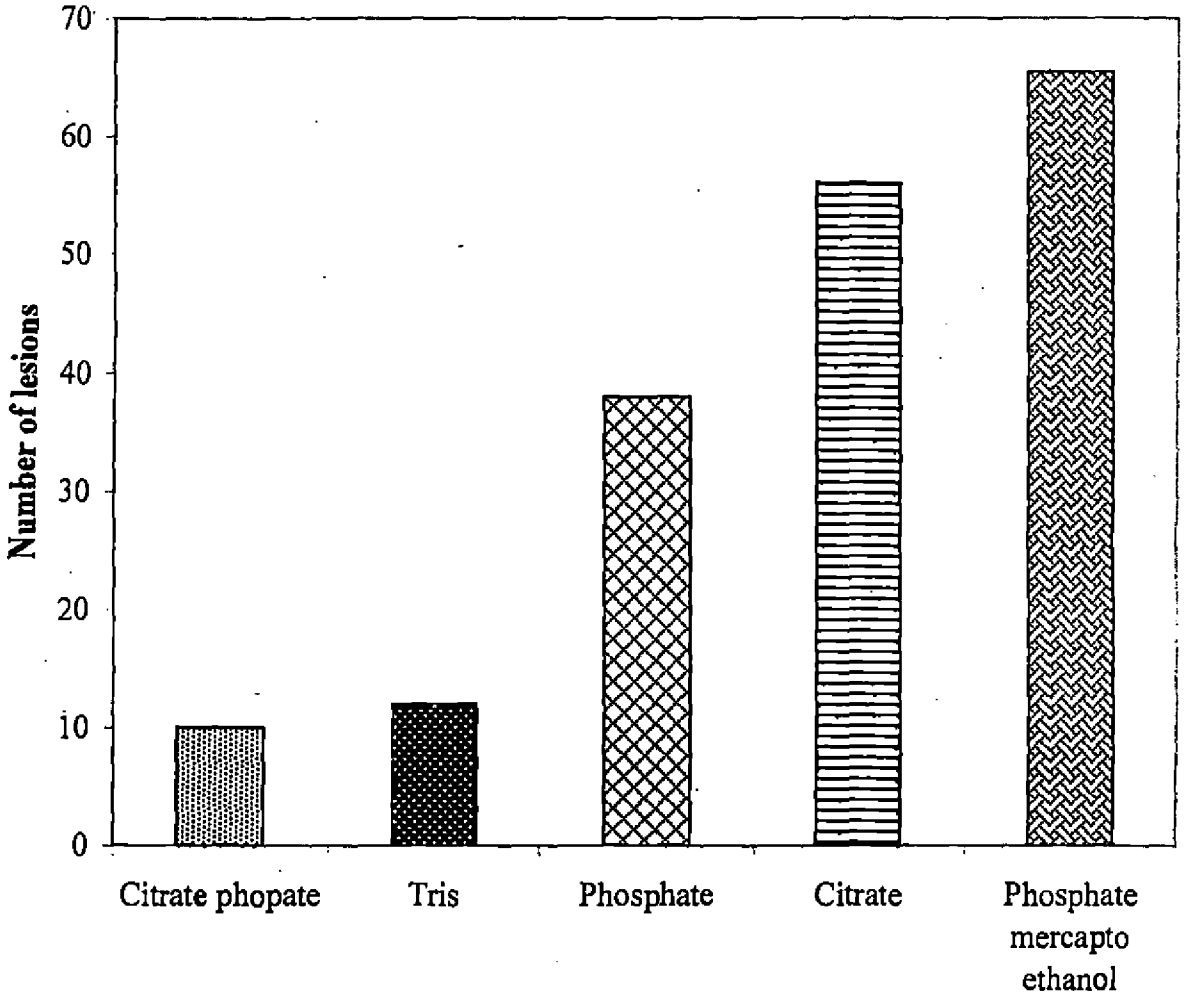


Fig. 1. Effect of different buffers on infectivity of tospovirus isolate from cowpea on *C. amaranticolor*

(Table 2, Fig.1). Extraction of sap for inoculation on cowpea seedlings was done using 0.01M phosphate buffer (pH 7.2). Symptom was observed as chlorotic spots five days after inoculation in the case of cowpea and was systemically transmitted (Plate 14). In *Chenopodium amaranticolor* symptom was observed as local lesions seven days after inoculation (Plate 15) with 87% transmission ,(Table 3).

4.3.2 Insect Transmission

Insect transmission studies were carried out using *Aphis craccivora* and *Thrips palmi* to find out whether tospo virus was transmitted by the insects. The results indicated that the virus was readily transmitted by both aphids and thrips. Even though thrips were reported as the major vector of this virus, the observations in the present study showed that *A. craccivora* was more efficient than *Thrips palmi* in transmission (Table 4).

4.3.3 Seed Transmission

Symptoms were not observed on seedlings obtained from seeds of infected cowpea plants. The per cent seed transmission was found to be nil.

4.3.4 Graft Transmission

Graft transmission studies were carried out in cowpea cv. Pusa Komal (Plate 16). Infected cowpea shoots showing typical symptoms were wedge grafted to 30 day old healthy plants, and the grafted plants were grown in insect proof glass house and observed for symptom development. Symptoms appeared after 6-10 days of grafting (Table 5).

4.4 PHYSICAL PROPERTIES

4.4.1 Dilution End Point (DEP)

Maximum number of lesions were obtained for the undiluted sap (22) and minimum for 10^{-2} dilution. The data indicated that the dilution end point of the virus was between 10^{-2} and 10^{-3} (Table 6, Fig.2).

Table 3 Mechanical transmission of tospovirus isolate in cowpea

No. of plants Inoculated	No. of plants established	No. of plants infected	Per cent transmission
25	23	20	87%

Table 4 Insect transmission of tospovirus isolate in cowpea

No. of plants Inoculated with aphids	No. of aphids released / plant	No. of plants infected	Per cent transmission
10	10	5	50%
No. of plants Inoculated with thrips	No. of Thrips released / plant	No. of plants infected	Percent transmission
10	10	3	30%

Table 5 Graft transmission of tospovirus isolate in cowpea

No. of plants grafted	No. of plants established	No. of plants infected	Per cent transmission
7	6	3	50%

Plate 16. Graft transmission

Table 6 Dilution end point (DEP) of tospovirus isolate from cowpea in *Chenopodium amaranticolor*

Sl. No.	Dilutions	Mean number of lesions
1	0	22
2	10^{-1}	14.5
3	10^{-2}	3.0
4	10^{-3}	0
5	10^{-4}	0
6	10^{-5}	0
7	10^{-6}	0

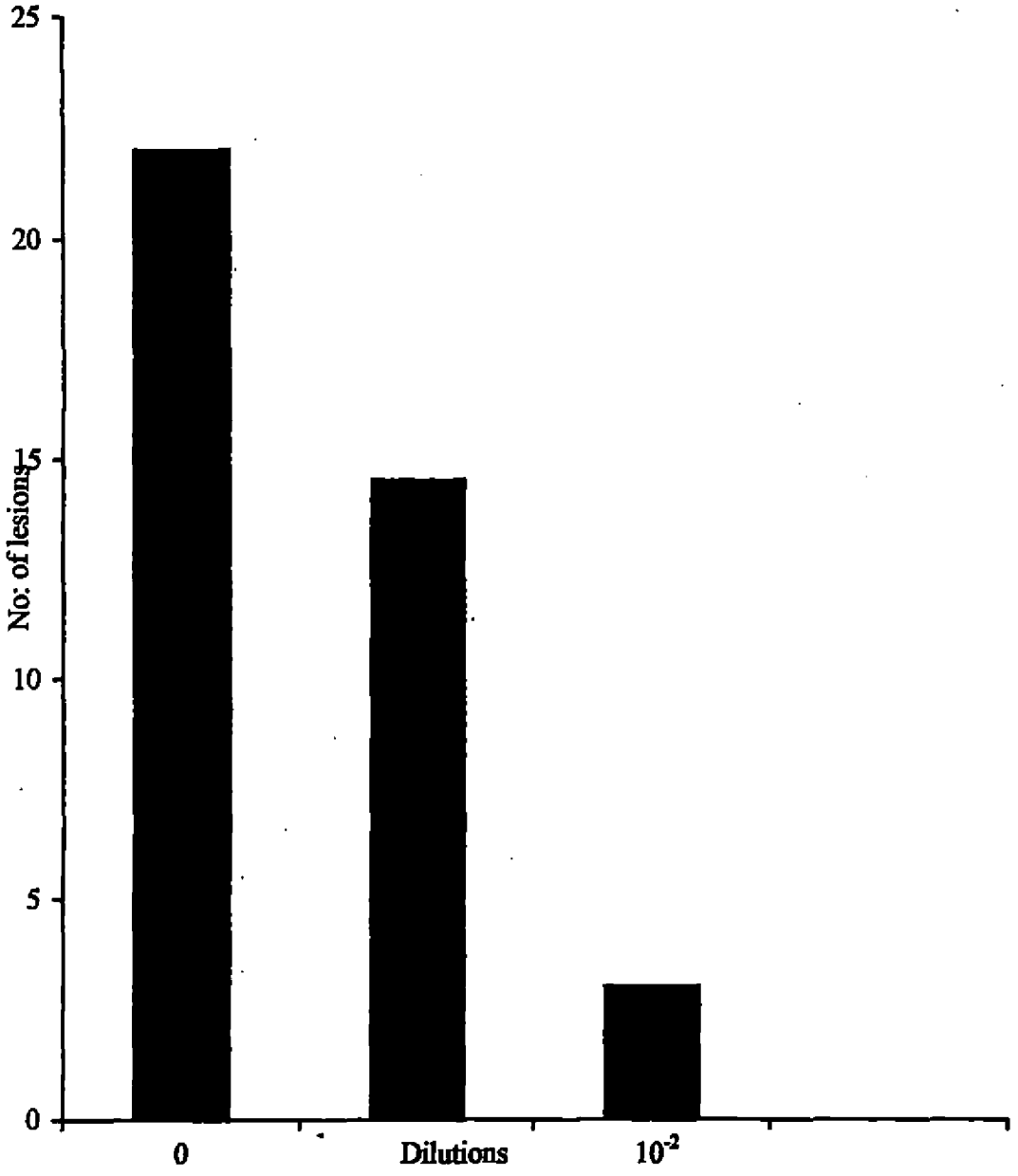


Fig. 2 Dilution end point (DEP) of tospovirus isolate from cowpea on *C. amaranticolor*

4.4.2 Thermal Inactivation Point (TIP)

The results indicated that the virus was inactivated at a temperature range between 50-55°C(Table 7, Fig.3)

4.4.3 Longevity *in vitro* (LIV)

The results indicated that the virus was infective for 6h at refrigerated condition and up to 8h at room temperature (Table 8, 9 Fig.4).

4.5 IMMUNODETECTION

4.5.1 Enzyme Linked Immunosorbent Assay of the Virus

DAC ELISA

Polycolonal antibodies for tospovirus and watermelon silver mottle virus were used as detecting antibodies. The results of the experiment revealed that both the antibodies gave high reactivity towards the virus isolate. The absorbances of the diseased sample against both the antiserum were more than three times than that of healthy sample. The healthy sample recorded an average absorbance of 0.5 at 405 nm. The average absorbance of diseased samples against both the antibodies was 2.0 at 405 nm. The reactivity of the antibody for WSMV towards the virus revealed the association of WSMV tospo virus with necrosis disease of cowpea (Table, 10 Plate, 17).

