CHARACTERIZATION OF BUD NECROSIS VIRUS INFECTING TOMATO

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2011

DECLARATION

I hereby declare that this thesis entitled **"Characterization of bud necrosis virus infecting tomato"** 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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Certified that this thesis entitled "**Characterization of bud necrosis virus infecting tomato**" is a record of research work done independently by Ms. S. SIMI. (2009-11-128) under my guidance and supervision and that it has not previously formed the basis for the award of any degree, fellowship or associateship to her.

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Introduction

1. INTRODUCTION

Tomato (*Lycopersicon esculentum* Mill.) is an important commercial vegetable crop in India cultivated in Uttar Pradesh, Bihar, Maharashtra, Karnataka, Assam, Punjab and West Bengal in an area of 5.72 lakh hactare producing 95.96 lakh tonnes (NHB database, 2009). Tomato is a good source of vitamins like A and C, minerals such as iron, phosphorous, organic acids, essential amino acids and dietary fibers.

Among the viral diseases, bud necrosis disease of tomato caused by a Tospovirus is rising to alarming proportions in Kerala and becoming a limiting factor in tomato cultivation. Natural infection of Tospovirus on tomato crop characterized by severe necrosis of leaves, petioles, buds, stems and fruits were recorded at experimental farms of IARI, New Delhi and Kerala Agricultural University, Vellayani (Jain et al., 2002). Early infection leads to a crop loss upto 100 per cent (Kumar and Irulappan, 1991). Moreover the presence of a wide host range for the vector as well as for the virus ensures abundant inoculum in nature resulting in fast spread of the disease.

Tospoviruses are a highly cosmopolitan group of viruses. Severe yield losses associated with Tospoviruses belonging to family Bunyaviridae have been reported worldwide in tomato, pepper, lettuce, celery, potato, tobacco, peanut, pea and ornamentals such as dahlia, chrysanthemum, gerbera, impatiens and iris (Soler et al., 2003). Tospovirus infect important vegetable and other crops in the tropical, subtropical and temperate regions worldwide (Whitfield et al., 2005). They are found to infect 35 plant families including Solanaceae, Asteraceae, Leguminaceae, Brassicaceae, and Bromiliaceae (Momol and Pernezny, 2006). Such a wide host range places them amongst the most economically important plant pathogen in the world at present (Hemalatha et al., 2008)

The genus Tospovirus, family Bunyaviridae, includes viruses with enveloped, quasi-spherical particles of 80–120 nm diameter and a tripartite, single-stranded RNA genome (Hemalatha et al., 2008). They are exclusively vectored by several species of thrips in a circulative and propagative manner (Moyer, 1999). In India three distinct Tospovirus species namely Groundnut bud necrosis (GBNV) (Reddy et al., 1992) and Groundnut yellow spot virus (GYSV) (Satyanarayana et al., 1998) from groundnut and

Watermelon bud necrosis (WBNV) (Jain et al., 1998) from watermelon have been reported.

The wide and overlapping host range of Tospoviruses, emergence of resistance breaking strains, relationship with polyphagous thrips, and difficulties in predicting their outbreaks pose challenges to development and implementation of effective management programmes. Further only very little attempt has been made so far to identify the bud necrosis virus infecting tomato in Kerala. To protect the crop from the devastating losses, caused by Tospovirus outbreaks, continued vigilance is required and also a need to identify and characterize this emerging virus problem.

Taking into consideration, the above aspects and also the importance of Tospovirus in the cultivation of tomato in our state, this study was undertaken to investigate the symptomatology, physical characteristics of the virus, host pathogen interaction, serological and molecular diagnosis of the virus and also to develop effective management strategies against the bud necrosis virus infecting tomato.

Review of Literature

2. REVIEW OF LITERATURE

2.1 SYMPTOMATOLOGY

The symptoms induced by Tospoviruses are highly varied and include ring spots, line patterns, wilting, stunting, silvering, mottling, bronzing, chlorosis, necrosis and a range of leaf and stem lesions (German et al., 1992). Adam and Kegler (1994) observed that the nature of symptoms depends on the tospovirus species, the virulence of the virus strain and the environmental conditions.

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).

Jain et al. (2002) reported that the natural infection of tospovirus on mungbean (*Vigna radiata*) and cowpea (*Vigna unguiculata*) and tomato (*Lycopersicon esculentum*) is characterized by severe necrosis of leaves, stem, growing point, buds, pods and fruits. Soler et al. (2003) reported that in TSWV (tomato spotted wilt virus) infected tomato plants the younger leaves turn violet, but the seriousness of the disease in most cases is due to stem necrosis, which causes complete loss of production. Umamaheswaran et al. (2003) isolated a tospovirus from tomato plants showing bronzing and severe necrosis of leaves and growing buds.

Ghotbi et al. (2005) reported necrotic local lesions on *Chenopodium amaranticolor* and *Vigna unguiculata* and systemic leaf deformation and mosaic symptoms on *Nicotiana benthamina*, when the plants were artificially inoculated with tospovirus. Hassani et al. (2005) reported that the symptoms on tomato consisted of systemic chlorotic and necrotic spots on leaves and yellow rings on fruits and the plants generally showed growth reduction due to TSWV infection.

Hemlatha et al. (2008) observed that tospoviruses induce symptoms like chlorotic and necrotic ring spots on leaves, necrosis of growing tip, tip wilting and yellowing.

2.2 HOST RANGE

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, greengram, cowpea and soyabean based on bioassay, serology and nucleic acid hybridization. Hosts of TSWV include horticultural crops such as tomato, pepper, lettuce, celery, potato, tobacco, peanut, pea and ornamentals such as dahlia, chrysanthemum, gerbera, impatiens and iris (Soler et al., 2003).

The experimental host range studies of tomato tospovirus isolate was done and plant species belonging to Amaranthaceae, Chenopodiaceae, Fabaceae and Solanaceae families were identified as hosts of the virus. TSWV was found to infect *Arachis hypogea, Vigna mungo, V. radiata, V. unguiculata, Macrotyloma uniflorum* and *Physalis floridana* (Umamaheswaran et al., 2003).

Ghotbi et al. (2005) reported the occurrence of tospoviruses in many ornamentals and weed species. Whitfield et al. (2005) reported that Tospovirus infect important vegetable and other crops in tropical, subtropical and temperate regions worldwide.

Jain et al. (2006) reported natural infection of tospoviruses on three cucurbitaceous (*Cucumis sativus, Luffa acutangula, Citrullus lanatus*) and three fabaceous (*Vigna unguiculata, Phaseolus vulgaris, Dolichos lablab*) vegetable crops in India.

Momol and Pernezny (2006) reported that TSWV can infect 35 plant families including Solanaceae, Asteraceae, Leguminaceae, Brassicaceae and Bromiliaceae.

Biswas et al. (2007) reported the mixed infection of gemnivirus, tospovirus and urdbean leaf crinkle complex under field conditions.

The ability of tospovirus isolate to cause severe losses on a broad range of crops, places them amongst the most economically important plant pathogens in the world at present (Hemalatha et al., 2008).

2.3 TRANSMISSION STUDIES

2.3.1 MECHANICAL TRANSMISSION

Tospovirus infected sample was used for mechanical inoculation on indicator hosts like, cowpea (Pusa Komal) using 0.05 M potassium phosphate buffer pH 7.0 containing sodium sulfite (Bhat et al., 2001). Umamaheswaran et al. (2003) reported mechanical inoculation of the virus isolate from infected tomato plants to cowpea (*Vigna unguiculata* cv Pusa Komal) under primary leaf stage under glass house condition using 0.01 M potassium phosphate buffer (pH 7.2) containing 0.1% β-mercaptoetanol.

Saritha and Jain (2007) reported the mechanical inoculation of GBNV isolated from mungbean from the experimental fields of IARI on local lesion host *Vigna unguiculata* cv. Pusa Komal using 0.1 M phosphate buffer, containing 0.1% mercaptoethanol and celite as abrasive. Tomato tospovirus isolates collected from tomato growing fields around Bangalore were maintained in green house by mechanical inoculation to *Nicotiana benthamiana* plants wherein the sample was ground in 50 mM potassium phosphate buffer, pH 7.5 containing 0.02 M mercaptoethanol and inoculated on *N. benthamiana* seedlings dusted with celite (Hemlatha et al., 2008). Pissawan et al. (2008) performed the mechanical inoculation of tospovirus to diagnostic host plants ie *Nicotiana glutinosa* and *V. unguiculata* using cold 0.05 M sodium phosphate buffer pH 7.0 with 0.2 % mercaptoethanol added prior to inoculation.

2.3.2 SEED TRANSMISSION

Ghanekar et al. (1979) reported that, of nearly 6000 seeds collected from tospovirus infected plants, none of the seedlings developed disease symptoms and results indicated that the virus is not seed borne. Antignus et al. (1997) reported that no virus transmission was found in seeds collected from TSWV infected vegetable and ornamental crops.

Pappu et al. (1999) reported that in grow out tests of symptomatic and asymptomatic peanut plants infected with TSWV, none of the plants showed TSWV

infection when assayed by ELISA. Gera et al. (2000) collected mature seeds from TSWV infected tomato and capsicum fruits. The seedlings produced from the seeds of infected fruits were germinated and grown for 6 weeks and tested for TSWV weekly upto two months. No visible symptoms were observed in tomato or capsicum and further, they also gave negative results in ELISA. These results indicated that the virus was not seed borne. Tospoviruses are mechanically transmissible with varying degrees of difficulty but there is no evidence that they are seed transmitted (Pappu et al., 2009).

2.3.3 GRAFT TRANSMISSION

Roy and Gupta (1977) reported that top necrosis disease of pea from India is caused by TSWV and could be transmitted through graft and sap inoculation. Ghanekar et al. (1979) transmitted tospovirus causing groundnut bud necrosis effectively to healthy groundnut plants by grafting. Solomon-Black burn and Barker (2001) reported that plants can be graft inoculated with TSWV using infected scions, but although this is a more reliable method, it is more laborious. Ariyaratne et al. (2004) studied transmission of TSWV through graft union, by grafting virus infected tomato scions on healthy root stocks.

Chick pea plants positive for TSWV were used as inoculum for mechanical and graft transmission to chickpea cv. Amethyst in the glasshouse. All produced severe shoot tip symptoms of wilting and necrosis or small chlorotic leaves (Thomas et al., 2004). Accoto et al. (2005) evaluated tomato hybrids engineered for TSWV resistance and the immunity to TSWV infection in 30 hybrids was confirmed using mechanical inoculation and grafting. Adkins and Baker (2005) reported successful graft transmission of TSWV in desert rose plants (*Adenium obesum*) a member of Apocyanaceae family.

2.4 PHYSICAL PROPERTIES

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

Ghanekar et al. (1979) reported that TSWV remained infective in buffered sap

(Phosphate buffer, pH 7.0) of groundnut at a dilution of 10^{-2} to 10^{-3}. The infectivity was retained after storage for four hours at room temperature ($30\,^0$C) and for 10 minutes at 40^0C but not at 45^0C.

TSWV has an *in vitro* thermal inactivation point of 46^0C, one of the lowest among plant viruses (Francki, 1980). Mali and Pahl (1980) found that TSWV remained infective in buffered sap of groundnut at a dilution of 10^{-3} but not at 10^{-4}. The TIP was between 45^0C-50^0C and LIV was found to be 1-2 h at room temperature.

Silveira junior et al. (1985) identified the pathogen from *Sechium edule* as TSWV from its host range and properties *in vitro*. The TIP was found to be between 45^0C to 50^0C. DEP was 10^{-3} to 10^{-4} and longevity at room temperature was less than 2 h.

Roggero and Pennazio (1997) studied the effect of *in vivo* of temperatures that are near the *in vitro* thermal inactivation point of TSWV in tobacco plants systemically infected with the virus. The isolate was infective after 10 minutes at 44^0C but not at 46^0C. Further the virus was infective in plants treated for 1h at 50^0C. However, the virus was inactivated in plants held for 24 h at $45\,^0$C with light, but not after 14 h at 45^0C in the light.