4.5.2 Dot Immunobinding Assay (DIBA)

DIBA was also conducted to detect the virus causing necrosis in cowpea. DIBA was done using polyclonal antibody for WSMV. Antiserum for WSMV was used for the detection of cowpea tospo isolate since WSMV is a closely

/as assessed by

Coloured spots

ate 18).

Table 7 Thermal Inactivation Point (TIP) of tospovirus isolate from cowpea in *Chenopodium amaranticolor*

Sl. No.	Temperature (°C)	Mean number of lesions
1	Control	30
2	35	20
3	40	17
4	45	10
5	50	3
6	55	0
7	60	0

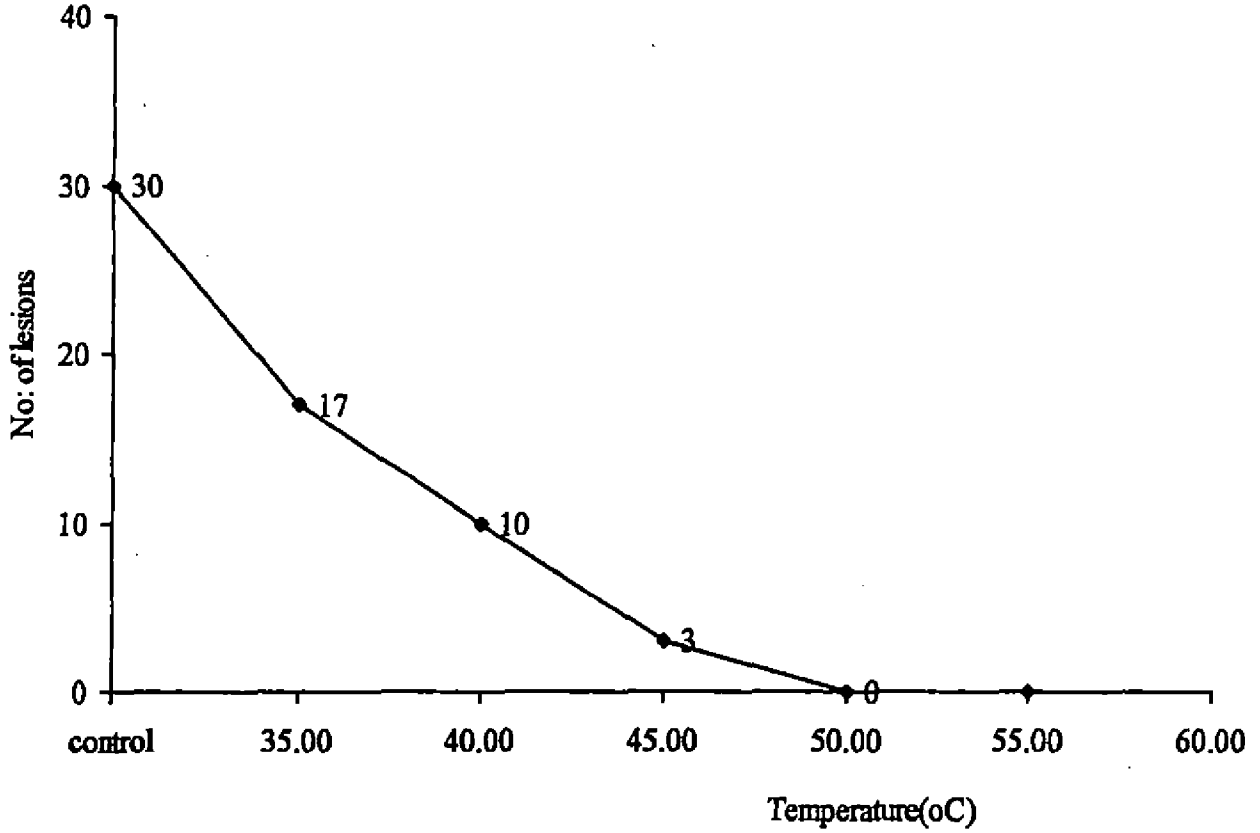


Fig.3 Thermal inactivation point (TIP) of tospovirus isolate from cowpea on *C. amaranticolor*

Table 8 Longevity *in vitro* (LIV) of tospovirus isolate from cowpea in *Chenopodium amaranticolor* at room temperature (28±2°C)

Sl. No.	Aging in hours	Mean number of lesions
1	0	30
2	2	10
3	4	10
4	6	3
5	8	0
6	24	0

Table 9 Longevity *in vitro* (LIV) of tospovirus isolate from cowpea in *Chenopodium amaranticolor* under refrigerated condition (8°C)

Sl. No.	Aging in hours	Mean number of lesions
1	0	30
2	2	14
3	4	12
4	6	11
5	8	5
6	24	0

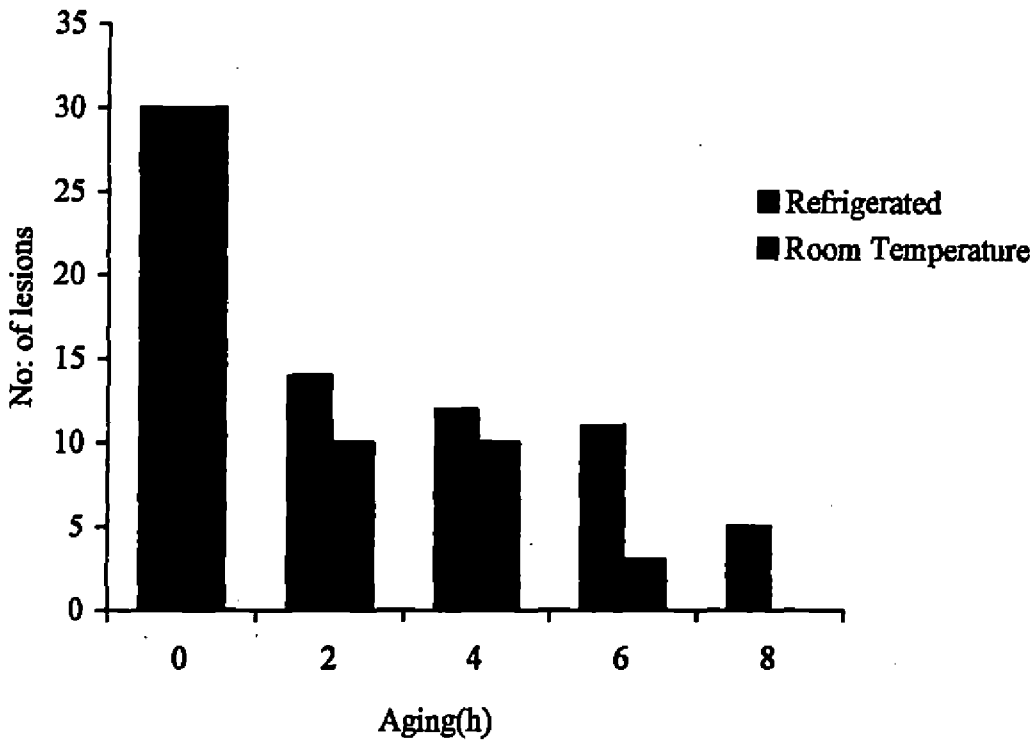
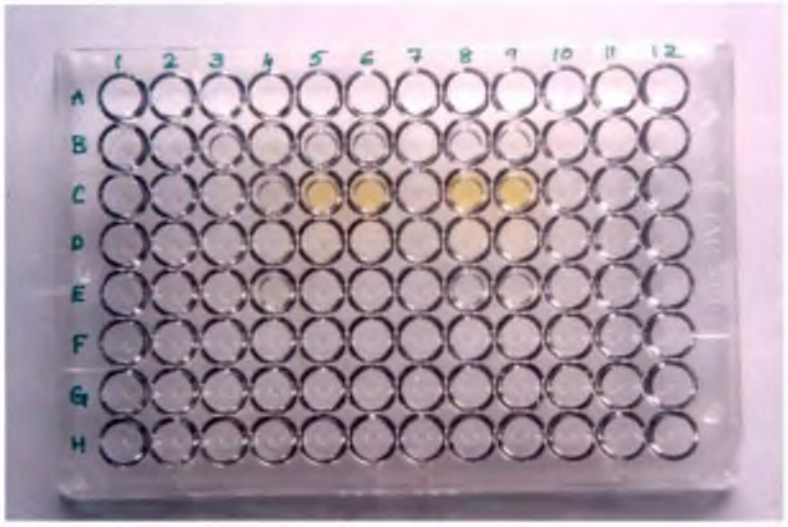


Fig: 4 Longevity *in vitro* (LIV) of tospovirus isolate from cowpea in *C. amaranticolor* at room temperature (28±2°C) and under refrigerated condition (8°C)

Table 10 Reaction of tospovirus isolate from cowpea using direct antigen coating ELISA

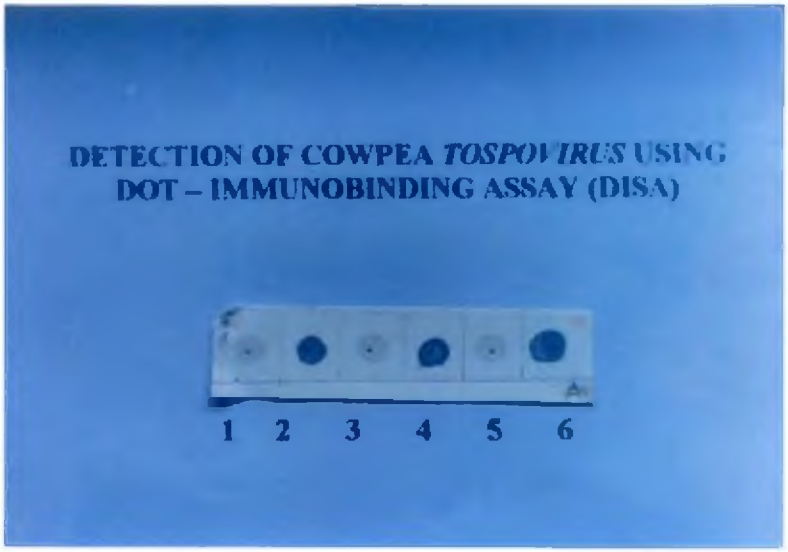
Samples	Polyclonal antibody tested	Absorbance at 405nm
Healthy Sample 1	Tospo	0.065
Healthy Sample 2	WSMV	0.034
Diseased Sample 1	Tospo	2.0
Diseased Sample 2	WSMV	2.0



Diseased sample – C
C6, C8 and C9

Healthy sample – B5
B6, B8 and B9

Plate 17. Reaction of tospovirus isolate from cowpea using DAC - ELISA



Diseased sample – 2,
4, and 6

Healthy sample – 1,
and 5

Plate 18. Detection of cowpea tospovirus using DIBA

4.6 BIOCHEMICAL CHANGES OF HOST PATHOGEN INTERACTION

4.6.1 Estimation of Total Carbohydrate

The result indicated that there was a decrease in carbohydrate level in inoculated plants of cowpea cv-Pusa Komal (Table 11,Fig.5). In the case of uninoculated control a value of 38.6 mg g^{-1} was recorded at one day after inoculation (DAI). There after the level increased to 41 mg g^{-1}at fifteen days after inoculation (DAI). The value decreased from 39 mg g^{-1} at five days after inoculation to 37.5 mg g^{-1} at ten days after inoculation in inoculated plants. After that the carbohydrate level increased to 39mg g^{-1} at fifteen days after inoculation in the tospo virus infected plants.