2.5 HOST - PATHOGEN INTERACTION

2.5.1 BIOCHEMICAL ANALYSIS

2.5.1.1 CARBOHYDRATES

Umamaheswaran (1996) found that the level of carbohydrate was significantly lower in susceptible varieties of cowpea when inoculated with cowpea aphid borne virus (CABMV). Sutha et al. (1998 a) studied the changes in concentration of chemical constituents in tomato caused by TSWV infection and revealed that there is 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 levels increased in infected plants at all stages of analysis. Sutha et al. (1998 b) reported that

TSWV infection reduced the concentration of total, non-reducing and reducing sugars of tomato fruits.

Mali et al. (2000) reported that infection of yellow mosaic virus in mothbean resulted in reduction of total soluble carbohydrate in susceptible plants when compared to resistant genotype. Manickam et al. (2000) reported that the increase in non- reducing sugars in TSWV inoculated plants was less.

Sugarcane plants infected with sugarcane yellow leaf virus showed abnormal starch accumulation in mesophyll and bundle sheath cells compared to healthy cowpea plants (Yan et al., 2008). Sinha and Srivastava (2010) reported increased total sugar and starch contents in finger millet plants infected by mottle streak virus.

2.5.1.2 CHLOROPHYLL

Sutha et al. (1998 a) investigated the biochemical changes caused in tomato following infection with TSWV and reported that chlorophyll, xanthophylls and carotene contents were reduced in infected plants. Sutha et al. (1998 b) reported that TSWV infection reduced the concentration of carotene of tomato fruits. In contrast lycopene content was increased.

The most obvious symptom of systemic virus infection is the mosaic pattern on the leaves. Yellowing and chlorosis is the characteristic sign of the altered photosynthetic activity. Virus infection effects photosynthesis in a complex manner, depending on the particular host-virus combination (Almasi et al., 2001)

Peanut leaf chlorophyll was consistently lower in leaves with symptoms like chlorosis caused by TSWV infection compared to those without any symptoms, irrespective of the cultivars (Rowland et al., 2005).

Total chlorophyll, chlorophyll a and chlorophyll b were found to be lower in mungbean plants infected by mungbean yellow mosaic virus, estimated in healthy and diseased leaves of mungbean plants at 15, 30, 45, 60 and 75 days by recording absorbance at 663 and 645 nm (Momol and pernezny, 2006).

Farooq et al. (2007) reported that chlorophyll content in tomato leaves of healthy plants was significantly higher compared to TSWV infected plants in different varieties tested at early, mid and late stages of infection. The percent reduction of plant

chlorophyll content was higher in early stage of infection followed by mid and late stages of infection. Reduction range for early stage infection was 36-26 % and that of mid and late stages of infection was 13-18% and 4-8% respectively depending on tomato varieties.

Arpita and Subrata (2008) investigated changes in biochemical components in mesta plants due to infection with yellow vein mosaic virus and found a gradual reduction in green pigments like chlorophyll a, b and total c hlorophyll at different stages of pathogenesis. There was alteration of the ratio between chlorophyll a and b which affects the photosynthetic efficiency. Singh and Shukla (2009) reported that the total chlorophyll, chlorophyll a, chlorophyll b and carotenoid contents were lower in papaya ring spot virus infected tissues. Saveetha et al. (2010) reported that chlorophyll pigments a and b as well as total chlorophyll were reduced due to mottle streak virus in finger millet plants.

2.5.1.3 PHENOL

The total phenols in the leaves of *Nicotiana tabacum* was significantly increased as a consequence of tobacco mosaic virus infection (TMV). Their levels reached a maximum between 60 and 156 hr after inoculation (Tanguy and Martin, 1972). Kato et al. (1993) extracted and characterized two phenolic compounds from cowpea leaves infected with cucumber mosaic virus.

Sutha et al. (1997) found that both total phenol and ortho- dihydroxy phenol increased in TSWV infected plants. Sutha et al. (1998 b) reported that TSWV infection reduced ortho- dihydroxy phenol contents of tomato plants. During symptom development, phenols decreased in susceptible plants whereas in resistant varieties they accumulated. (Thimmaiah, 1999).

Meena et al. (2008) investigated the changes in phenol (total and ortho-dihydroxy phenols) in chilli (*Capsicum annuum*) due to infection of gemni virus and they found that total phenol was significantly high in diseased leaf as compared to healthy leaf. The increased quantity of total phenolics in infected plant parts is attributed to resistance of the plants against the pathogen. Saveetha et al. (2010) while investigating physiological changes due to virus infection reported increased phenol

contents in finger millet plants infected by mottle streak virus.

2.5.1.4 PROTEIN

Manickam et al. (2000) studied the impact of application of a foliar spray of antiviral proteins (AVP's) *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 in cowpea plants. It was found that the increase in total soluble protein was higher in TSWV inoculated plants.

Protein content was found to be higher in alfafa plants infected with alfafa mosaic virus. Protein content was 37.25% in infected plants compared to a lower 35.3% in healthy pants (Yardimci et al., 2006). Arpita and Subrata (2008) observed the protein contents to be low in mesta plants infected with yellow vein mosaic compared to control. They also observed higher contents of free amino acids indicating that the disease might have caused the denaturation or breakdown of proteins as well as polypeptide chains and bound amino acids. Selman et al. (2008) analysed the changes in the free amino acids in TSWV inoculated leaves of tomato plants and found that an increase in total free amino acids by 150-180% in the inoculated leaves.

Ashfaq et al. (2010) reported that urdbean leaf crinkle virus infected plants appeared to have increased total soluble protein contents at 15 and 30 days after inoculation, more likely to be due to increased level of viral proteins in the plant. Saveetha et al. (2010) reported increased soluble protein contents in finger millet plants due to virus infection. Sinha and Srivaastava (2010) reported increased protein contents in mungbean plants infected by mungbean yellow mosaic virus.

2.5.1.5 DEFENCE RELATED ENZYMES

Fritig et al. (1973) observed that during hypersensitive reaction of tobacco to TMV, higher phenylalanine ammonia-lyase activity was detectable before the symptoms were visible and a sharp increase occurs at time of appearance and during subsequent development of the local necrotic lesions.

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 black eye cowpea mosaic virus. The enhanced phenol synthesis and peroxidase activity in various host parasitic combination is correlated with disease resistance (Ghosal et al., 2004). Devanathan et al. (2005) observed high peroxidase (PO) activity in bunchy top banana virus infected cultivars of banana.

Enhanced peroxidase activity and polyphenol oxidase activities was found to occur in diseased leaves as compared to healthy leaves of chilly infected with gemnivirus (Meena et al., 2008). Vera Quecini et al. (2007) investigated the metabolic changes caused by TSWV in petunia hybrids and *Nicotiana tabacum* and found increased peroxidase activity at local lesion sites.

Ashfaq et al. (2010) reported that at all stages of sampling, non-significantly higher peroxidase activity was observed in healthy leaves of susceptible genotype than in resistant genotypes of urdbean inoculated with urdbean leaf crinkle virus (ULCV). The activity of PO increased with the passage of time in both the genotypes but there was significant increase of total PO activity in resistant cultivar at 15 and 30 days of inoculation, whereas increase remained non-significant in the diseased leaves of susceptible plants. These results are in agreement with Clarke et al. (2002) and Karthikeyan et al. (2007) who also observed significant increase in peroxidase activity in *Phaseolus vulgaris* and *Vigna mungo* plants after inoculation with white clover mosaic virus (WCIMV) and urdbean leaf crinkle virus (ULCV) respectively.

2.5.2 SDS-PAGE (SODIUM DO-DECYL POLYACRYLAMIDE GEL ELECTROPHORESIS

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.

Satyanarayana et al. (1996) performed PAGE of purified nucleocapsid protein of peanut yellow spot virus (PYSV), a distinct Tospovirus species. The results revealed a protein with a molecular mass of 29 kDa.

Cortes et al. (1998) characterized iris yellow spot virus (IYSV) a Tospovirus species and estimated the size of IYSV, N-protein of IYSV had a molecular weight of 30 kDa and is larger than the N-proteins of TSWV (28.8 kDa) but still smaller than that of Watermelon silver mottle virus (WSMV).

SDS-PAGE analysis of virus purified from Tospovirus infected *Nicotiana benthamina* leaves showed the presence of bands G1, G2 and N protein as expected of purified Tospoviruses (Hemalatha et al., 2008)

2.5.3 ISOZYME ANALYSIS

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 cowpea aphid borne mosaic virus (CABMV) susceptible cowpea varieties and found five isoforms of polyphenol oxidase in susceptible varieties, Sharika and Malika, whereas other two genotypes Co-6 and Pallichal local expressed only four iso-PPO’s.

Arpita and Jain (2008) reported isozyme analysis of peroxidase and polyphenol oxidase by native PAGE, which showed alteration in the activities of different enzymes due to infection by yellow vein mosaic. PPO bands were found to be hyperactive in diseased plants in comparison with control plants.

The electrophoretic studies in chilly infected with Gemnivirus revealed three iso peroxidase bands in diseased leaves, whereas two in healthy leaves. The Rm values in diseased leaves were 0.22, 0.46 and 0.87, whereas in case of healthy plants it was 0.25 and 0.46 (Meena et al., 2008).

2.6 IMMUNOLOGICAL DIAGNOSIS

2.6.1 ELISA (ENZYME LINKED IMMUNOSORBENT ASSAY)

De Avila et al. (1990) performed ELISA using polyclonal and monoclonal

antibodies for the detection of Tospoviruses. The virus causing bud necrosis disease of peanut in India failed to react with the antisera of TSWV and Impatiens necrotic ring spot virus in ELISA, hence on the basis of serological differences the virus that caused bud necrosis disease in India is distinct and appears to be restricted to Asia (Reddy et al., 1992). Adam and Kegler (1994) reported that the most frequently used technique for serologically based diagnosis of Tospoviruses is double antibody sandwich ELISA (DAC-ELISA).

Bhat et al. (2001) observed that in DAC- ELISA, of the seven different tospovirus antiserum treated, only groundnut bud necrosis virus (GBNV) and watermelon silver mottle virus (WSMV) showed positive reaction with the blackgram, cowpea, greengram and soyabean samples tested.

Jain et al. (2002) reported that symptomatic leaf samples of cowpea, mungbean and tomato reacted positively with polyclonal antiserum against nucleocapsid protein of groundnut bud necrosis virus in DAC-ELISA. Further Tospovirus isolated from tomato reacted positively to polyclonal antisera of WSMV and GBNV (Umamaheswaran et al., 2003).

Jain et al. (2005) reported that the antiserum to the nucleocapsid protein of GBNV at 1:4000 dilution detected successfully the natural infection of GBNV and watermelon bud necrosis virus in a wide range of cucurbitaceous, leguminous and solanaceous hosts from different locations.

Hemalatha et al. (2008) reported that virus isolates from infected tomato samples were confirmed to be Tospovirus by ELISA using polyclonal antibodies to GBNV.

2.6.2 DOT IMMUNOBINDING ASSAY (DIBA)

Hsu and Lawson (1991) while investigating direct tissue blotting for detection of TSWV in impatiens found that when biotinylated mouse monoclonal antibody was used, DIBA was nearly eight times more sensitive than ELISA. Bhat et al. (2001) reported successful dot-blot hybridization of healthy and infected blackgram, greengram and soyabean with nucleocapsid protein gene probe of GBNV. Both DIBA method and modified DIBA method using alkaline phosphatase conjugated

IgG against TSWV successfully identified TSWV from leaves of pepper, tobacco, tomato and egg plant showing necrotic symptoms (Takaaki et al., 2003). Anjaneya et al. (2008) reported that necrosis affected tomato plants showed positive reaction with groundnut bud necrosis virus (GBNV) polyclonal antiserum in DIBA indicating that Tospovirus is serologically related to GBNV.