4.6.2 Estimation of Chlorophyll

The samples were estimated for chlorophyll a, chlorophyll b and total chlorophyll at different days after inoculation. Chlorophyll was seen increasing gradually in both inoculated and uninoculated plants (Table 12). The chlorophyll content was less in inoculated plants compared to healthy control. Chlorophyll a for healthy plants increased from 0.73 mg g^{-1} at one DAI to 1.26mg g^{-1} at 15 DAI, where as in inoculated plants it increased from 0.53 mg g^{-1} at one DAI to 1.09 mg g^{-1} at 15 DAI. Chlorophyll b increased from 0.06 mg g^{-1} at one DAI to 0.38 mg g^{-1} at 15 DAI in healthy and in diseased the content decreased from 0.33 mg g^{-1} to 0.32 mg g^{-1}. Total chlorophyll for healthy control was 1.64 mg g^{-1} at 15 DAI and it was only 1.42 mg g^{-1} in inoculated plants.

4.6.3 Estimation of Protein

. The result indicated that inoculation of cowpea plants with tospovirus caused a significant difference in total soluble protein content. The total soluble protein content was found higher in case of inoculated plants compared to uninoculated control (Table 13, Fig.7). In the uninoculated control plants the protein content recorded was 646 µg g^{-1} at one DAI where as in inoculated leaves it was 1121 µg g^{-1}. The protein content was found maximum at 10DAI (765 µg g^{-1}) in uninoculated control plants. Where as in inoculated plants the value found to

Table 11 Changes in total carbohydrate content of cowpea leaves in response to tospovirus inoculation

Days after inoculation	Change in carbohydrate content mg g^{-1} fresh weight of tissue	
	Healthy	Diseased
1	38.6	36.8
5	36	39
10	37.6	37.5
15	41.0	39.0

CD- Not Significant

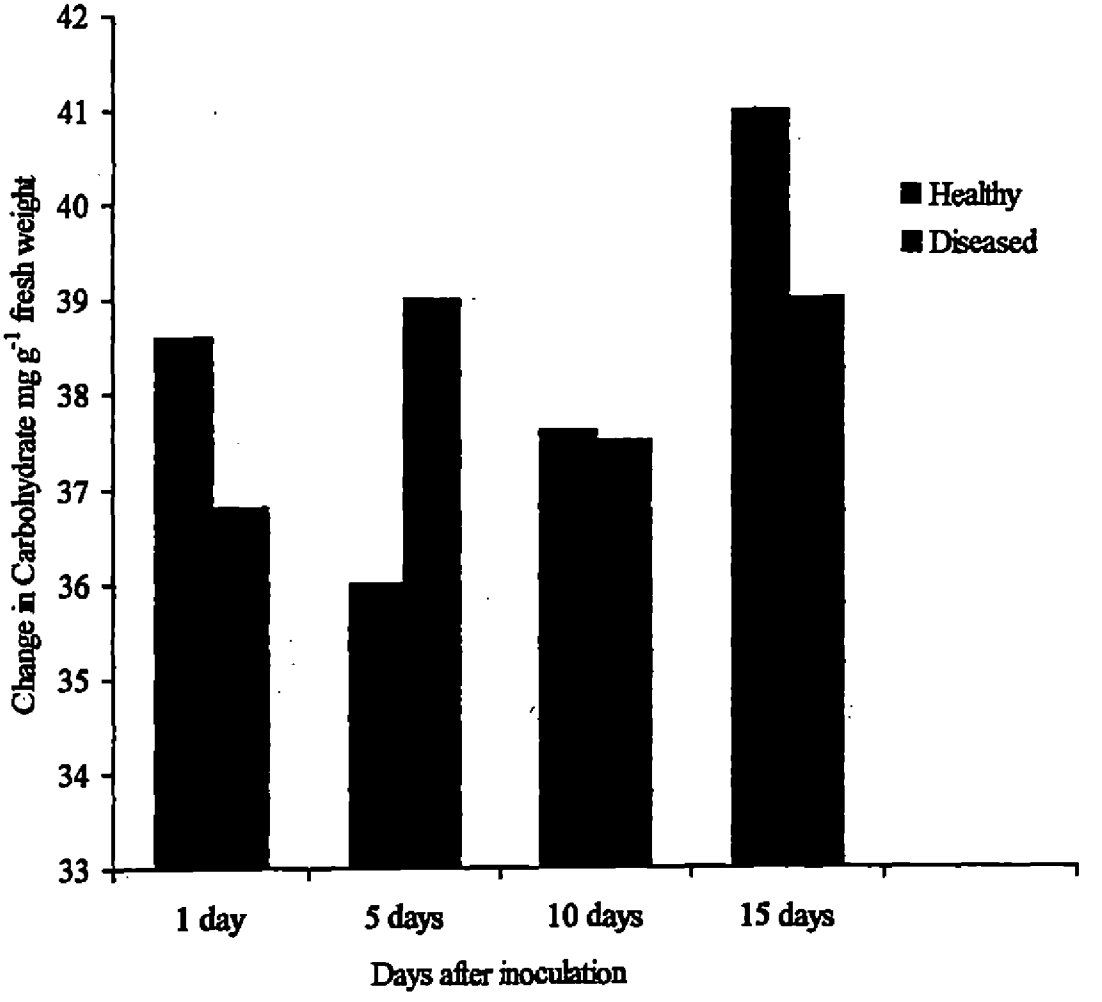


Fig.5 Changes in total carbohydrate content of cowpea leaves in response to tospovirus inoculation

Table 12. Changes in Chlorophyll content of cowpea leaves in response to tospovirus inoculation

Days after inoculation	Changes in Chlorophyll content (mg g^{-1} fresh weight)					
	Healthy			Inoculated		
	a	b	Total	a	b	Total
1	0.73	0.06	0.84	0.53	0.33	0.83
5	0.66	0.18	0.85	0.73	0.08	0.75
10	0.85	0.24	1.09	0.95	0.25	1.20
15	1.26	0.38	1.64	1.09	0.32	1.42

CD- values

Chlorophyll- a	Chlorophyll- b	Total Chlorophyll
A - 3.27	A - NS	A - NS
B - 3.13	B - 5.65	B - 4.08
AB - 4.44	AB - 7.99	AB - 5.78

*Healthy X Inoculated- A, Days- B, Healthy X Inoculated X Days- AB, NS- Non Significant.

Table 13 Changes in Total soluble protein content of cowpea leaves in response to tospovirus inoculation

Days after inoculation	Change in soluble protein content (µg g^{-1} fresh weight of tissue)	
	Healthy	Inoculated
1	646	1121
5	490	583
10	765	993
15	513	1110

CD- values: A – 191.2 B -147.1 AB - 208

*Healthy X Inoculated- A, Days- B, Healthy X Inoculated X Days- AB

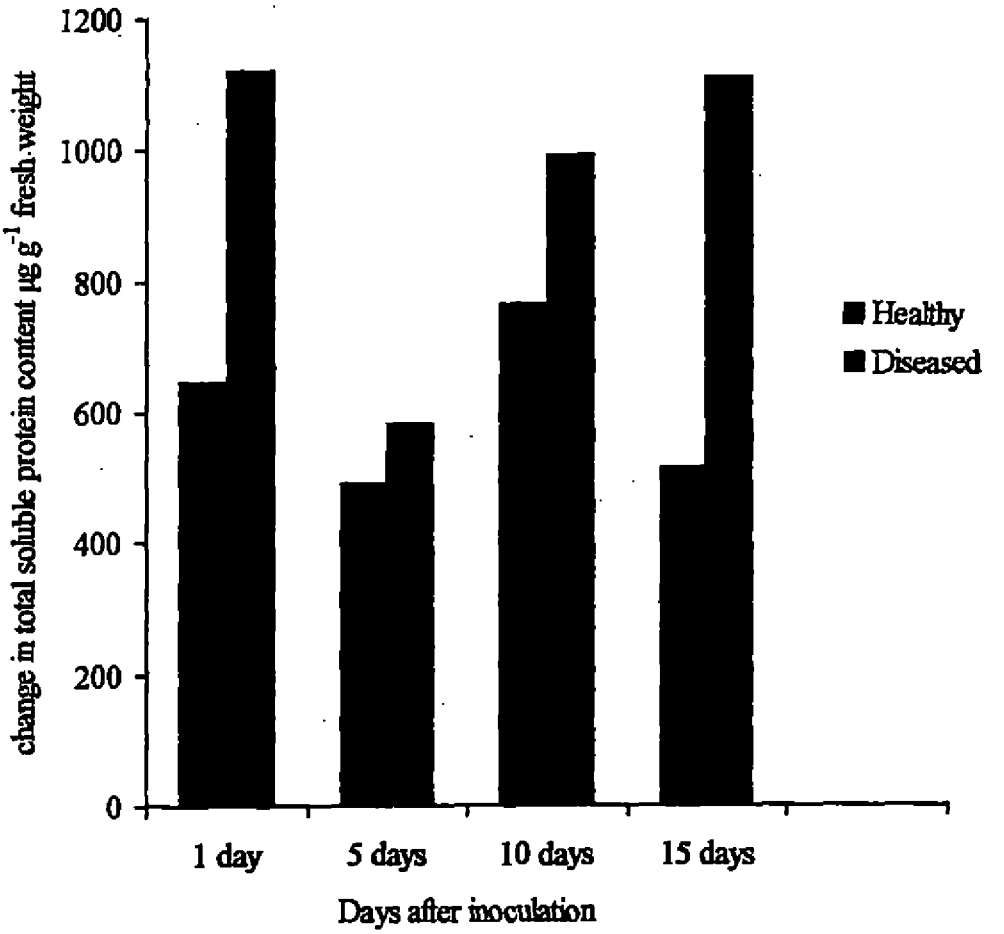


Fig. 6 Changes in total soluble protein content of cowpea leaves in response to tospovirus inoculation

be 1121 μg g^{-1} and 1110 μg g^{1} at one day and 15 DAI respectively which was found to be on par.

4.6.4 Estimation of Phenol

The results indicated significant difference in phenol contents between inoculated and uninoculated plants of susceptible cowpea cv-Pusa Komal (Table 14, Fig.6). The phenol content was found to be more in inoculated plants. Inoculation of tospo virus increased the total phenolics of inoculated plants from 36.8 μg g^{-1} at one DAI to 81.5 μg g^{-1} at 10 DAI. There after it decreased to 23.8 μg g^{-1} at 15 DAI. In uninoculated plants the value was maximum at 5 DAI (56μg g^{-1}). Thereafter it declined to 21.6μg g^{-1} at 15 DAI.

4.6.5 Defence Related Enzymes

4.6.5.1 Peroxidase

There was a progressive decrease in peroxidase activity with age of the plant, in both inoculated and uninoculated plants. Peroxidase activity was observed to be maximum at one DAI in both healthy control (2.0 min^{-1} g^{-1}) and inoculated plants (5.3 min^{-1} g^{-1}) (Table 15, Fig.8). The activity was found higher in inoculated than uninoculated leaves of cowpea plant. The value decreased from 5.3 min^{-1} g^{-1} at one DAI to 0.2 min^{-1} g^{-1} at 15 DAI in inoculated plants and in uninoculated plants the peroxidase activity decreased from 2.0 min^{-1} g^{-1} at one DAI to 0.5 min^{-1} g^{-1} at 15 DAI .

4.6.5.2 Polyphenol Oxidase

The results showed that there was significant difference between inoculated and uninoculated plants and the activity was found higher in the inoculated plants(Table 16,Fig .9). Enzyme activity recorded a maximum value of 0.4 min^{-1} g^{-1} and 0.5min^{-1} g^{-1} at 5 DAI in uninoculated and inoculated plants respectively. The value decreased from 0.5 min^{-1} g^{-1}at one DAI to 0.25 min^{-1} g^{-1} at 15 DAI in inoculated plants and in uninoculated plants the enzyme activity decreased from 0.14 min^{-1} g^{-1} to 0.02 min^{-1} g^{-1}.