DIBA was done by coating nitrocellulose strips with leaf extracts of *Nicotiana benthamina* prepared in 50 mM phosphate buffer saline, pH 7.5 (1g/ml). The color was developed by incubating the membrane with 0.05 M sodium citrate buffer containing 0.01% - 0.03% hydrogen peroxide and diaminobenzidine (DAB) (Hemalatha et al., 2008)

Esmaalifar et al. (2009) reported successful detection of tomato samples infected with TSWV using DIBA.

2.7 MOLECULAR DIAGNOSIS

Okuda and Hanada (2001) investigated the RT-PCR (reverse transcription polymerase chain reaction) procedures for the detection of multiple species of Tospovirus from plant tissues.

Jain et al. (2002) cloned and sequenced the N gene from cowpea, mungbean and tomato Tospovirus isolates. Umamaheswaran et al. (2003) observed that when RT-PCR was performed using primer pair derived from the Nucleocapsid protein gene sequence of GBNV and WSMV, an amplicon of 800 bp was visualized on analysis of the PCR products by 1% agarose gel electrophoresis.

Akram et al. (2004) determined and compared the nucleotide and amino acid sequences of the movement protein (Nsm) genes of five isolates of GBNV originating from different hosts and parts of India such as cowpea and tomato from Kerala, Groundnut from TamilNadu and Potato from Madhya Pradesh.

RNA template for RT-PCR was extracted from Tospovirus infected and healthy cowpea tissues using RNeasy kit according to manufacturer's instructions (Saritha and Jain, 2007). Hemalatha et al. (2008) reported the extraction of viral RNA from tospovirus infected tomato by Phenol-chloroform method and size of RNA was estimated by formaldehyde agarose gel electrophoresis wherein the N-gene was

amplified by RT-PCR in thermal cycler subjecting it to 94^{0}C for 4 minutes, Followed by 30 cycles of 94^{0}C for 30 seconds, 52^{0}C for 1 minute and final extension at 72^{0}C for 10 minutes and resulted in a product of 831 bp.

Sivaprasad and Gubba (2008) isolated total RNA from *Nicotiana sp.* infected with TSWV and subjected it to reverse polymerase chain reaction using primers specific to nucleocapsid gene of TSWV. Venkatesan et al. (2009) cloned and characterized the nucleocapsid protein (N) gene of Tospovirus devastating tomato crop in South Indian state of Tamilnadu and observed high identity of the cloned sequence to a peanut bud necrosis virus (PBNV) tomato isolate and PBNV peanut isolate and identified the Tospovirus as an isolate of PBNV.

2.8ELIMINATION OF VIRUS THROUGH MERISTEM CULTURE

Novak and Maskova (1979) studied the effect of phytohormones on growth and development of apical shoot tip of tomato (*Lycopersicon esculentum* Mill.). Mirghis et al. (1995) reported efficient plantlet regeneration in tomato from meristems. Meristem culture allows plants to be freed from viruses, viroids, mycoplasma, bacteria and fungi. Several reasons such as absence of plasmodesmata in the meristamatic domes, faster cell division, competition between synthesis of nucleoproteins for cellular division and viral replication and presence of inhibition substances make meristem useful source to obtain virus free plantlets (Grout, 1999)

Benefits of meristem culture has been demonstrated for disease elimination in many crops including potato (Bittner et al., 1989), garlic (Conci and Nome, 1991), Peanut (Moris et al, 1997), Sugarcane (Balamuralikrishnan et al., 2002) and tomato (Alam et al., 2004).

For meristem culture shoot meristem consisting of the apical dome with on or two leaf primordia is isolated using sterile hypodermic needle and scalpel under a dissecting microscope (Alam et al., 2004).

Sheeja et al. (2004) assessed the in vitro culture response of three varieties of tomato for optimum callus induction and plantlet regeneration. Callus induction was achieved within 7-10 days and plantlet regeneration was observed in 20 days across varieties. Further the presence of 2.0 mg/L BAP produced maximum shootlets and

addition of IAA 1.0 mg/L was found essential to induce longer roots.

Sharmin et al. (2008) reported meristem culture for developing an efficient protocol of production of egg plant clones and found that surface sterilization of shoot tips with 0.1% $HgCl_2$ for 3 minutes duration is the most effective. Further found that among the different concentrations of BAP, 2.0 mg/L BAP showed better performance than other treatments.

Materials and Methods

3. MATERIALS AND METHODS

3.1 SYMPTOMATOLOGY

Tomato leaves showing chlorotic and necrotic ring spotting and terminal bud necrosis were collected from field and the culture of the virus was maintained by repeated transfers on *Chenopodium amaranticolor* by mechanical inoculation using 0.01M phosphate buffer (Appendix-I) (pH 7.2) containing 0.1% 2-mercaptoethanol at 24-32°C.

3.2 HOST RANGE

To determine the host range of tospovirus infecting tomato, the plants belonging to species of different families were inoculated by sap inoculation. Ten seedlings of each species were inoculated. The inoculated plants were examined for the development of symptoms. Inoculated plant species without symptoms were back inoculated to *Chenopodium amaranticolor*.

3.3 TRANSMISSION

3.3.1 MECHANICAL TRANSMISSION

Sap transmission was conducted using 0.01 M phosphate buffer (pH 7.2). In all sap inoculation studies carborundum powder was used as abrasive. The extract was taken from young leaves showing necrotic ring spotting symptom. One part of the leaf tissue was homogenized 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 carborundum powder. Test plants were inoculated by using finger dipped in the inoculum and 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 eighth leaf of the local lesion host at 8-15 leaf stage was chosen for inoculation. The plants were kept for 6-7 days for the development of symptoms.

3.3.2 SEED TRANSMISSION

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

3.3.3 GRAFT TRANSMISSION

Small shoots showing bud necrosis, necrotic ring spotting and stem lesions 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 tomato plants.

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 tomato 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*. Three replications were maintained for each treatment. The inoculated leaves were 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 tomato as mentioned in the above experiment. Three ml of sap was pipetted into a thin walled glass test tube. Care was taken not to smear the upper part of the test tube. This was placed in a water bath with thermostat arrangement. The level of water was maintained three cm above the level of sap in the tubes. Three ml aliquots of the sap were treated for ten minutes each at 35, 40, 45, 50, 55 and 60°C. After each treatment, the tubes were removed and cooled immediately. Control was kept at room temperature (28 ± 2°C). The samples were inoculated on three fully opened leaves of *C. amaranticolor*. 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 above and the homogenate was filtered through a thin layer of cotton. It was divided into two parts and taken in two test tubes and closed with cotton wool. One tube was kept at room temperature (28 ± 2° C) and other in a refrigerator maintained at 8° C. The homogenate was pippetted into a mortar @ 400 µl for inoculating 3 leaves of C. *amaranticolor* and used for inoculation at specific periods of 2, 4, 6, 8, 12, 24, 48 and 72 hrs, maintaining

three replications for each treatment. The leaves inoculated immediately after inoculation was treated as control. The plants were observed for the development of local lesions.

3.5 HOST PATHOGEN INTERACTION

Biochemical analysis of healthy and diseased plants was carried out. Tomato plants were graft inoculated at one, five, ten, fifteen and thirty 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 performed in native PAGE.

3.5.1 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 5 ml 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.

3.5.2 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.

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

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

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

3.5.3 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) (Appendix-IV) 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.

3.5.4 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 in a boiling water bath. 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. The reaction mixture 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.

3.5.5 ESTIMATION OF DEFENCE RELATED ENZYMES

3.5.5.1 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) (Appendix-I) 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 of 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.

3.5.5.2 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.

3.5.5.3 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) (Appendix - IV) 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 were 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.

3.5.6 SDS- PAGE

Electrophoretic separation of soluble protein of cowpea leaves were carried out as per the procedure described by Laemelli (1970).

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 refrigeration.

e) Electrode buffer pH 8.3

Tris base - 6.0 g

Glycine - 28.8 g

SDS - 2.0 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

SDS 20 % (w/v) - 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

SDS 10 % - 0.2 ml

Acrylamide stock - 8.0 ml

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

10 per cent 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

SDS 10 % - 0.2 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. Immediately after electrophoresis the gel was removed from the glass plates and incubated in the staining solution for overnight with uniform shaking. Then the gel was transferred to the destaining solution. The protein appeared as bands and the gel was photographed after placing it on a transilluminator (Appligene Model White / UV TMW-20).

3.5.7 ELECTROPHORETIC ANALYSIS OF ISOZYME

Electrophoresis of protein extracts from plant tissues using different kinds of support media and buffer systems (Appendix V) 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 tomato 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 supernatant was used for isozyme analysis. The protein content was adjusted in each sample to the 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 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) Stacking 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 and 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 O– dianisidine HCl for 30 minutes at room temperature. The gel was transferred to 0.1 M hydrogen peroxide until visible bands were developed.

3.6 IMMUNO DETECTION

3.6.1 DIRECT ANTIGEN COATING – ENZYME LINKED IMMUNOSORBENT ASSAY (DAC- ELISA)

The detection of tospovirus was done using ELISA . Diseased samples were taken from field. 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 of 1:5 (w/v). The homogenate was centrifuged at 5000 rpm for 10 minutes at 4 °C and samples were added to the wells. 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 mentioned 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 *p*-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 (BIORAD, Model 680 Microplate reader). (Appendix II).

3.6.2 DOT IMMUNO BINDING ASSAY (DIBA) FOR THE DETECTION OF TOSPOVIRUS

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

Procedure

- Tissue was extracted in antigen extraction buffer (1:10 w/v) and filtered 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) in 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).

3.7 MOLECULAR DIAGNOSIS

Molecular diagnosis using polymerase chain reaction (PCR) was performed for the detection of the bud necrosis virus infecting tomato. RNA was extracted using Ultra clean plant RNA isolation kit (Imperial Bio-Medic: Cat#13300-20).

RT-PCR protocol for the detection of TSWV

Primers used:

SL.No.	Primer name	Sequence (5'...........3')	Annealing temperature
1	Tospovirus F	ATGTCTAAC GTYAAGCARCTC	56^0C
	Tospovirus R	TTACAATTCCAGCGAAGGACC	

A PCR mixture was prepared containing specific primer pairs (Okuda and Hanada

,2001). The PCR reaction mixture for each reaction contained 10 mM each of dNTPs, 1 µM of each primer, 2.5 mM $MgCl_2$, 0.5 units of Taq DNA polymerase and template DNA in a total volume of 50 µl.

Reverse transcription was performed at 50^0C for 40 minutes followed by a 95^0C for 15 minutes in a PCR thermocycler (BIORAD). Thermocycler conditions were 35 cycles of denaturation at 94^0C for 30 seconds, annealing at 56^0C for 1 minute, elongation at 72^0C for 1 minute, followed by a final elongation of 72^0C for 30 minutes.

Horizontal gel electrophoresis was performed to view the amplicons obtained after RT-PCR. For 20 µl of sample, 3µl of loading dye was added and mixed. These were then loaded on a 1% agarose gel (w/v) in 1xTAE (Tris acetate EDTA) buffer, containing 8 µl ethidium bromide. Electrophoresis at 75 V for 1 h was done and the gel was viewed with an UV transilluminator.

3.8 MERISTEM CULTURE OF TOMATO

3.8.1 Source of explants

Explants were obtained from tomato plants infected by tospovirus in the field.

3.8.2 Culture media

Standard procedures (Murashige and Skoog, 1962) were followed for the preparation of the plant tissue culture media. Stock solutions of macro, micro, minor nutrients, iron and vitamins were prepared by dissolving adequate quantities of each chemical as per Murashige and Skoog (Appendix VI). stock solutions of the growth regulators BA and IAA were maintained separately. After mixing appropriate quantities of the stock solutions, the pH of the medium was adjusted to 5.7 using 0.1 N NaOH/HCl. and the volume were made up using double distilled water, Agar was added at the rate of 6.3 g l^{-1} for solidification of the medium. The medium was heated to mix the agar. Then the medium was dispensed to the culture vessels at the rate of 15 ml per culture tube. The test tubes were plugged with non absorbent cotton. Medium was sterilized by autoclaving. It was allowed to cool to room temperature and stored in culture room until used.

3.8.3 Preparation of the sterile tissue

The meristem portion was separated from the infected plant, washed in tap water and cut into small sizes. These were soaked in 2% laboline for 30 minutes and again washed with distilled water for three times, kept in sterile distilled water. The explant was then transferred to 0.08% mercuric chloride for 5 minutes and washed in three changes of sterile distilled water.

3.8.4 Dissection and explant preparation

Using sterile forceps the disinfected meristem were transferred to the stage of

dissection microscope previously wiped with alcohol and meristem of 2 mm size was removed using sterile blade. The whole process was carried out in the laminar air flow chamber.

3.8.5 Inoculation of the explant

Inoculation of the excised sterile meristem was done in a laminar air flow chamber. The excised meristem was inoculated into sterile medium.

3.8.6 Incubation of cultures

The culture tubes were placed in dark for 24 h and then kept in incubation room at 24 ± 4^0C giving a photoperiod of 16 h.

3.8.7 Induction media

Murashige and Skoog medium (MS media) with BA at the rate of 1 mg l^{-1} (Appendix VI) was used for induction.

3.8.8 Virus indexing of tissue culture plants

Virus indexing of tissue culture plants was performed using DAC-ELISA as described in para 3.6.1 using polyclonal antiserums for WSMV.

Results

4. RESULTS

4.1 SYMPTOMATOLOGY

Symptoms on naturally infected tomato plants were observed on leaves, stem, growing tips and fruits. The infected young leaves showed small necrotic ring spots, which later turned into necrotic patches leading to the death of the infected leaves (Plate 1a & 1b). Bronzing was also seen on the infected leaves. The growing tips and buds were observed to be severely affected with systemic necrosis (Plate 2). There was complete death of the growing tips. Dark brown streaks were also observed on the stem of infected plants (Plate 3). On mature fruits the symptoms were seen as yellow rings and also orange red discoloration in a concentric pattern (Plate 4). Stunting of plants was also noticed.

4.2 HOST RANGE

Host range studies were conducted with 12 plant species belonging to seven families. The result showed that eight species belonging to four families ie., Chenopodiaceae , Fabaceaea, Solanaceae and Cucurbitaceae produced symptoms of virus disease (Table 1, Plate 5-9).

4.3 TRANSMISSION

4.3.1 MECHANICAL TRANSMISSION

The virus was transmitted successfully through mechanical inoculation to the local lesion host *C. amaranticolor* using 0.01M phosphate buffer (pH 7.2). Symptoms were observed as local lesions seven days after inoculation (Plate 10). There was 100 % transmission.

Table 1. Host range studies of tospovirus isolates from tomato

SL. No.	Host	Symptoms	Percent transmission
1.	AMARANTHACEAE		
	Amaranthus tricolor	NS	-
2.	CHENOPODIACEAE		
	Chenopodium amaranticolor	Chs, NLL	100
	Chenopodium quinoa	Chs, NLL	50
3.	CUCURBITACEAE		
	Cucumis melo var acidulus	Chs	45
	Benincasa hispida	Chs	30
4.	FABACEAE		
	Vigna unguiculata cv Pusa	Chs, NE, BN, SI,	85
	Komal	NLL	30
	Canavalia gladiata	Chs	70
	Vigna radiata	Chs, SI, BN	
5.	SOLANACEAE		
	Capsicum annuum	NS	-
	Lycopersicon esculentum	NL, SI, BN	90
6.	APOCYNACEAE		
	Catharanthus roseus	NS	-
7.	MALVACEAE		
	Abelmoschus esculentus	NS	-

*NS – No symptoms, Chs – Chlorotic spots, NLL – Necrotic local lesions, SI – Systemic infection, BN – Bud necrosis, NE – Necrosis

Plate 1a

Plate 1. Necrotic ring spots on tomato leaves

Plate 1 b

Plate 2. Bud necrosis in tomato

Plate 3. Stem necrosis on tomato plants

Plate 4. Yellow concentric rings on tomato fruit.

Plate 5. Chlorosis on cowpea cv Pusa Komal

Plate 6. Necrotic local lesions on cowpea cv Pusa Komal

Plate 7. Chlorotic spots on sword bean

Plate 8. Chlorosis on *Cucumis melo* var acidulus

Plate 9. Chlorosis on *Benincasa hispida*

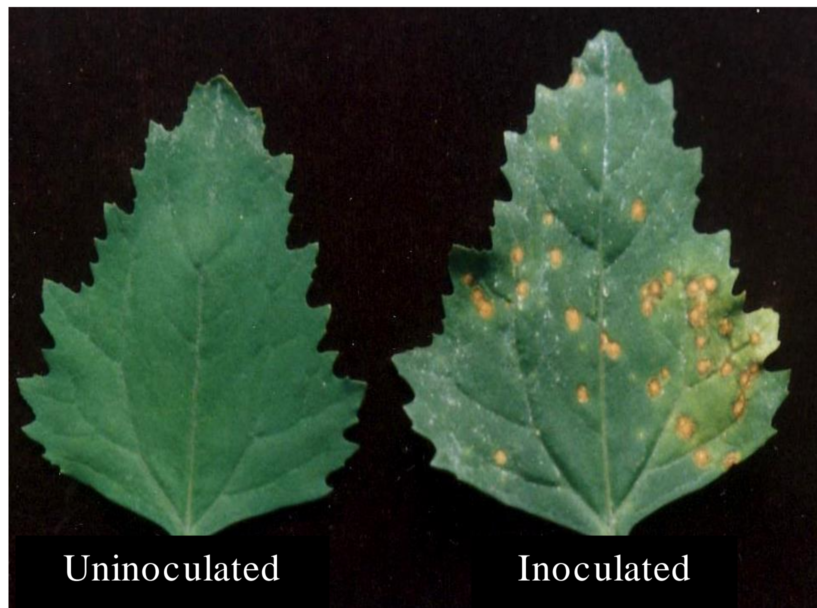


Plate 10. Necrotic local lesions on *Chenopodium amaranticolor*

4.3.2 SEED TRANSMISSION

Symptoms were not observed on seedlings raised from the seeds of infected tomato plants upto 90 days. The per cent seed transmission was found to be nil.

4.3.3 GRAFT TRANSMISSION

Graft transmission studies were carried out by wedge grafting of scion obtained from tospovirus infected tomato plants on the healthy root stock of tomato plants. Grafted plants were grown in insect proof glass house and observed for symptom development. Symptoms appeared after 8-10 days of grafting in new sprouts of the root stock (Plate 11 & 12).

4.4 PHYSICAL PROPERIES

4.4.1 DILUTION END POINT (DEP)

Maximum number of lesions on *C. amaranticolor* was obtained for the undiluted sap (46) and no lesions were observed for 10^{-4} dilution. The data showed that the dilution end point of the virus was between 10^{-3} and 10^{-4} (Table 2).

4.4.2 THERMAL INACTIVATION POINT

Maximum number of lesions on *C. amaranticolor* was observed at 30^0C and at 55^0C no lesions were noticed. The results indicated that the virus was inactivated at a temperature range between 50^0C and 55^0C (Table 3).

Table 2. Dilution end point (DEP) of tospovirus isolate from tomato in *C. amaranticolor*

Sl. No.	Dilutions	Mean number of lesions
1	Control	46
2	10^{-1}	16
3	10^{-2}	3
4	10^{-3}	1
5	10^{-4}	0
6	10^{-5}	0

Table 3. Thermal Inactivation Point (TIP) of tospovirus isolate from tomato in *C. amaranticolor*

Sl. No.	Temperature (°C)	Mean number of lesions
1	30	35
2	35	23
3	40	17
4	45	8
5	50	6
6	55	0
7	60	0

Plate 11. Graft transmission in tomato

Plate 12. Symptoms on graft inoculated tomato

4.4.3 LONGEVITY INVITRO

The results showed that the virus was infective for 24 h at refrigerated condition and upto 8 h at room temperature (Table 4).

4.5 HOST PATHOGEN INTERACTION

4.5.1 BIOCHEMICAL ANALYSIS

4.5.1.1 ESTIMATION OF TOTAL CARBOHYDRATE

The results showed that the carbohydrate levels in inoculated plants were lower compared to the uninoculated tomato plants. In case of uninoulated control plants carbohydrate level was found to decrease from 9.13 mg g^{-1} at one day after inoculation (DAI) to 7.2 mg g^{-1} at 5 DAI, which increased to 8.9 mg g^{-1} at 15 DAI and then to 9.1 mg g^{-1} at 30 DAI. Whereas in case of inoculated plants the carbohydrate content was 6.4 mg g^{-1} at one DAI which decreased to 6.1 mg g^{-1} at 5 DAI, then it increased to 6.4 mg g^{-1} at 15 DAI and 8.6 mg g^{-1} at 30 DAI in tospovirus inoculated tomato plants (Table 5).

4.5.1.2 ESTIMATION OF CHLOROPHYLL

The samples were estimated for chlorophyll a, chlorophyll b and total chlorophyll at different days after inoculation. The content of chlorophyll a, b and total chlorophyll was found to be lower in inoculated tomato plants as compared to the healthy tomato plants. In case of healthy plants chlorophyll a content increased from 1.83 mg g^{-1} at 1 DAI to 2 mg g^{-1} at 30 DAI and chlorophyll b decreased from 0.68 mg g^{-1} at1 DAI to 0.28 mg g^{-1} at 30 DAI. Total chlorophyll was 2.54 mg g^{-1} at 1 DAI which decreased to 2.31 mg g^{-} at 30 DAI in case of uninoculated healthy plants. Whereas in case of tospo virus inoculated tomato plants, chlorophyll a content decreased from 1.6 mg g^{-1} at 1 DAI to 1.3 mg g^{-1} at 30 DAI. Chlorophyll b content decreased from 0.56 mg g^{-1} at 1 DAI to 0.39 mg g^{-1} at 30 DAI and the content of total chlorophyll was found to decrease from 2.29 mg g^{-1} at 1 DAI to 1.76 mg g^{-1} at 30 DAI (Table 6).

Table 4. Longevity *in vitro* (LIV) of tospovirus isolate from tomato in *C. amaranticolor*

Sl. No.	Aging in hours	Mean number of lesions	
		(8°C)	(28±2°C)
1	0	30	30
2	2	14	10
3	4	10	10
4	6	9	3
5	8	8	0
6	12	1	0
7	24	0	0
8	48	0	0
9	72	0	0

Table 5. Changes in total carbohydrate content of tomato leaves in response to tospovirus inoculation

Days after inoculation	*Change in carbohydrate content mg g^{-1} fresh weight of tissue	
	Healthy	Inoculated
1	9.13	6.4
5	7.2	6.1
15	8.9	6.4
30	9.1	8.6

Healthy X Inoculated - 0.56, Days - 0.79, Healthy X Inoculated X Days - 0.39

* Mean of three replications

Table 6. Changes in Chlorophyll content of tomato 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	1.83	0.68	2.54	1.6	0.56	2.29
5	1.6	0.28	1.93	1.0	0.4	1.44
15	1.9	0.4	2.38	1.3	0.45	1.86
30	2.0	0.28	2.31	1.3	0.39	1.76

	Chlorophyll- a	Chlorophyll- b	Total Chlorophyll
Healthy X Inoculated	9.99	9.14	0.74
Days	0.14	0.12	0.10
Healthy X Inoculated X Days	7.06	6.46	5.29

* Mean of three replications

4.5.1.3 ESTIMATION OF PHENOL

The results indicated significant difference in the phenol contents of inoculated and uninoculated tomato plants. The phenol content was found to be more in inoculated plants. The total phenol content was found to be highest at 15 DAI in case of both healthy and inoculated plants. It was 28.66 $\mu g\ g^{-1}$ in healthy and 37.33 $\mu g\ g^{-1}$ in inoculated plants. Thereafter the phenol contents were found to decline (Table 7).