Table 14 Changes in Phenol content of cowpea leaves in response to tospovirus inoculation

Days after inoculation	Change in Phenol content (µg g^{-1} fresh weight of tissue)	
	Healthy	Inoculated
1	20.80	36.6
5	55.00	34.0
10	37.20	81.5
15	21.60	23.8

CD- values A – 6.92 B -1.41 AB - 16.14

*Healthy X Inoculated- A, Days- B, Healthy X Inoculated X Days- AB

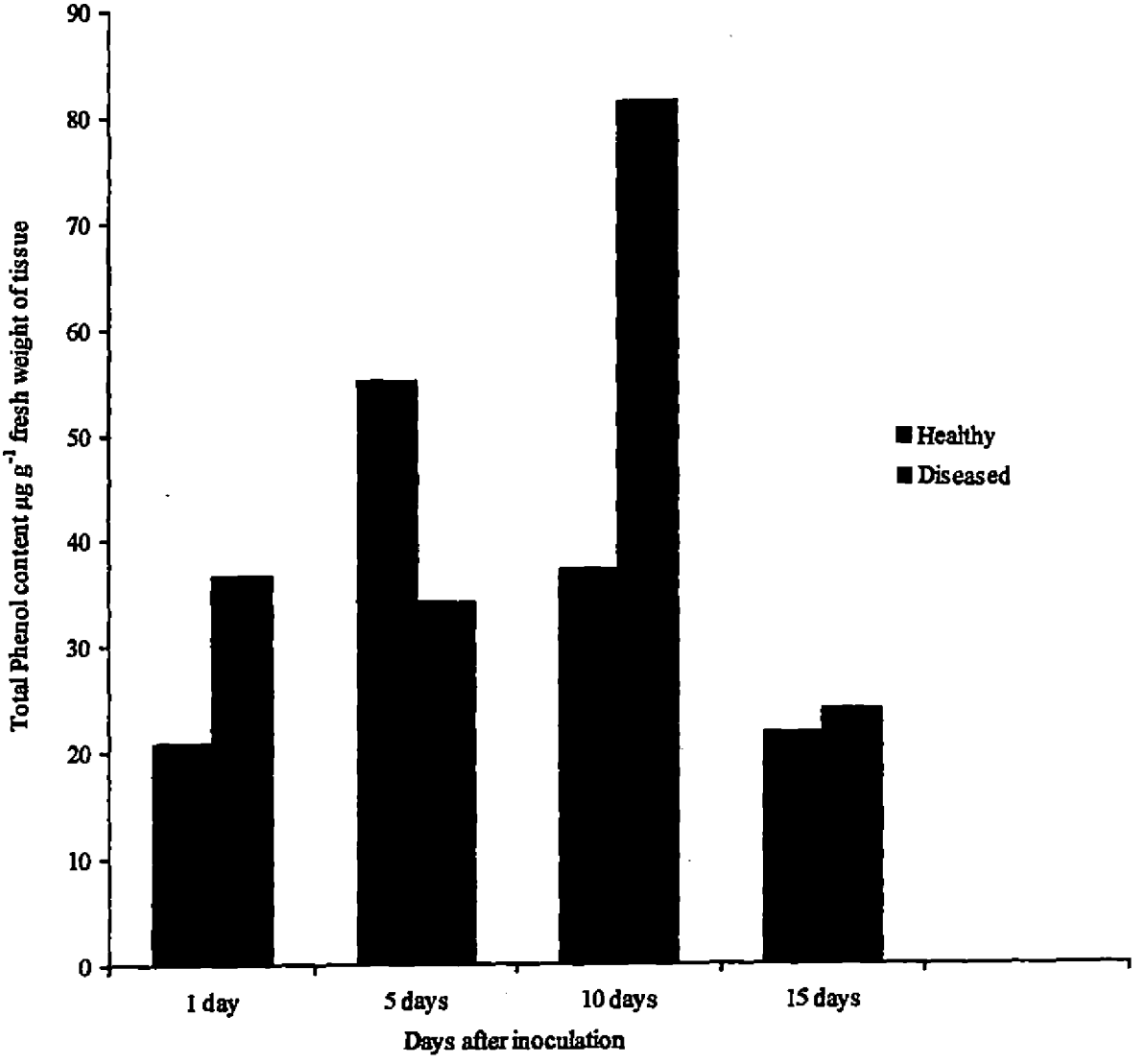


Fig. 7 Changes in total Phenol content of cowpea leaves in response to tospovirus inoculation

Table 15 Changes in peroxidase activity of cowpea leaves in response to tospovirus inoculation

Days after inoculation	Peroxidase activity(changes in absorbance $min^{-1}g^{-1}$ fresh weight)	
	Healthy	Inoculated
1	2.0	5.3
5	1.0	1.5
10	0.4	0.9
15	0.5	0.2

Table 16 Changes in Polyphenoloxidase activity of cowpea leaves in response to tospovirus inoculation

Days after inoculation	Polyphenol oxidase activity(changes in absorbance $min^{-1}g^{-1}$ fresh weight)	
	Healthy	Inoculated
1	0.14	0.5
5	0.4	0.5
10	0.04	0.4
15	0.02	0.25

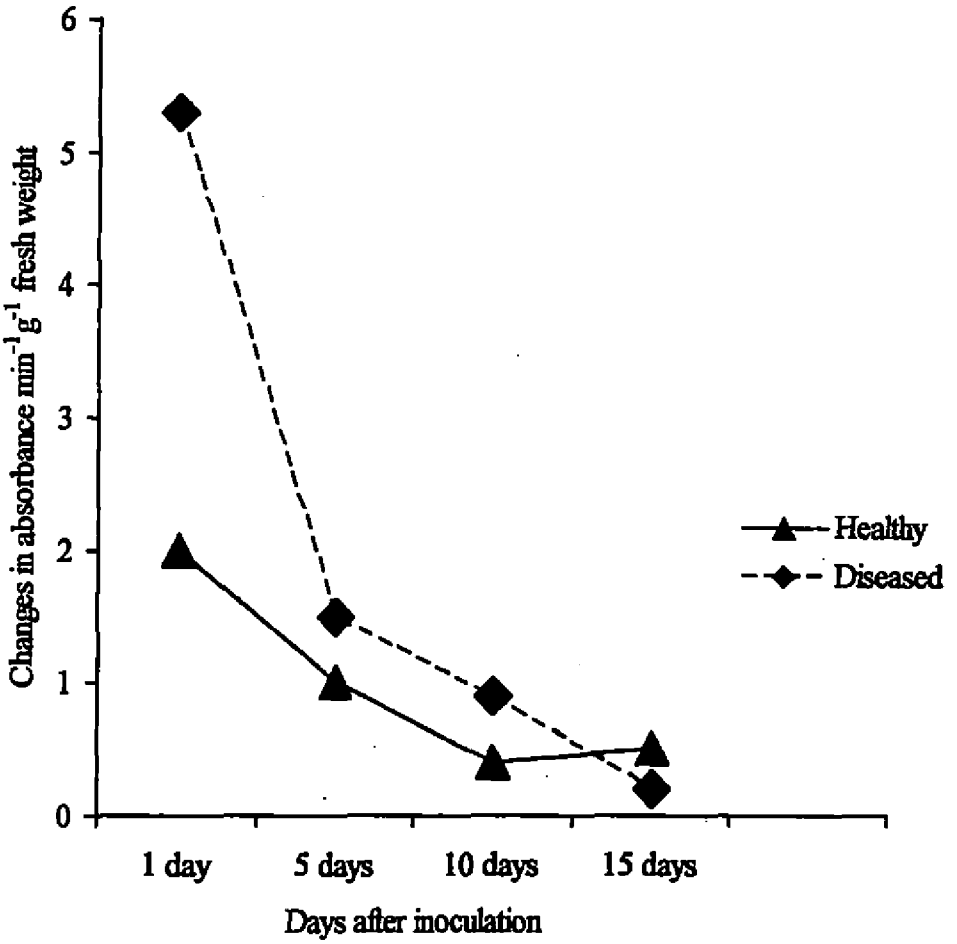


Fig.8 Changes in peroxidase activity in cowpea plants in response to tospovirus inoculation

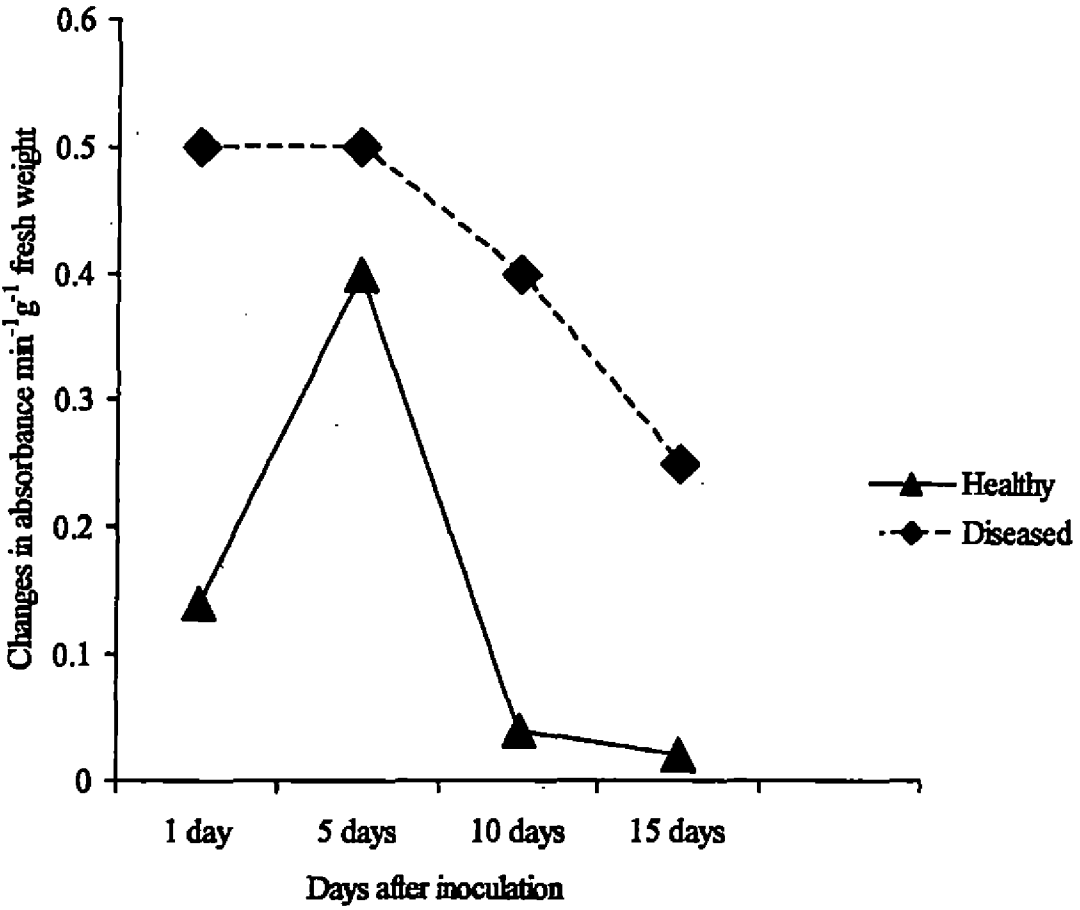


Fig.9 Changes in polyphenol oxidase activity in cowpea plants in response to tospovirus inoculation

4.6.5.3 Phenyl Alanine Ammonia-lyase

The results showed an increase in enzyme activity initially and decreased later in inoculated plants (Table 17,Fig .10) . Maximum value of 56.4 $\mu g\ g^{-1}\ min^{-1}$ was attained at 5 DAI in inoculated plants. Thereafter the value decreased to 41.5 $\mu g\ g^{-1}\ min^{-1}$ at 15 DAI. In the case of healthy control maximum value (56.4 $\mu g\ g^{-1}\ min^{-1}$) was recorded at 15 DAI. In the healthy control the value increased from 47.3 $\mu g\ g^{-1}\ min^{-1}$ at one DAI to 52.29 $\mu g\ g^{-1}\ min^{-1}$ at 5 DAI and then decreased to 49.8$\mu g\ g^{-1}\ min^{-1}$ at 10 DAI. Thereafter increased to 56.4$\mu g\ g^{-1}\ min^{-1}$ at 15 DAI.