4.5.1.4 ESTIMATION OF PROTEIN

The total soluble protein content was estimated in tospovirus inoculated and healthy tomato plants. The results showed higher protein contents in inoculated plants compared to the healthy plants. The protein content recorded in the healthy plants was 29.66 $\mu g\ g^{-1}$ at 1 DAI and increased to a maximum of 33 $\mu g\ g^{-1}$ at 15 DAI. In case of inoculated plants the protein content was 31.33 $\mu g\ g^{-1}$ at 1 DAI which increased and reached a maximum value of 45.66 $\mu g\ g^{-1}$ at 15 DAI. Thereafter lower protein content was recorded (Table 8).

4.5.1.5 DEFENCE RELATED ENZYMES

4.5.1.5.1 PEROXIDASE

Peroxidase activity was found to be higher in tospovirus inoculated plants compared to healthy uninoculated plants. The activity of the enzyme was found to be the highest at 5 DAI (200 $min^{-1}\ g^{-1}$) in case of inoculated plants whereas a lower value of 66.33 $min^{-1}\ g^{-1}$ was obtained in case of control plants. The activity of peroxidase enzyme was later found to decrease (Table 9).

4.5.1.5.2 POLYPHENOL OXIDASE

There was a significant difference between the activity of polyphenol oxidase in inoculated and uninoculated plants. Activity of the enzyme was found to be higher in tospo virus inoculated plants. The maximum level of activity of the enzyme

Table 7. Changes in Phenol content of tomato leaves in response to tospovirus inoculation

Days after inoculation	*Change in Phenol content (μg g^{-1} fresh weight of tissue)	
	Healthy	Inoculated
1	28.00	26.33
5	10.66	12.66
15	28.66	37.33
30	10.00	16.83

Healthy X Inoculated - 0.94 Days - 1.33, Healthy X Inoculated X Days - 0.66
* Mean of three replications

Table 8. Changes in Total soluble protein content of tomato leaves in response to tospovirus inoculation

Days after inoculation	*Change in soluble protein content (μg g^{-1} fresh weight of tissue)	
	Healthy	Inoculated
1	29.66	31.33
5	28.66	33.00
15	33.00	45.66
30	28.33	39.00

Healthy X Inoculated - 4.65, Days - 6.58, Healthy X Inoculated X Days - 3.29

* Mean of three replications

Table 9. Changes in peroxidase activity of tomato leaves in response to tospovirus inoculation

Days after inoculation	*Peroxidase activity(changes in absorbance $min^{-1}g^{-1}$ fresh weight)	
	Healthy	Inoculated
1	39.00	49.33
5	66.33	200.00
15	61.33	151.00
30	42.00	49.66

Healthy X Inoculated -15.37, Days - 21.74 , Healthy X Inoculated X Days - 10.87

* Mean of three replications

Table 10. Changes in Polyphenol oxidase activity of tomato leaves in response to tospovirus inoculation

Days after inoculation	*Polyphenol oxidase activity(changes in absorbance $min^{-1}g^{-1}$ fresh weight)	
	Healthy	Inoculated
1	1.40	5.16
5	0.16	0.60
15	0.06	0.25
30	1.76	3.66

Healthy X Inoculated - 0.65, Days - 0.92, Healthy X Inoculated X Days -0.46

* Mean of three replications

(5.16 min^{-1} g^{-1}) was found in inoculated plants at 1 DAI. Later the enzyme activity was found to decrease from 0.6 min^{-1} g^{-1} at 5 DAI to 0.25 min^{-1} g^{-1} at 15 DAI. There after there was an increase in the activity to 3.66 min^{-1} g^{-1} at 30 DAI was noticed. In case of uninoculated plants the activity decreased initially to 0.06 min^{-1} g^{-1} at 15 DAI but was found to increase to 1.76 min^{-1} g^{-1} at 30 DAI (Table 10).

4.5.1.5.3 PHENYL ALANINE AMMONIA-LYASE

PAL activity was higher in inoculated plants compared to the healthy plants. The activity was found to initially increase and then decrease in case of both uninoculated and inoculated plants. In healthy plants PAL activity was 0.8 µg g^{-1} min^{-1} at 15 DAI which was decreased to 0.7 µg g^{-1} min^{-1} at 30 DAI. In the case of inoculated plants a maximum PAL activity of 3.07 µg g^{-1} min^{-1} was found at 15 DAI, thereafter it was reduced to 1.5 µg g^{-1} min^{-1} at 30 DAI (Table 11).

4.5.2 SDS-PAGE (SODIUM DODECYL POLYACRYLAMIDE GEL ELECTROPHORESIS

Protein profiles of uninoculated healthy tomato plant samples and tospovirus infected plant samples were analysed. Protein profile of healthy samples produced six different proteins and the virus infected samples produced nine separate proteins with different molecular weight. Out of the nine proteins six proteins were identical to that of the healthy and three extra protein bands were obtained. Molecular weights of these extra bands were estimated using protein markers loaded along with the samples. The three extra novel proteins in the diseased samples were with molecular weight 28, 15 and 12 kDa respectively. The extra proteins found in the inoculated plants were induced in the plant due to the virus infection (Plate 13).

Table 11. Changes in Phenylalanine ammonia- lyase activity of tomato leaves in response to tospovirus inoculation

Days after inoculation	*Phenylalanine ammonia-lyase activity(changes in absorbance µg $g^{-1} min^{-1}$ fresh weight)	
	Healthy	Diseased
1	0.46	3.05
5	0.57	2.10
15	0.80	3.07
30	0.70	1.50

Healthy X Inoculated - 1.43, Days - 2.02 , Healthy X Inoculated X Days - 1.01

* Mean of three replications

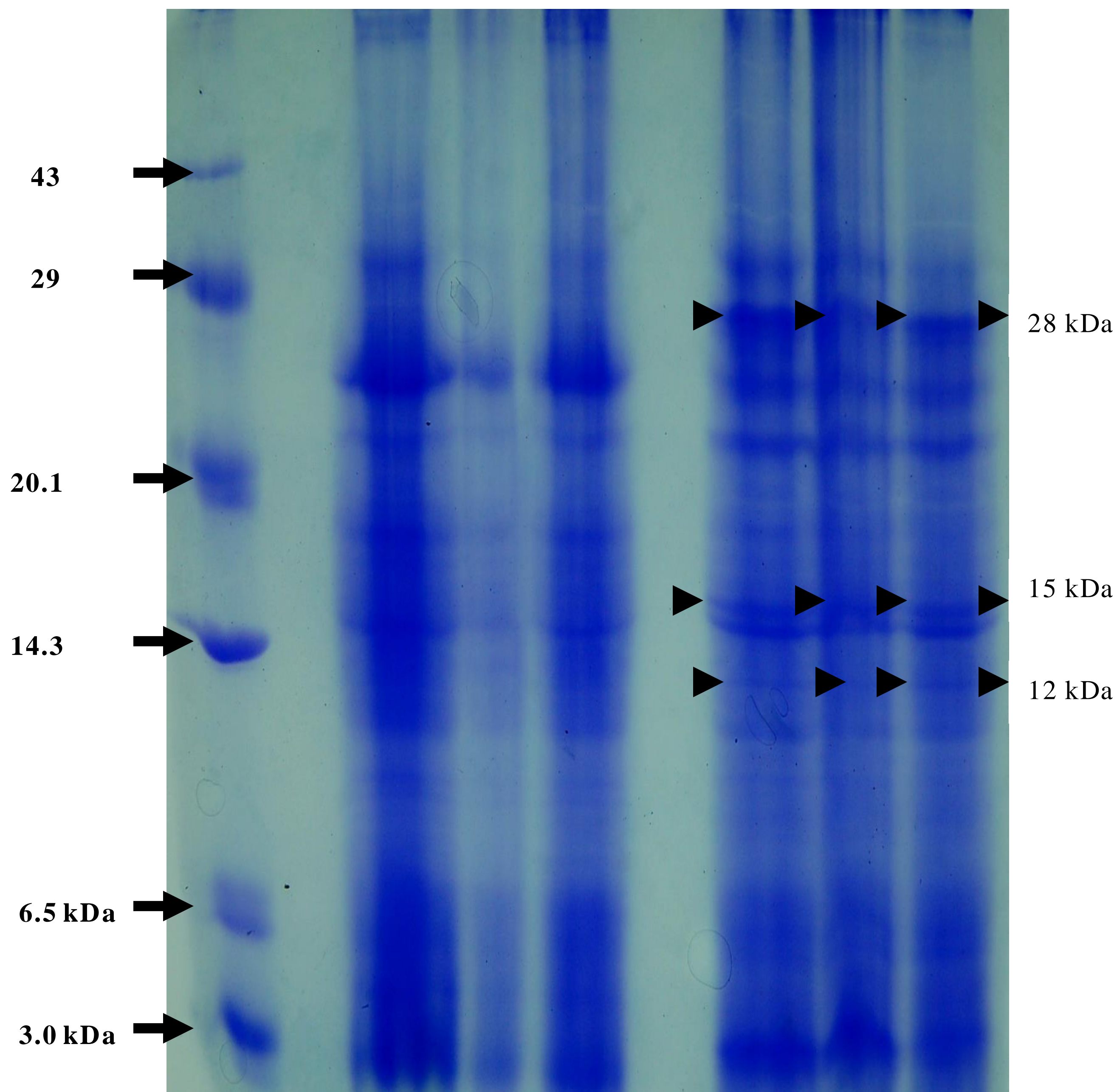


Plate 13. Protein profile of tomato

4.5.3 ISOZYME ANALYSIS

Native polyacrylamide gel electrophoresis was carried out for isozyme analysis of polyphenol oxidase. The experiment was performed to find out the presence of isozymes and their intensity in both virus inoculated and healthy tomato plants. Isozyme analysis of PPO produced three isoforms in both healthy and inoculated plants. Same banding patterns were observed. These isoforms are with relative mobility (Rm) values of 0.60 and 0.77 respectively. The activity of the two isoforms expressed, were more prominently noticed in the inoculated plants compared to the uninoculated control (Table 12, Plate 14).

Table 12. Rm value of Polyphenol oxidase

Iso PPOs	Healthy control	Inoculated
Iso PPO1	0.60	0.60
Iso PPO2	0.77	0.77

4.6 IMMUNOLOGICAL AND MOLECULAR DIAGNOSIS

4.6.1 ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA)

Polyclonal antibodies for TSWV and WSMV were used for the detection of the virus causing the bud necrosis disease of tomato. The absorbance was measured at 405 nm in an ELISA reader (BIORAD Microplate reader 680). The results of the experiment revealed that the virus causing the bud necrosis disease of tomato failed to react with the antisera specific to TSWV but showed positive reaction with the antisera of WSMV. The absorbance of the diseased sample against the antisera of WSMV was more than 3 times than that of the healthy sample. The average absorbance of diseased samples against the antisera of WSMV was 0.81 at 405 nm and only 0.016 against the antisera of TSWV. The high reactivity of the antibody for WSMV towards the virus revealed the