4.7 ELECTROPHORETIC SEPARATION OF SOLUBLE PROTEINS

Protein profiles of uninoculated healthy cowpea plant produced 4 proteins and the inoculated or virus affected cowpea plants produced 7 proteins. Out of 7 proteins 4 proteins were identical to that of healthy and 3 extra bands were obtained. Molecular weight of 3 extra bands of inoculated leaves were estimated using the molecular markers loaded along with the samples. The 3 extra novel proteins were with molecular weight 28, 15 and 6.2 kDa respectively. The extra proteins found may be induced due to virus infection (Fig.11, Plate 19).

4.8 ELECTROPHORETIC ANALYSIS OF ISOZYMES

Native polyacrylamide gel electrophoresis was carried out for isozyme analysis of peroxidase and polyphenol oxidase.The experiment was performed to find out the presence of these isozymes and their intensity in both inoculated and uninoculated cowpea plants. Isozyme analysis of polyphenol oxidase (PPO) produced 4 isoforms of PPO in both healthy and virus affected plants. Both healthy and affected plants showed same banding pattern. These isoforms are with relative mobility (Rm) values, 0.03, 0.37, 0.54 and 0.62. Isoforms with Rm value 0.54 (IsoPPO2) was expressed more prominently in the inoculated plants compared to uninoculated plants (Table 18, Fig.12, Plate 20).

Isozyme analysis of Peroxidase produced only one isoform(IsoPO1) in virus infected plants. No isoform was expressed on healthy cowpea plants (Table 19, Fig. 12, Plate 21).

Table 17 Changes in Phenylalanine ammonia- lyase activity of cowpea leaves in response to tospovirus inoculation

Days after inoculation	Phenylalanine ammonia-lyase activity(changes in absorbance $\mu g\ g^{-1}min^{-1}$ fresh weight)	
	Healthy	Diseased
1	47.30	48.09
5	52.29	56.40
10	49.80	53.53
15	56.44	41.50

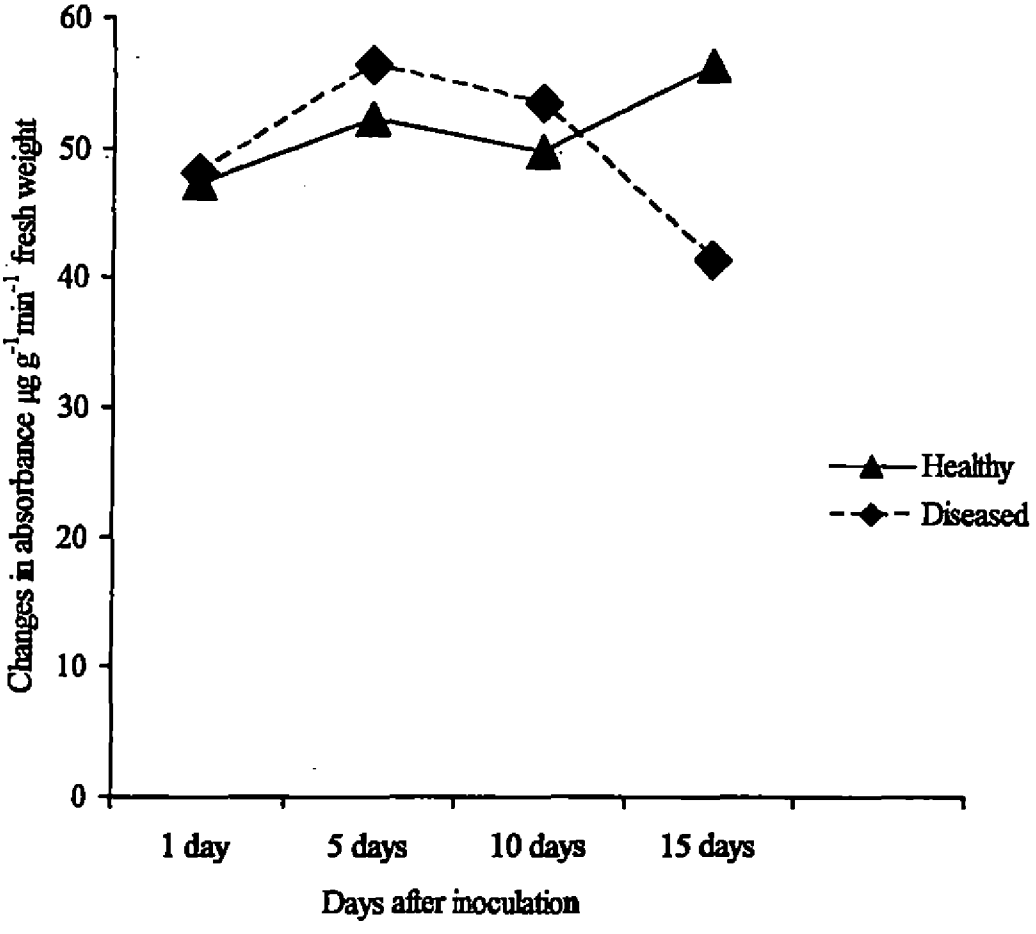


Fig.10 Changes in phenylalanine ammonia-lyase activity in cowpea plants in response to tospovirus inoculation

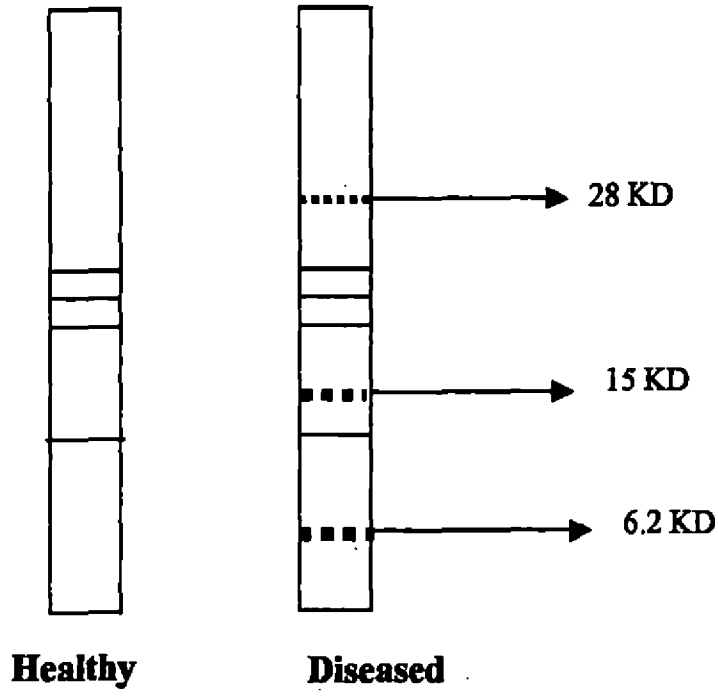


Fig. 11 Protein profile studies of healthy and diseased cowpea plants

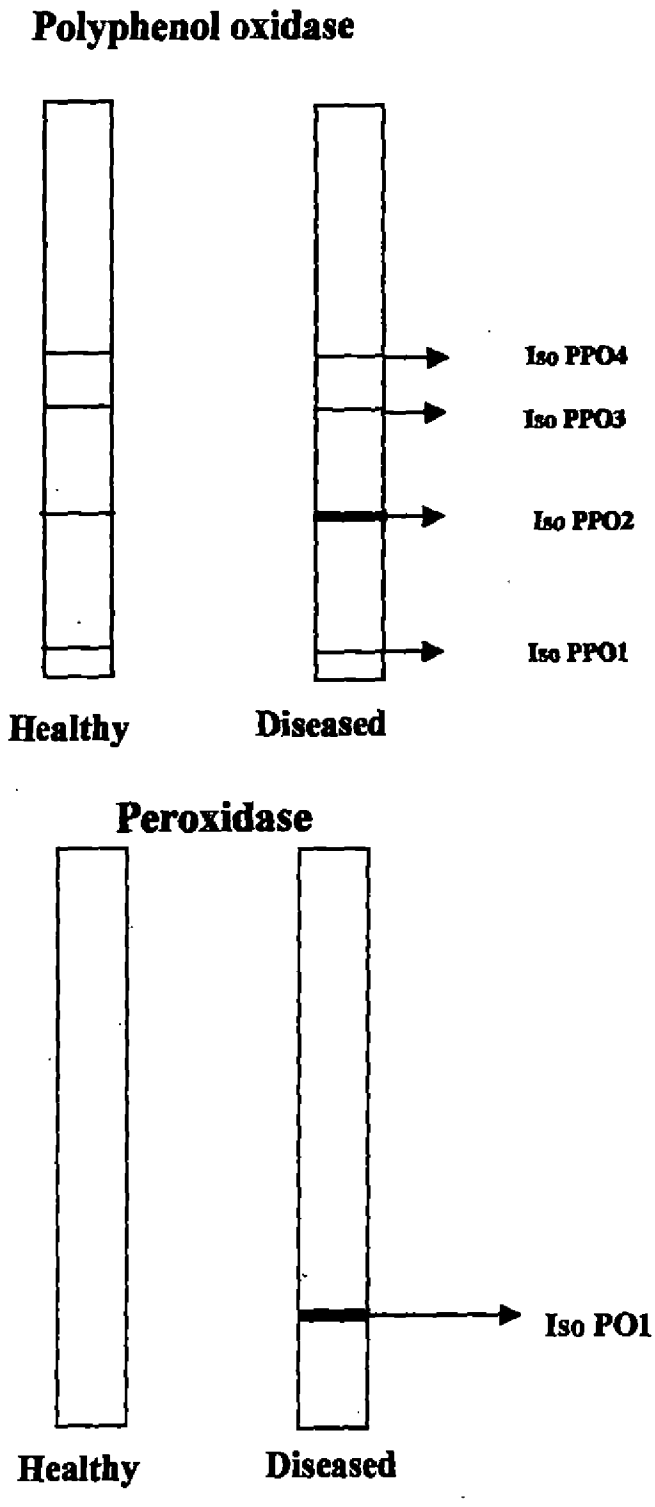
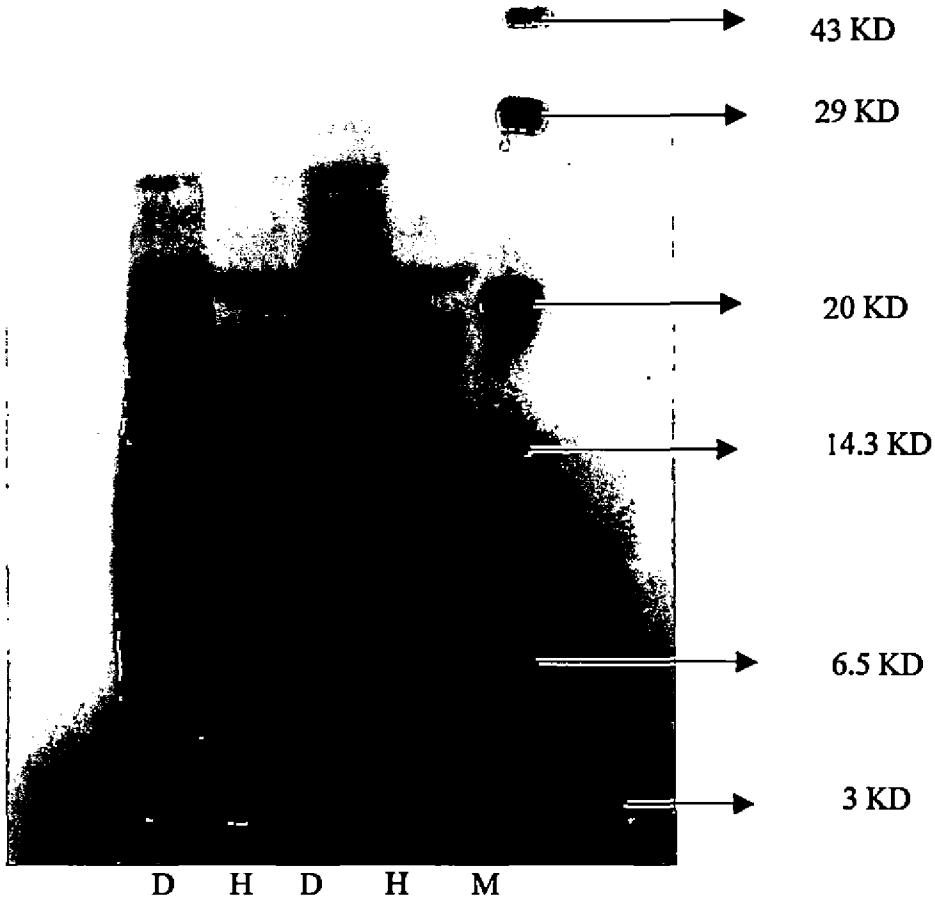