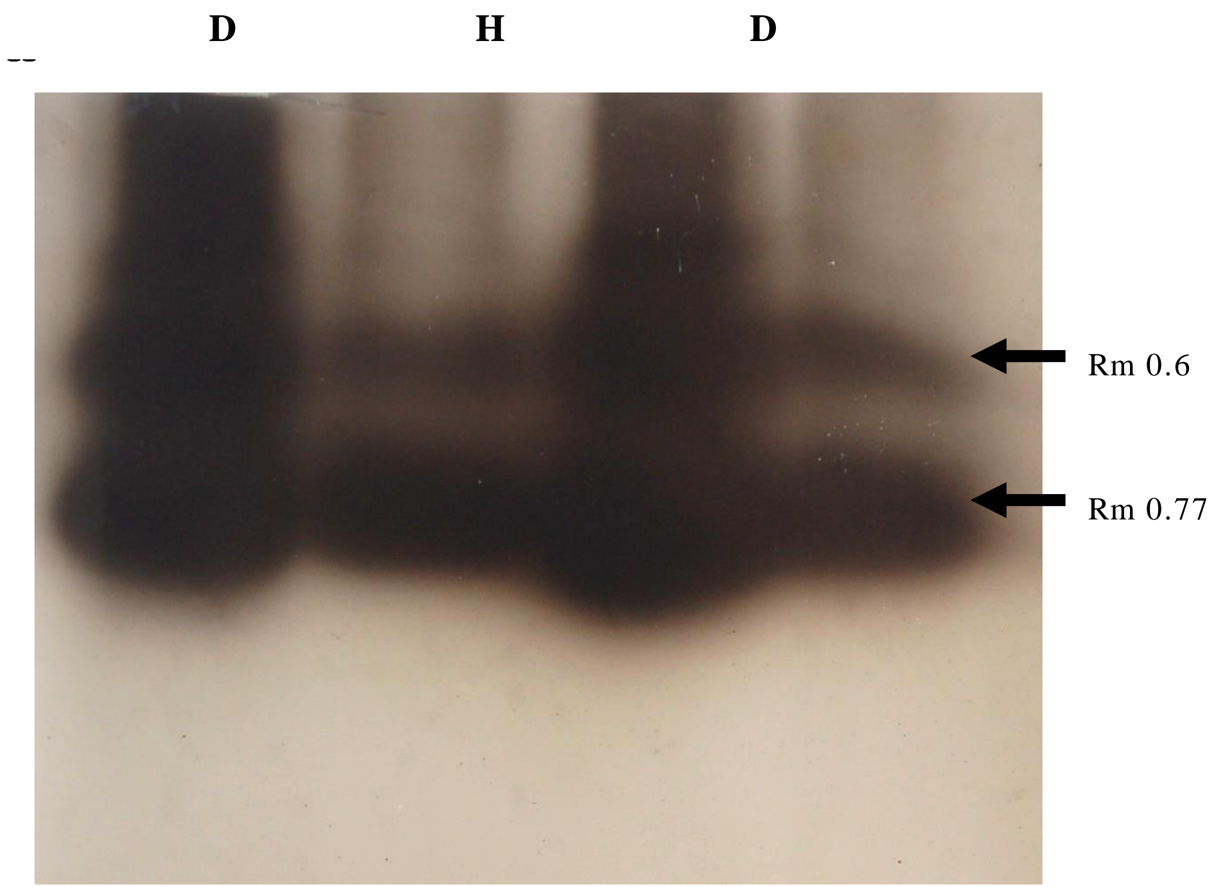


Plate 14 . Polyphenol oxidase profile in tomato

close serological relationship of WSMV with the bud necrosis disease of tomato (Table 13, Plate 15).

Table 13. Reaction of tospovirus isolate from tomato using direct antigen coating ELISA

Samples	*Absorbance at 405nm		
	TSWV	WSMV	Remarks
Leaves from healthy Sample	0.012	0.076	Sample were found positive to WSMV
Leaves from diseased Sample	0.016	0.813	

* Mean of three replications

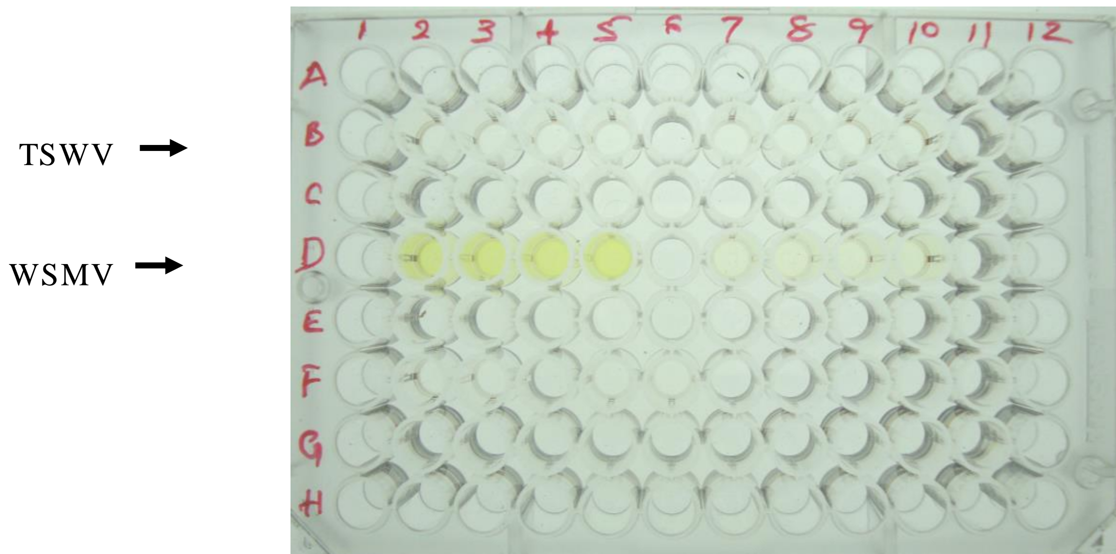
4.6.2 DOT IMMUNOBINDING ASSAY (DIBA)

DIBA was also conducted to detect the virus causing the bud necrosis of tomato. DIBA was done using polyclonal antibody for WSMV as it is a closely related tospovirus and was confirmed by DAC- ELISA. The result of the experiment was assessed by visual observation by comparing the intensity of the purple colour. Purple coloured spots were recorded on the point were diseased samples were loaded indicating a positive reaction (Plate 16).

4.6.3 MOLECULAR DIAGNOSIS

Molecular diagnosis using PCR was performed for the detection of the tospovirus infecting tomato.

Plate 15. Reaction of tospovirus isolate from tomato using DAC - ELISA

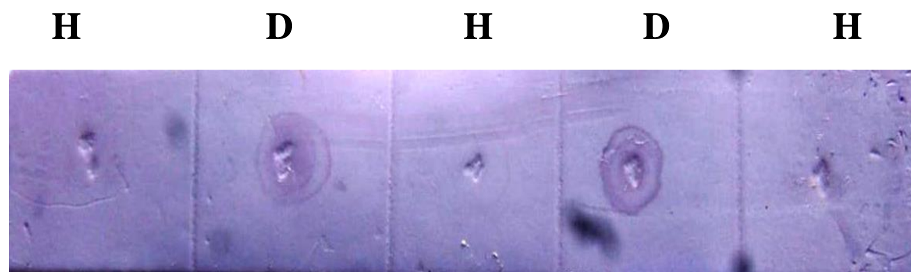


Diseased sample – B2, B3, B4, B5, D2, D3, D4 and D5

Healthy sample – B7, B8, B9, B10, D7, D8, D9 and D10

Buffer – F2, F3, F5 and F6

Plate 16. Detection of tospovirus in tomato using DIBA



H – Healthy sample
D – Diseased sample

PCR product

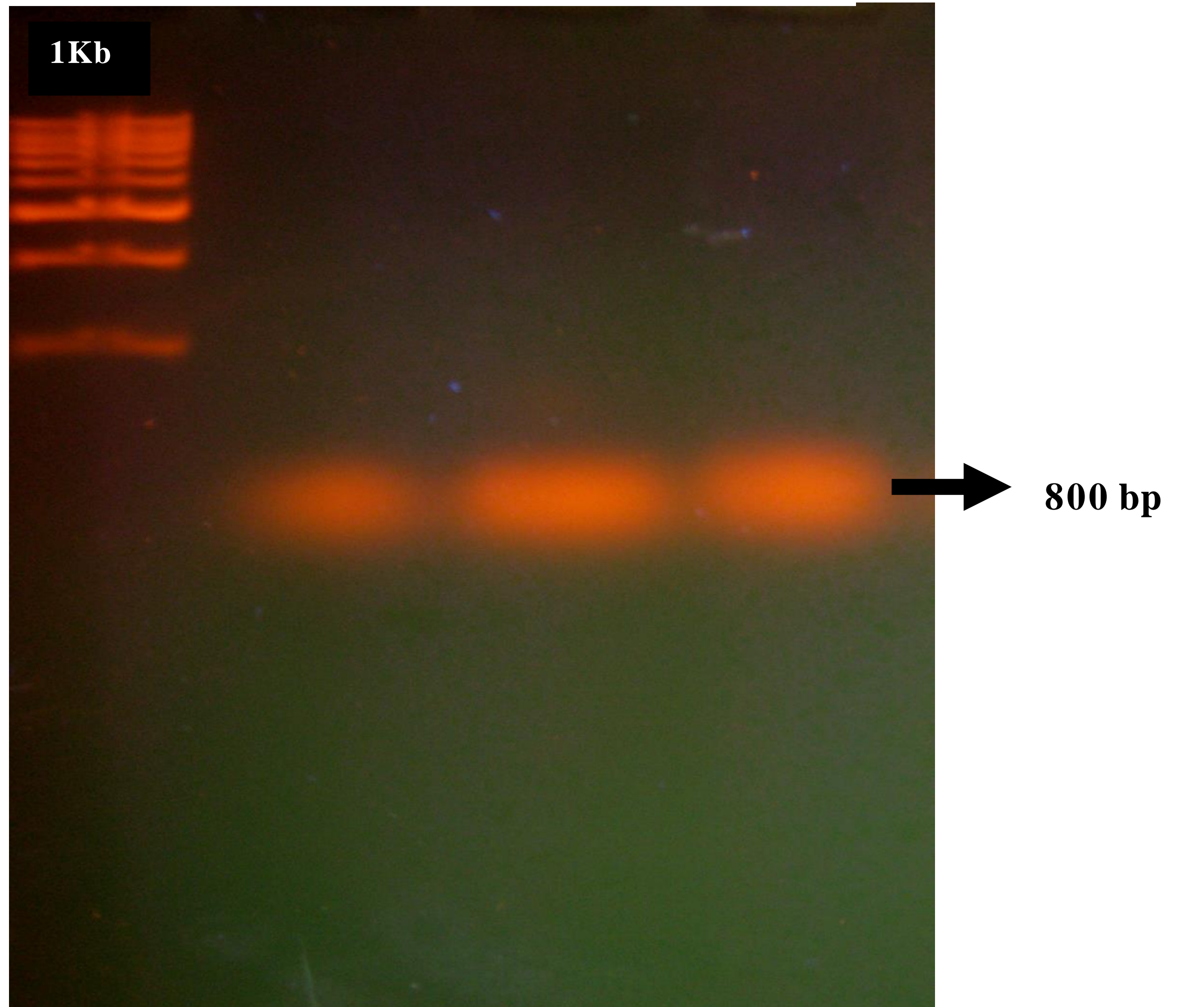


Plate 17. RT-PCR : Detection of bud necrosis virus infecting tomato

Primer specific for the tospovirus was used for diagnosing virus infected samples. PCR product of amplicon size of about 800 bp was observed (Plate 17).

4.6 ELIMINATION OF VIRUS THROUGH MERISTEM CULTURE

The meristem of bud necrosis infected tomato plant was regenerated into plantlets, which were then subjected to DAC- ELISA to detect the presence of the tospovirus. The absorbance of plantlet regenerated from healthy and infected meristem were found to be 0.13 and 0.12 respectively which was on par with the healthy field sample but lower than that of the infected field sample used as the positive control which recorded an absorbance of 0.81. Thus meristem culture of tomato was found to be successful in eliminating the virus and for producing virus free plantlets (Plate 18-22, Table 14).

Table 14. Reaction of plantlets regenerated from meristem of tospovirus infected plants in DAC- ELISA

Sl. No.	Sample	*Absorbance at 405 nm (WSMV)	Remarks
1.	Meristem from healthy tomato plants	0.11	Plantlets regenerated from the meristem of infected plants was free of the bud necrosis virus.
2.	Meristem from infected tomato plants	0.12	
3.	Infected field sample	0.81	
4.	Healthy field sample	0.13	

* Mean of three replications

Plate 18 . Callus produced from tomato meristem

Plate 19 . Multiple shoots regenerated from tomato meristem

Plate 20 . Subculturing of tomato shoots developed by meristem culture

Plate 21. Rooting of tomato shoots developed by meristem culture

Discussion

5. DISCUSSION

Tomato (*Lycopersicon esculentum* Mill.) is an important commercial and dietary vegetable crop in India. Various diseases and pests are major constraints in increasing production of the crop. Among the different diseases, bud necrosis disease of tomato caused by a tospovirus is rising to alarming proportions in Kerala and is causing severe damage to the crop. Tospoviruses belonging to the family Bunyaviridae are a highly cosmopolitan group and have been reported worldwide infecting a broad range of crops and hence considered to be the most economically important plant pathogens in the world at present. Taking into consideration the importance of tospovirus in the cultivation of tomato in our state, this study was undertaken to identify and characterize the virus and to obtain more information on the etiology and management of the disease.