Fig. 12 Zymogram of healthy and diseased cowpea plants



D – Diseased, H – Healthy, M – Molecular marker

Plate 19. Protein profile of healthy and diseased cowpea plants

ISOZYME ANALYSIS

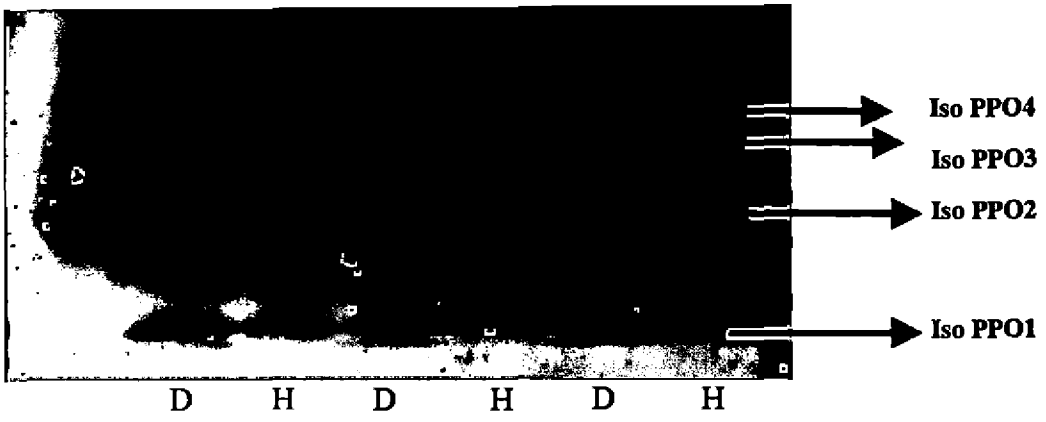


Plate 20. Activity of poly phenol oxidase enzyme

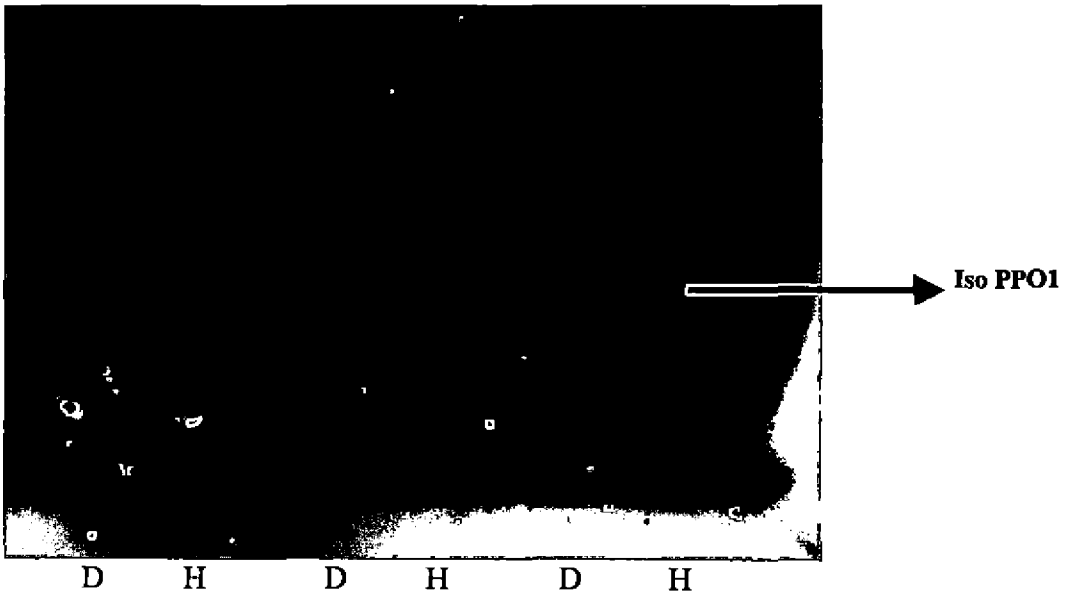


Plate 21. Activity of peroxidase enzyme

D – Diseased
H - Healthy

Table 18 Rm value of Polyphenol oxidase

Iso PPOs	Healthy control	Inoculated
Iso PPO1	0.03	0.03
Iso PPO2	0.37	0.37
Iso PPO3	0.54	0.54
Iso PPO4	0.62	0.62

Table 19 Rm value of peroxidase

Iso POs	Healthy control	Inoculated
Iso PO1	-	0.65

Discussion

5. DISCUSSION

Cowpea [*Vigna unguiculata* (L.) Walp.] is an important vegetable and grain crop cultivated in Kerala. Diseases and pests are major constraints in increasing the production of the crop. Among the pathogens, viruses infect cowpea at all stages of growth of the plant and are a major threat to its cultivation. Tospoviruses belonging to the family Bunyaviridae are causing substantial losses world wide to many crops. Tospovirus infecting cowpea are reported to be causing severe damage in many farmers fields. The study was undertaken to identify and characterize the virus and to obtain more information on the etiology and management of the disease.

The characteristic symptoms of the virus disease under study were chlorotic spots on newly emerging trifoliate leaves, necrosis of the growing point of the plant leading to severe stunting and profuse growth of short axillary shoots giving a bushy appearance. Severe infections led to death of the plant. Initial symptoms appeared as chlorotic spots which later became necrotic. Veinal necrosis and bud necrosis were also observed on the infected plant. These symptoms were found to be similar to those caused by tospovirus reported earlier. Francki *et al.* (1981) reported that tospoviruses cause necrosis on several plants, chlorosis, ring patterns, mottling, silvering, stunting and local lesions .The symptoms vary depending on the virus isolate, host plant, time of year and environment. Bhat *et al.* (2001) reported necrosis of the growing point, severe stunting, profuse growth of short axillary shoots on field infected cowpea plants. Natural infections of tospovirus characterized by stem, leaf and bud necrosis on cowpea, mung bean and tomato was reported by Jain *et al.* (2002). Localized as well as systemic infections characterized by chlorotic or necrotic lesions, veinal and systemic necrosis in tospo inoculated cowpea plants cv Pusa Komal were reported by Umamaheswaran *et al.* (2003). Thus, the comparison of symptoms produced by the virus under study with those reported revealed that all these viruses are same or related.

Host range studies of the virus with 24 plant species belonging to seven families indicated that the virus under study produced symptoms on 12 species belonging to 5 families *viz;* Chenopodiaceae, Amaranthaceae, Leguminosae, Solanaceae and Malvaceae. It was found that tospovirus causing necrosis disease produced systemic symptoms on *Gomphrena globosa, Vigna unguiculata, Arachis hypogaea, Cajanus cajan. Glycine max,Vigna radiata, Lypcopersicon esculentum,Datura stramonium and local lesions on Chenopodium amaranticolor Chenopodium quinoa & Chenopodium album.* None of the cucurbitaceous species tested were infected . Ghanekar *et al.* (1979) reported similar symptoms on ground nut infected by tomato spotted wilt virus (TSWV) and the study showed that the virus induced chlorotic or necrotic local lesions in *Cajanus cajan, Chenopodium amaranticolor, Chenopodium quinoa, Gomphrena globosa, Nicotiana rustica, Nichotiana tabacum, Petunia hybrida* and chlorotic and necrotic spots followed by systemic infection in *Datura stramonium, Glycine max, Lycopersicon esculentum, Vigna radiata, Vigna mungo, Vigna unguiculata* cv C-152 and *Vinca rosea.* Moyer (1999) reported that tospoviruses belonging to family Bunyaviridae caused substantial losses world wide to crops such as groundnut, potato, tobacco,vegetables and ornamentals. Bhat *et al.* (2001) identified tospovirus isolates associated with black gram, green gram, cowpea and soybean based on bioassay, serology and nucleic acid hybridization. Umamaheshwaran *et al.* (2002) ascertained the experimental host range of tomato isolate of tospovirus. The virus could infect 15 out of 20 different hosts tested belonging to four families but none of the cucurbitaceous species tested were infected. The virus caused only chlorotic and necrotic lesions on plant species belonging to Amaranthaceae and Chenopodiaceae families. Both localized as well as systemic infections were observed in plants of Fabaceae and Solanaceae . From the present investigation it was found that tospovirus causing necrosis disease of cowpea possessed wide host range. Disease incidence is initiated by the presence of initial inoculum at some source . So it is anticipated that all these hosts such as groundnut, tomato etc.

could act as alternate or collateral hosts of virus. This may lead to severe infection on cowpea plants if grown along with these crops.

The virus could be transmitted very efficiently by mechanical means. Different buffers such as 0.1M citrate buffer (pH 6.2) 0.1M citrate phosphate buffer (pH 7.0), 0.01M phosphate buffer (pH7.0),) 0.1M tris buffer (pH 7.2) and 0.01M Phosphate buffer (pH 7.2) containing 0.1 per cent 2-mercaptoethanol were used to compare the effectiveness on transmitting the virus. The virus was sap transmitted efficiently to cowpea cv.Pusa Komal as well as to the local lesion host, *Chenopodium amaranticolor* by these buffers out of which 0.01M phosphate buffer (PH7.2) containing 0.1 per cent mercaptoethanol produced maximum number of lesions (65.5) on *C. amaranticolor*. This buffer was most effective and was used for sap inoculation in cowpea plants. Chlorotic spots were produced on young trifoliate leaves five days after inoculation. Local lesions were observed seven days after of inoculation on *Chenopodium amaranticolor*. Several authors have reported the effective transfer of this virus using phosphate buffer containing 2-mercaptoethanol. Ghanekar *et al.* (1979) reported mechanical transmission of tomato spotted wilt virus using 0.05M potassium phosphate buffer containing 0.02M 2-mercaptoethanol. Bhat *et al.* (2002) reported the maintenance and propagation of tospovirus using 0.1M phosphate buffer (pH 7.2) containing 0.1 per cent 2-mercaptoethanol. Umamaheshwaran *et al.* (2003) also reported mechanical transmission using 0.01M Potassium phosphate buffer containing 0.1 per cent 2-mercapto ethanol. Phosphate buffer retained the infectivity of the virus for long periods compared to other buffers used for inoculation. Maximum level of mechanical transmission(87%) was obtained in the present study. Phosphate buffer containing 0.1 per cent 2 mercaptoethanol was found to be the most efficient buffer for transmission compared to other buffers tested. This is in agreement with the earlier findings.