5.1. SYMPTOMATOLOGY

The characteristic symptoms of the disease under study were chlorotic ring spots on emerging leaves, necrosis of the growing point, necrotic lesions on the stem and yellow and orange coloured rings on the fruits. Initially the symptoms appeared as small chlorotic ring spots on the leaves which later turned necrotic. The necrotic spots later coalesced to form necrotic patches which finally lead to the death and shedding of the affected leaves. Severe bud necrosis is another important symptom caused by the tospovirus.

These symptoms were found to be similar to those caused by tospovirus reported earlier. German et al. (1992) reported that symptoms induced by tospoviruses are highly varied and include ring spots, line patterns, wilting, stunting, silvering, mottling, bronzing, chlorosis, necrosis and a range of leaf and stem lesions. Adam and Kegler (1994) reported that the nature of symptoms depends on the tospovirus species, the virulence of the virus strain and the environmental conditions. Jain et al. (2002) reported that the natural infection of tospovirus on mungbean, cowpea and tomato is characterized by severe necrosis of leaves, stem, growing point, buds, pods and fruits. Soler et al. (2003) reported that in TSWV (tomato spotted wilt virus) infected tomato plants the younger leaves turn violet and severe stem necrosis is also seen.

Umamaheswaran et al. (2003) reported that tospovirus infection leads to bronzing and severe necrosis of leaves and growing buds in tomato plants.

Hassani et al. (2005) reported that the symptoms on tomato consisted of systemic chlorotic and necrotic spots on leaves and yellow rings on fruits and the plants generally showed growth reduction due to TSWV infection. Hemalatha et al. (2008) also reported symptoms like chlorotic and necrotic ring spots on leaves, necrosis of growing tip, tip wilting and yellowing induced by tospovirus.

In the present study symptoms such as chlorotic ring spots on emerging leaves, necrosis of the growing point, necrotic lesions on the stem and yellow and orange coloured rings on the fruits. Initially the symptoms appeared as small chlorotic ring spots on leaves which later turned necrotic. The necrotic spots coalesced to form necrotic patches which finally lead to the death and shedding of the affected leaves. Severe bud necrosis was also observed. The symptoms observed in tomato were found to be similar to earlier reports of tospovirus infection on tomato.

5.2 HOST RANGE

Host range studies of the virus with 12 plant species belonging to seven families were done and 8 species belonging to 4 families ie Fabaceae, Solanaceae, Cucurbitaceae and Chenopodiaceae produced symptoms on mechanical inoculation. Systemic symptoms on *Vigna unguiculata* cv Pusa Komal, *Vigna radiata, Lycopersicon esculentum, Cucumis melo, Benincasa hispida and Canavalia gladiata.* Local lesions were observed on *Chenopodium amaranticolor, Chenopodium quinoa* and *Vigna unguiculata* cv Pusa komal. Umamaheswaran et al. (2003) reported that species belonging to Amaranthaceae, Chenopodiaceae, Fabaceae and Solanaceae families are hosts of tospovirus and was found to infect *Arachis hypogea, Vigna mungo, Vigna radiata, V. unguiculata, Macrotyloma uniflorum* and *Physalis floridana.* Jain et al. (2006) reported natural infection of tospoviruses on three cucurbitaceous (*Cucumis sativus, Luffa acutangula, Citrullus lanatus*) and three Fabaceous (*Vigna unguiculata, Phaseolus vulgaris, Dolichos lablab*) vegetable crops in India. From the present investigation it was observed that the tospovirus causing bud necrosis disease of tomato has a wide host range and hence the different hosts like cowpea, sword bean, culinary

melon, green gram etc could act as alternate or collateral hosts of the virus and may lead to severe infection of tomato plants if grown along or near these crops.

5.3 TRANSMISSION STUDIES

There are many reports indicating the successful transfer of tospovirus using 0.01 M phosphate buffer (pH 7.2) containing 0.1 per cent 2-mercaptoethanol. Bhat et al. (2001) reported mechanical transmission of tospovirus to indicator hosts like, cowpea (Pusa Komal) using 0.05M potassium phosphate buffer pH 7.0. Umamaheswaran et al. (2003) also reported mechanical inoculation of the virus isolate from tospo virus infected tomato plants to cowpea (*Vigna unguiculata* cv Pusa Komal) under primary leaf stage using 0.01 M potassium phosphate buffer (pH 7.2) containing 0.1% β-mercaptoetanol. Saritha and Jain (2007) reported the mechanical inoculation of GBNV isolate on local lesion host *Vigna unguiculata* cv. Pusa komal using 0.1 M phosphate buffer, containing 0.1% mercaptoethanol and celite as abrasive. Hemalatha et al. (2008) reported efficient transmission of tospovirus isolates to *Nicotiana benthamiana* using 50 mM potassium phosphate buffer, pH 7.5 containing 0.02 M mercaptoethanol. Pissawan et al. (2008) have reported the successful transfer of tospovirus to diagnostic plants like *Nicotiana glutinosa* and *V. unguiculata* using cold 0.05 M sodium phosphate buffer pH 7.0 with 0.2 % mercaptoethanol.

In the present investigation the bud necrosis virus was very efficiently transmitted by mechanical means using 0.01 M phosphate buffer (pH 7.2) containing 0.1 per cent 2-mercaptoethanol with carborundum powder as abrasive to both cowpea cv. Pusa Komal as well as to the local lesion host, *C. amaranticolor*. Local lesions were observed seven days after inoculation on *C. amaranticolor*. Necrotic lesions were observed on cowpea cv. Pusa Komal 5 days after inoculation. The findings of the present study are in line with previous reports (Bhat et al. 2001, Umamaheswaran et al., 2003, Saritha et al., 2007, Hemalatha et al., 2008 and Pissawan et al., 2008).

The bud necrosis virus infecting tomato was not found to be transmitted through the seeds of tomato. No symptoms were noticed in the seedlings raised from infected tomato plants. Similar results were reported earlier by many authors. Ghanekar et al. (1979) reported that none of the seedlings raised from seeds collected from tospovirus

infected plants developed disease symptoms and hence not seed borne. Antignus et al. (1997) reported that no virus transmission was found in seeds collected from tospovirus infected vegetable and ornamental crops. Gera et al. (2000) also reported that seedlings produced from the seeds of TSWV infected tomato plants showed no visible symptoms of tospovirus infection and further gave negative results in ELISA indicating that the virus is not seed borne. Thus finding of the present investigation is in accordance with previous reports(Ghanekar et al.,1979, Antignus et al., 1997, Gera et al., 2000)

Successful graft transmission has been reported by different researchers. Roy and Gupta (1977) reported 100 % graft transmission of TSWV in pea. Ghanekar et al. (1979) reported successful graft transmission of the tospovirus causing groundnut bud necrosis to healthy groundnut plants. Solomon-Blackburn and Barker (2001) reported that plants can be graft inoculated with TSWV successfully. Ariyaratne et al. (2004) proved successful graft transmission of tospovirus in tomato by grafting virus infected scions on healthy root stocks.

In the present study also, successful graft transmission of the bud necrosis virus was observed as reported by many authors. Tospovirus isolate from infected tomato plants was found to be successfully transmitted to healthy tomato plants by grafting of infected shoots to healthy root stocks by wedge and cleft grafting. Symptoms were observed on the new sprouts of the root stock as chlorotic rings.

5.4 PHYSICAL PROPERTIES

Physical properties are characteristic for a virus. Dilution end point (DEP) thermal inactivation point (TIP) and longevity *in vitro* (LIV) were studied in the present investigation. The virus recorded a DEP in the range of 10^{-3} to 10^{-4} TIP of $50^{0}C$ to $55^{0}C$, and LIV of 8 h at room temperature ($28\pm2^{o}C$) and 24 h at refrigerated condition ($8^{0}C$) (Fig 1- 3). Similar results were reported in case of DEP and TIP by Mali and Pahl (1980).

Silveira et al. (1985) also reported the physical properties of tospovirus from *Sechium edule*. The TIP was between $45^{0}C$ to $50^{0}C$. DEP was 10^{-3} to 10^{-4} and longevity at room temperature was less than 2h. DEP and TIP were found to be in line with those

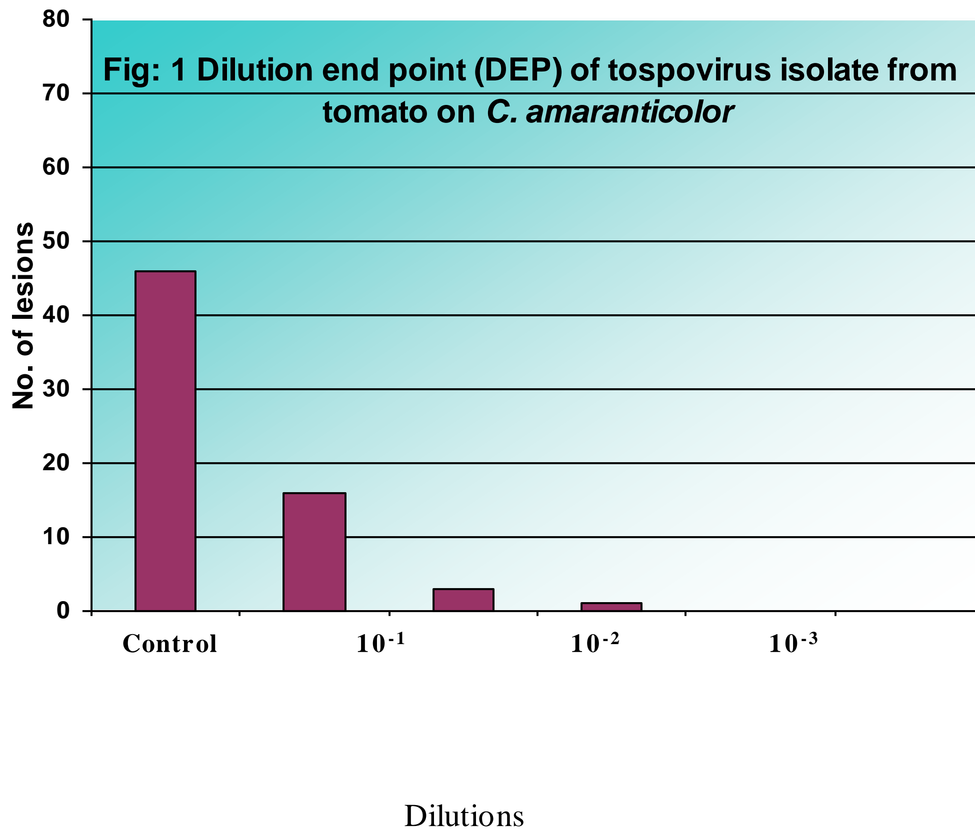


Fig: 1 Dilution end point (DEP) of tospovirus isolate from tomato on C. amaranticolor
No. of lesions
80
70
60
50
40
30
20
10
0
Control
10-1
10-2
10-3
Dilutions