Aphids are major pests of cowpea in Kerala. Insect transmission of tospovirus causing necrosis disease of cowpea was tried using *Aphis craccivora*

and *Thrips palmi* as these insects were reported to be the major insect vectors of tospoviruses. It was found that both aphids and thrips transmit the virus. In the present study aphids and thrips could transmit the virus at the rate of 50 per cent and 30 per cent respectively . Thrips transmission has been reported by many authors. Ghanekar *et al.* (1979) reported transmission of tomato spotted wilt virus (TSWV) by thrips (*Scirtothrips dorsalis*). Gofflot and Verhoyen (1990) reported that TSWV occurrence in Belgium was related to significant increase in *Frankliniella occidentalis* populations. Sakurai (2004) reported dark form of *Frankliniella schultzei* transmitting TSWV in Paraguayan tomato fields. No previous report of aphids transmission was recorded. In the present investigation *Aphis craccivora* was found to be the most efficient vector than thrips.

The virus under study was not transmitted through the seeds of cowpea. No symptom was noticed on seedlings raised from seeds on infected cowpea plants. The non transmission of virus through seeds was reported earlier. Ghanekr *et al.* (1979) reported that TSWV causing bud necrosis of ground nut was not seed borne. Mali *et al.* (1979) reported that tospovirus was not transmitted through seeds. Mumford (1995) suggests that seed transmission does not occur among tospoviruses. Thus finding of present investigation is in accordance with the previous reports.

The tospovirus isolate under investigation could be transmitted through graft. The virus was transmitted to cowpea plants by wedge and cleft grafting. Roy and Gupta (1977) also reported 100% graft transmission of TSWV in pea plants. The graft transmission of TSWV in groundnut plants was further proved by Ghanekar *et al.* (1979).

Physical properties are distinct to a particular virus. Thermal inactivation point (TIP), dilution end point (DEP) and longevity *in vitro* (LIV) were studied in the present investigation . The virus recorded a TIP of 50 – 55°C, DEP in the range of 10^{-2}-10^{-3} and LIV for 6 h at room temperature (28±2°C) and 8 h at refrigerated condition (8°C) (Table,8,9 and Fig.4) . The TIP and DEP were similar with those reported by Mali and Pahl (1980). Silveria Junior *et al.* (1985) reported

physical properties of virus isolated from *Sechium edule*. TIP was recorded as 45-50°C, DEP as 10^{-3} to 10^{-4} and LIV at room temperature was less than 2 h. TIP and DEP are in line with the result obtained in the present study but difference was observed in LIV.

ELISA is one of the quickest techniques to detect and characterize a virus. With a view to identify the virus causing necrosis disease in cowpea, ELISA test was conducted using polyclonal antibodies of TSWV and WSMV received from Dr. Jain, R.K. , Principle Scientist, Advanced Centre for Plant Virology, IARI, New Delhi. The result indicated that there was high reactivity of the virus isolate to both the antibodies. So it was confirmed that necrosis disease of cowpea was caused by a tospovirus. The reactivity towards WSMV ascertained the association of its close relationship with tospovirus, which caused the necrosis disease of cowpea. The average absorbance value recorded for diseased sample (2.0) was more than that of healthy sample (0.05). Cho *et al.* (1986) reported that ELISA could be used as a method of choice for the diagnosis and detection of tospovirus in plants and thrips. Avila *et al.* (1990) conducted serological differentiation of 20 isolates of tomato spotted wilt virus using ELISA. The result indicated that polyclonal and monoclonal antiserum was an useful tool to differentiate TSWV isolates. Bhat *et al.* (2001) conducted DAC-ELISA for the detection and characterisation of tospovirus isolates from black gram, cowpea, green gram and soybean .Positive reaction was obtained only with antisera to GBNV and WSMV. The present investigation was also in accordance with the previous reports.

Dot immunobinding assay (DIBA) was conducted using antisera specific to WSMV. Positive reaction was observed with tospovirus isolate from cowpea . Since the relationship of WSMV and TSWV was ascertained by ELISA test, this result also confirms that the necrosis disease of cowpea was caused by tospovirus. Bananej *et al.* (1998) used DIBA for identification of tomato spotted wilt virus from tomato fields. Bhat *et al.* (2001) reported positive result, when dot-blot hybridization was conducted using necrosis disease of black gram, cowpea, green gram and soybean with NP gene probe of GBNV.

Biochemical changes due to virus infection were also studied. The present investigation revealed that there was no significant difference in the carbohydrate content between healthy and inoculated plants of cowpea plant. The carbohydrate content decreased from 39mg g^{-1} at 5 DAI to 37.5mg g^{-1} at 10 DAI. Thereafter it increases to 39 at 15 DAI in inoculated plants. In uninoculated plants an increase of carbohydrate content from 38.6mg g^{-1} at one DAI to 41 mg g^{-1} at 15 DAI was noticed. Even though there was a decrease in carbohydrate content at 10 DAI, when compared to 5 DAI, the value regained at 15 DAI . Many studies showed similar results regarding carbohydrate content due to virus infection. Decrease in carbohydrate concentration due to viral infection in susceptible cultivars of cowpea has been reported (Ramiah, 1978; Singh & Singh, 1984 and Mayoral *et al.*, 1989). Decreased photosynthesis and increased respiration occurring in virus infected tissues and lead to altered concentration of carbohydrates (Bhavani *et al.*, 1998). Sutha *et al.* (1998) reported that TSWV infection reduced the concentration of total, non reducing and reducing sugars and the reduction was more in initial stages of infection compared to later stages. It was also reported that in contrast to sugar concentration, the starch increased in infected plants at all stages of analysis. In the present study decrease in carbohydrate content was noticed in inoculated plants. The reduction at the level of carbohydrate might be due to the breakdown of carbohydrate accelerated during respiration in virus infected plants as suggested by Narayanaswamy and Ramakrishnan (1966).

Virus infections were reported to cause reduction in chlorophyll in susceptible plants. Kaur *et al.* (1991) reported that there was reduction in chlorophyll content in yellow mosaic infected soybean cultivars. Sutha *et al.* (1998) found that there was reduction in chlorophyll, xanthophyll and carotene in TSWV infected tomato plants. The present investigation indicated a reduction in chlorophyll content in inoculated plants. When compared to uninoculated plants, chlorophyll a was 1.26 mg g^{-1} at 15DAI in healthy plants where as it was only 1.09 mg g^{-1} at 15 DAI in inoculated plants. Chlorophyll b was 0.38 mg g^{-1} for healthy and it was 0.32 mg g^{-1} in inoculated plants. Total

chlorophyll recorded a value of 1.64 mg g^{-1} at 15 DAI in healthy but it was only 1.42 mg g^{-1} in inoculated plants .

Estimation of protein indicated significant difference in the level of protein between healthy and inoculated plants. The total soluble protein was found higher in case of inoculated plants. In the inoculated plants protein content was maximum at one DAI (112.1 µg g^{-1}). There was a slight decrease in protein content at 5 DAI and 10 DAI. There after it increased and became on par with the value at one DAI. In healthy control protein content was only 64.6 µg g^{-1} at one DAI and reached a maximum of 76.5µg g^{-1} at 10 DAI, but not the level that of inoculated plants. Higher protein content due to virus infection has been reported by many authors (Singh *et al.*, 1978., Singh & Singh ,1984 ., Singh and Singh, 1987 ., Yadav ,1988 ., Yadhav and Sharma ,1988., Mayoral *et al.*, 1989. and Patil and Sayyad ,1991). Manickam *et al.* (2000) reported that there was significant increase in RNA content in cowpea and greengram on inoculation with tospovirus. All these reports are in conformity with the results of the present investigation. The drastic increase in total protein content in the virus inoculated plants was due to increase in viral proteins and non-viral induced proteins occurring indirectly at the expense of normal host proteins directed by genes present in viral DNA .

There was significant difference in phenol between the healthy and inoculated plants. Phenol content was significantly higher in inoculated plants. Due to tospovirus infection phenol content increased from 36.8 µg g^{-1} at one DAI to 81.5 µg g^{-1} at 10 DAI and then decreased to 23.8 µg g^{-1}. Maximum value of 55µg g^{-1} was recorded at 5 DAI in healthy plants and thereafter decreased to 21.6 µg g^{-1} at 15 DAI . Ramiah (1978) observed that the total phenol content was increased in CABMV inoculated plants of susceptible cultivars of cowpea. Enhanced level of phenol content has been observed in hypersensitive cowpea leaves infected with tobacco ring spot virus. Sutha *et al.* (1997) found that both total and *ortho*-dihydroxy phenol increased in TSWV 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 infected moth bean.Accumulation of phenol observed 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 as reported by Sutha *et al.* (1997).

Investigation on changes in the activity of peroxidase, polyphenol oxidase and phenylalanine ammonialyase clearly indicated that there was difference in the activity of these defence related enzymes in healthy and inoculated plants of cowpea (cv- Pusa Komal). The enzyme was found higher in inoculated plants but gradually decreased. The decrease of the enzyme activity may be due to the susceptibility of the plant to the virus. Peroxidase activity was maximum (5.3 min^{-1} g^{-1}) at 1 DAI in inoculated plants. Thereafter it decreased to 0.2 min^{-1} g^{-1} at 15 DAI. In healthy control maximum enzyme activity recorded was only 2 min^{-1} g^{-1} at 1 DAI compared to 5.3 min^{-1} g^{-1} in tospo infected plants .

Polyphenol oxidase was found maximum at 5 DAI in both healthy (0.4 $min^{-1}g^{-1}$) and inoculated plants (0.5 $min^{-1}g^{-1}$) at 1 DAI. PPO decreased from 0.14 $min^{-1}g^{-1}$ at 1 DAI to 0.02 $min^{-1}g^{-1}$ at 15 DAI in healthy, while in inoculated plants it decreased from 0.5 $min^{-1}g^{-1}$ at 1 DAI to 0.25 $min^{-1}g^{-1}$ at 15 DAI .

PAL activity was maximum (56.4 $\mu g\ g^{-1}min^{-1}$) at 5 DAI in inoculated plants. The enzyme activity was higher for inoculated (56.4$\mu g\ g^{-1}min^{-1}$) to healthy (52.9 $\mu g\ g^{-1}\ min^{-1}$) at 15 DAI .

Khathri and Chenulu (1970) found higher peroxidase activity in inoculated susceptible cowpea varieties infected with cowpea mosaic virus. Umamaheshwaran (1996) reported that there was progressive increase in peroxidase, polyphenol oxidase and phenylalanine ammonia-lyase activity in inoculated susceptible varieties of cowpea due to CABMV infection . Verma and Prasad (1987) established that induced resistance was attributed to the enhanced activity of peroxidase and polyphenol oxidase enzymes. Mali *et al* (2000) reported that the activity of catalase, peroxidase and nitrate reductase enzymes decreased

with increasing intensity of disease, in the case of yellow mosaic disease of moth bean. The decreasing trend in enzyme activity of the present study may be due to increase in intensity of disease or may be due to high susceptibility of plant to the disease.

Infection of the plant with viruses was accompanied by the appearance of specific soluble proteins. PAGE protein was analysed in cowpea following inoculation with tospovirus causing nercosis disease. The expression of protein in the inoculated plants was more than in uninoculated control. Three extra bands were noticed in inoculated leaves with molecular weight of 28, 15 and 6.2 kDa . These three novel proteins were absent in control. Satyanarayana *et al.* (1996) performed SDS-PAGE of nucleocapsid protein of peanut yellow spot virus (PYSV), a distinct tospovirus species and obtained a protein with a molecular mass of 29 kDa. Cortes *et al.* (1998) reported that N.protein of TSWV was about 28.8 kDa which is less than IYSV (30 kDa). de Avila *et al.* (1993) analysed protein composition of a number of tospovirus isolates and reported that migration rates of different isolates correspond with a molecular mass of 29 and 28 kDa. All these studies are in accordance with the results of the present investigation. Among the three novel proteins obtained, the protein with molecular weight 28 kDa may be the N-protein of tospovirus.

Isozyme analysis of defence related enzymes peroxidase and polypenol oxidase were performed using native PAGE . The results indicated that there was no distinct polymorphism in PPO between inoculated and uninoculated control . Both of them produced same banding pattern. Four isoforms of Rm value 0.03, 0.37, 0.54 and 0.62 were obtained in both samples analysed . The isoform with migration rate 0.54 was more expressed in inoculated compared to uninoculated one . In the case of peroxidase, no isoforms were produced in uninoculated plants but an isoform with Rm value 0.65 was recorded in inoculated plants .

Novacky and Hampton (1967) separated isozymes from TMV infected tobacco and CMV or tobacco ring spot virus infected cowpea by disc electrophoresis and reported only quantitative changes in both host plants. The

present study also indicated quantitative changes as observed in PPO in one of the isoforms with Rm value 0.54. Umamaheshwaran (1996) indicated significant variation in peroxidase isozyme in resistant and susceptible cultivars. The present study revealed that a new isoform of Rm value 0.65 could be located in the inoculated plants when compared to healthy control.

Based on the results on symptomatology, physical properties, transmission, host range and serological properties the virus causing necrosis disease of cowpea was identified and confirmed as tospovirus.

Summary

6. SUMMARY

Studies were conducted on the necrosis disease of cowpea in Kerala.

The symptoms appeared as chlorotic spots in the newly emerged trifoliate leaves of cowpea. Typical symptoms appeared as necrosis, veinal necrosis, distortion and reduction in leaf size, and bud necrosis. Severe infection leads to the death of the plant.

The virus was found to have its host range confined to the members of the families Chenopodiaceae, Solanaceae, Leguminosae, Amaranthaceae, and Malvaceae. It produced systemic symptoms on *Gomphrena globosa, Vigna radiata, Vigna unguiculata ,Glycine max, Arachis hypogaea, Datura stramonium, Solanum melongena, Petunia hybrida and Lycopersicon esculentum*. Distinct chlorotic local lesions were produced by the virus on *Chenopodium amaranticolor, Chenopodium quinoa* and *Chenopodium album.*

The virus could be transmitted mechanically through sap extracted in 0.01M phosphate buffer (pH7.2) containing 0.1%2-mercaptoethanol. Insect transmission studies carried out with *Aphis craccivora* and *Thrips palmi* indicated high percentage of transmission by aphids compared to thrips. The per cent transmission obtained for *A. craccivora* and *Thrips palmi* were 50 and 30 respectively. The virus was not transmitted through seeds and could be transmitted through graft. Fifty per cent graft transmission was obtained in the present investigation.

Physical properties of virus *i.e.*, dilution end point, thermal inactivation point and longevity *in vitro* were investigated and it was found that the virus was inactivated at dilutions between 10^{-2} and 10^{-3} and at temperatures between 50 and 55°C. Longevity *in vitro* recorded was 6 h at 28±2°C and 8 h at 8°C.

The virus causing necrosis disease in cowpea was identified as tospo virus by serological analysis such as ELISA and DIBA. This virus isolate of cowpea showed close relationship with WSMV.

The biochemical changes due to virus infection were studied in healthy control and virus inoculated cowpea plants. There was no significant difference in carbohydrate content between uninoculated and inoculated plants. Chlorophyll content was found higher in healthy plants. There was an increase in protein content in inoculated plants compared to uninoculated plants. Phenol content was also higher in inoculated plants. A decrease in the activities of defence related enzymes with the age of the plant was noticed. However the activities of enzymes were found higher in inoculated plants compared to healthy control.

PAGE was carried out to analyse the protein profile of cowpea plants under inoculated and uninoculated conditions to investigate the presence of virus induced proteins. The experiment revealed 3 newly induced virus related proteins in the inoculated cowpea plants compared to healthy control. The proteins obtained were with molecular weight 28kDa, 15 kDa and 6.2 kDa.

Native polyacrylamide gel electrophoresis was performed for polyphenol oxidase(PPO) and peroxidase enzyme(PO). Both healthy and inoculated leaves produced same banding patterns. Out of 4 isoforms obtained in PPO, one isoform with Rm value 0.54 was more expressed in inoculated plants. Peroxidase showed only one isoform in inoculated leaves and no isoforms were observed in healthy.

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Appendices

APPENDIX - I

Buffers for Mechanical Inoculation

1. 0.1 M citrate buffer (pH 6.2)

   Stock solutions.

   A: 0.1 M solution of citric acid (19.21g in 1000ml)

   B: 0.1 M solution of sodium citrate (29.41 g in 1000 ml)

   7.2 ml of A is mixed with 42.8 ml of B and diluted, to total of 100 ml.

2. 0.1 M citrate-phosphate buffer (pH 7.0) stock solution.

   A: 0.1 M solution of citric acid (19.21 g in 1000 ml)

   B: 0.2 M solution of dibasic sodium phosphate (53.65 g of $Na_2HPO+7H_2O$ in 1000 ml)

   6.5 ml of A is mixed with 43.6 ml of B diluted to a total of 100 ml.

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

   B: 0.2 M solution of dibasic sodium phosphate (53.6 g of $Na_2HPO_4.12H_20$ in 1000 ml)

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

4. 0.1 M Tris buffer (pH 7.2)

   A: 0.2 M solution of Tris (24.2 g in 1000 ml)

   B: 0.2 M HCl

   50 ml of A is mixed with 44.2 ml B diluted to a total of 200 ml.

5. 0.01 M phosphate buffer containing 2 mercaptoethanol (pH 7.2)

   KH_2PO_4 – 800 g

   K_2HPO_4 – 2.2 g

Mercaptoethanol - 100µl

Made up to 100 ml in distilled water.

APPENDIX II

Buffers for ELISA

1 Phosphate buffer saline (1 x PBS) pH 7.4

$NaCl$	-	8 g
$Na_2HPO_4\ 2H_2O$	-	1.44 g
KH_2PO_4	-	0.2g
KCl	-	0.2g
Distilled water to make	-	1 Litre

2 Wash Buffer (PBS-T)

3 Coating buffer (Carbonate buffer pH 9.6)

Na_2CO_3	-	1.59g
$NaHCO_3$	-	2.93g
Distilled water to make	-	1 Litre

4 Enzyme conjugate diluent buffer (PBS-TPO)

Add 20 g PVP and 2 g ovalbumin to 1 l PBS-T

5 Antibody diluent buffer – Same as PBS-TPO

6 Substrate buffer (pH9.8)

Diethanol amine	-	97 ml
Distilled water	-	800 ml.

APPENDIX III

DOT – IMMUNOBINDING ASSAY (DIBA)

Chemicals and solutions

1. Stock buffer (Tris-buffer saline, TBS, pH 7.5)

   0.02 M Tris 48 g

   0.5 M NaCl 58.48 g

   Adjust the pH to 7.5 with 1 N Hcl and make upto 2 litre. This is used as Wash solution.

2. Antigen extraction buffer (TBS – 50 mM DIECA)

   Add 11.25 g diethyl dithiocarbonate (DIECA) to 1 litre TBS

3. Blocking solution (TBS-SDM)

   Add 5.0 g spray dried milk (SDM) to 100 ml TBS

4 Antibody and enzyme-conjugate diluent/buffer

   Same as TBS-SDM

5 Substrate buffer (pH 9.5)

   0.1 M Tris 12.11 g

   0.1 M NaCl 5.8 g

   5 mM $MgCl_2.6H_2O$ 1.01 g

   Adjust the pH to 9.5 with 1 N HCl and make up to 1litre.

6 Substrate solution

   Solution A

   Nitro Blue tetrazolium (NBT) 75 mg

   DMFA 1 ml

   Solution B

   Bromo chloro Indolyl phosphate (BCIP) 50 mg

   DMFA 1 mg

Store solutions A and B refrigerated in amber bottles. Add NBT to ---- mg/ml to the substrate buffer just before use.

7 Fixing solution (pH 7.5)

10 mM Tris	1.21 g
1 mM EDTA	0.29 g

Adjust the pH to 7.5 with 1 N HCl and make up to 1 litre

All buffers contain 0.02% sodium azide as a preservative.

APPENDIX IV

Buffers for enzyme analysis

0.1 M sodium acetate (pH 4.7)

Stock solutions

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

B: 0.2 M solution of sodium acetate (16.4 g of $C_2H_3O_2$ Na or 27.2 g of $C_2H_3O_2$ Na $3H_2O$ in 1000 ml).

22.7 ml of A is mixed with 27 ml of B, diluted to a total of 100 ml.

0.1 M Borate Buffer (pH 8.8)

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

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

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

CHARACTERIZATION OF A TOSPOVIRUS CAUSING NECROSIS DISEASE OF COWPEA [*Vigna unguiculata* (L). Walp.]

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Abstract of the
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ABSTRACT

Studies were conducted on the tospo virus causing necrosis disease of cowpea [*Vigna unguiculata* (L.) Walp.] in Kerala. This investigation was conducted to characterize the virus. The characteristic symptoms appeared as chlorotic spots, veinal and bud necrosis, distortion and reduction in leaf size. Host range studies were done and the virus was found to have its host range in the members of the families Chenopodiaceae, Solanaceae, Leguminosae, Amaranthaceae and Malvaceae.

The virus was mechanically transmitted through sap extracted in 0.01M phosphate buffer (pH 7.2) containing 2mercapto ethanol. The virus could be efficiently transmitted by the aphid vector, *Aphis craccivora* and *Thrips palmi*. The virus could be transmitted through graft but not through seeds. Thermal inactivation point was 50-55°C, dilution end point, 10^{-2}-10^{-3} and longevity *in vitro* for 4 h at room temperature (28±2°C) and 8 h under refrigerated conditions (8°C). The virus causing necrosis was identified as tospo virus by ELISA and DIBA. Virus was related to WSMV, a tospo isolate. Biochemical changes indicated a decrease in chlorophyll content in virus inoculated leaves compared to healthy control. There was no significant difference observed in carbohydrates. Increase in protein content was observed in inoculated cowpea plants. The phenol content was found more in inoculated leaves compared to healthy control. The level decreased during later stages. Peroxidase, polyphenol oxidase and phenyl alanine ammonialyase showed a decreasing trend with age in both inoculated and healthy plants. But it was comparatively high in inoculated plants. Native polyacrylamide gel electrophoresis performed for PPO and peroxidase revealed that there was four isoforms of PPO for both inoulated and uninoculated control. Only quantitative change in one of the isoform was observed in PPO. One isoform in peroxidase was observed in inoculated plant but no isoform was observed in healthy. PAGE analysis of proteins with samples extracted from diseased and healthy plants showed the presence of three novel proteins in diseased sample. One of the proteins molecular weight, 28 kDa co-relates with the N-Protein of TSWV reported earlier.