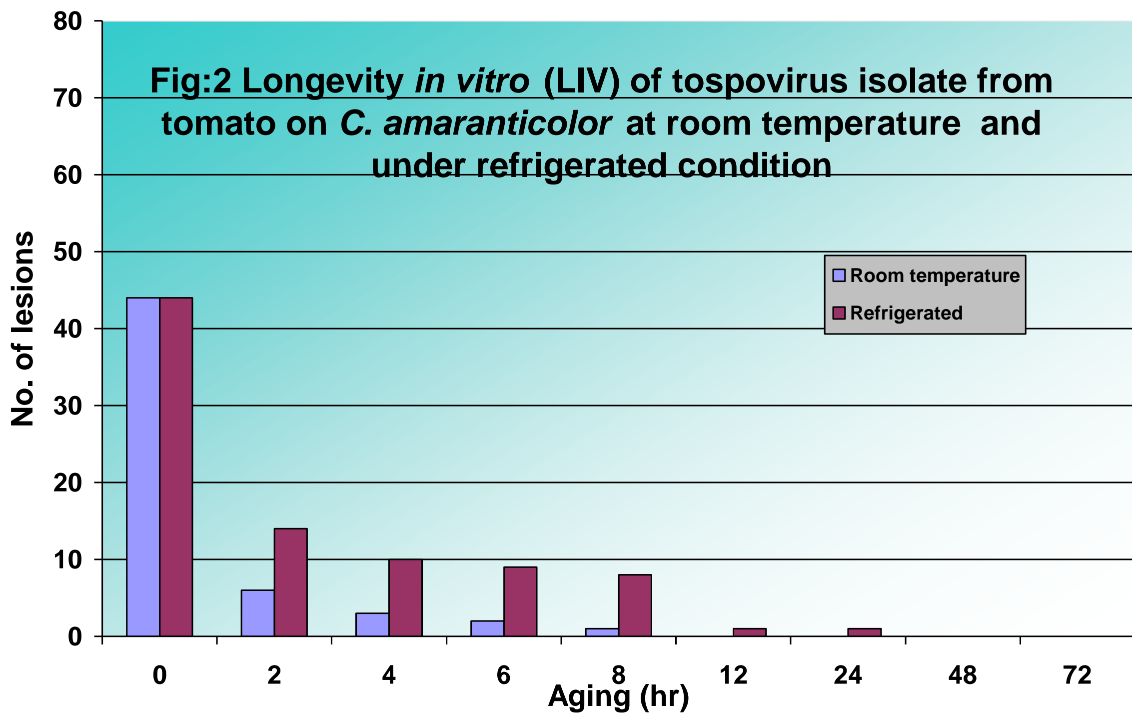


Fig:2 Longevity in vitro (LIV) of tospovirus isolate from tomato on C. amaranticolor at room temperature and under refrigerated condition
No. of lesions
80
70
60
50
40
30
20
10
0
Room temperature
Refrigerated
0
2
4
6
8
12
24
48
72
Aging (hr)

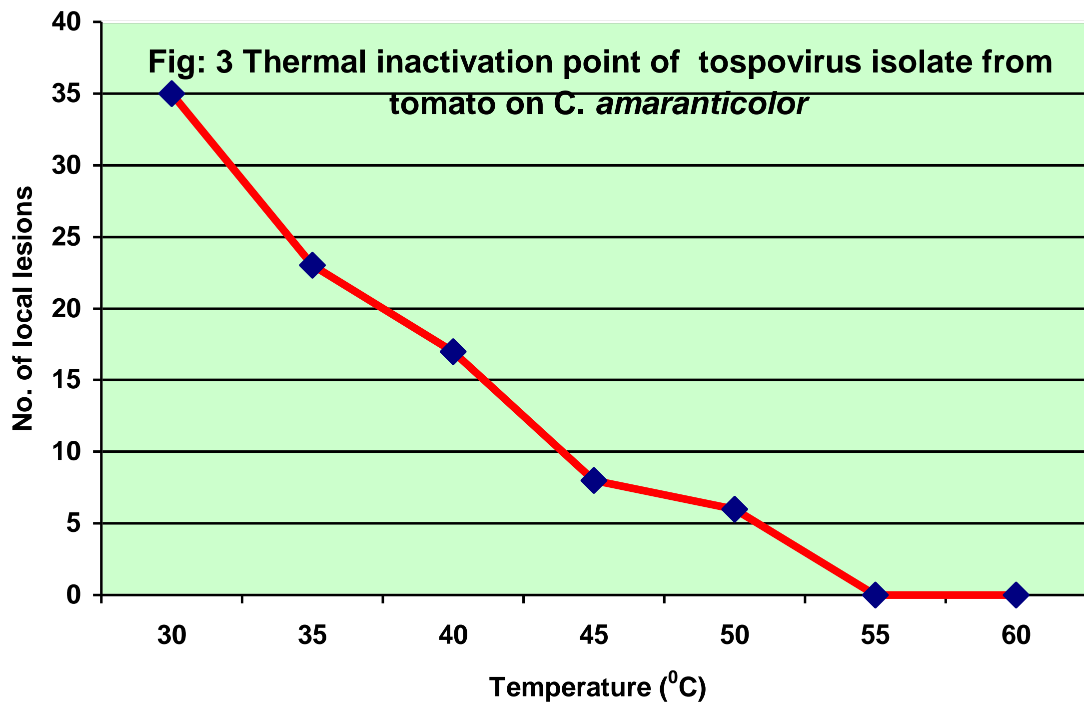


Fig: 3 Thermal inactivation point of tospovirus isolate from tomato on C. *amaranticolor*
No. of local lesions
40
35
30
25
20
15
10
5
0
30
35
40
45
50
55
60
Temperature (^{0}C)

obtained in the present study ie 10^{-3} to 10^{-4} but a difference was observed in the case of LIV which was found to be 8 hrs.

5.5 HOST PATHOGEN INTERACTION

Biochemical changes due to host pathogen interaction were also studied in the present investigation. A significant difference was observed in the total carbohydrate content of healthy and tospovirus inoculated plants. The level of the total carbohydrate content was lower in tospovirus inoculated tomato plants. In the case of uninoculated control carbohydrate level was found to decrease from 9.13 mg g^{-1} at one day after inoculation to 7.2 mg g^{-1} at 5 day after inoculation, which increased to 8.9 mg g^{-1} at 15 DAI and then to 9.1 mg g^{-1} at 30 DAI. Whereas in case of inoculated plants the carbohydrate content was 6.4 mg g^{-1} at one DAI which decreased to 6.1 mg g^{-1} at 5 DAI then it increased to 6.4 mg g^{-1} at 15 DAI and 8.6 mg g^{-1} at 30 DAI in tospo virus inoculated tomato plants (Fig 4). Several studies have shown similar results related to the carbohydrate content in virus infected plants. Umamaheswaran (1996) reported lower levels of total carbohydrate in cowpea inoculated with CABMV. Sutha et al. (1998 a) found that 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. Sutha et al. (1998 b) further reported that TSWV infection reduced the concentration of total sugars of tomato fruits. Mali et al. (2000) reported that infection of yellow mosaic virus in mothbean resulted in reduction of total soluble carbohydrate. The reduction in the level of carbohydrate was due to the breakdown of carbohydrate which is accelerated during respiration in virus infected plants as suggested by Narayanaswamy and Ramakrishnan (1966).

Virus infection has been reported to cause reduction in chlorophyll content in plants. Sutha et al. (1998 a) reported reduced chlorophyll content in tomato following infection with TSWV. Momol and Pernezny, (2006) reported reduction in total chlorophyll, chlorophyll a and chlorophyll b contents in mungbean plants infected by mungbean yellow mosaic virus. Farooq et al. (2007) reported that chlorophyll content in tomato leaves of healthy plants was significantly higher compared to TSWV infected plants. Arpita and Subrata (2008) reported gradual reduction in green pigments like

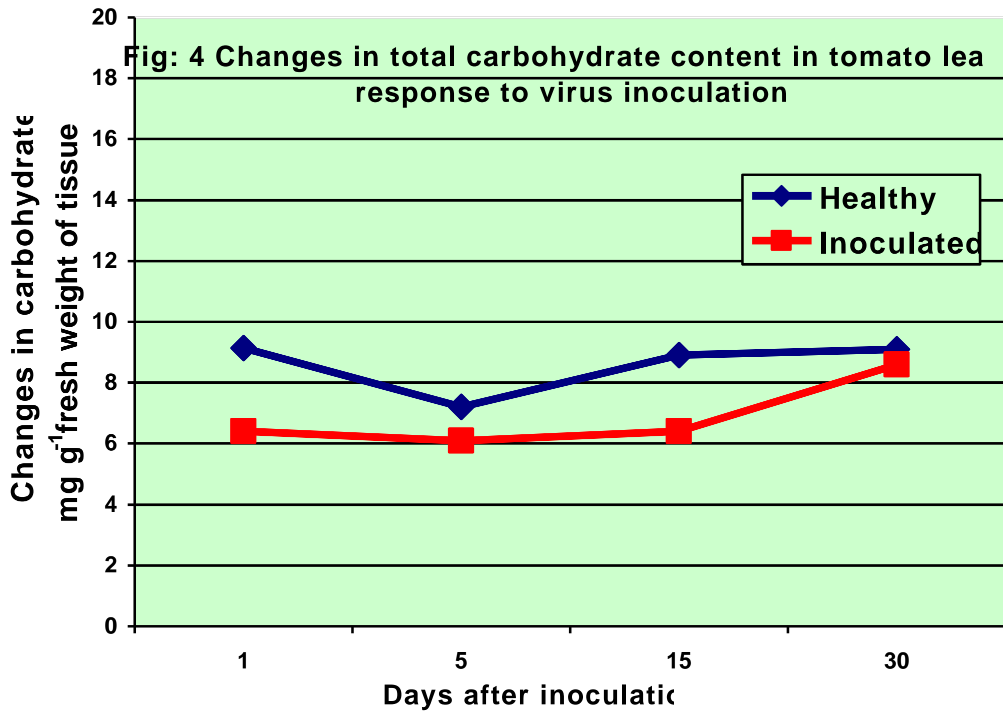


Fig: 4 Changes in total carbohydrate content in tomato lea
response to virus inoculation
Changes in carbohydrate
mg g-1 fresh weight of tissue
Healthy
Inoculated
20
18
16
14
12
10
8
6
4
2
0
1
5
15
30
Days after inoculatic

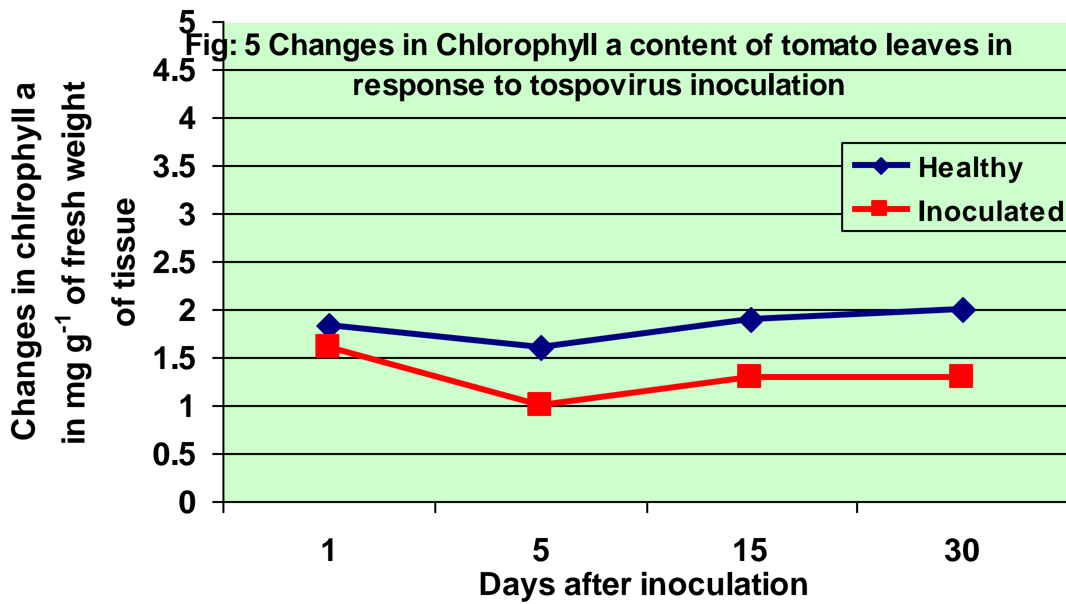


Fig: 5 Changes in Chlorophyll a content of tomato leaves in
response to tospovirus inoculation
Changes in chlrophyll a
in mg g-1 of fresh weight
of tissue
Healthy
Inoculated
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
1
5
15
30
Days after inoculation

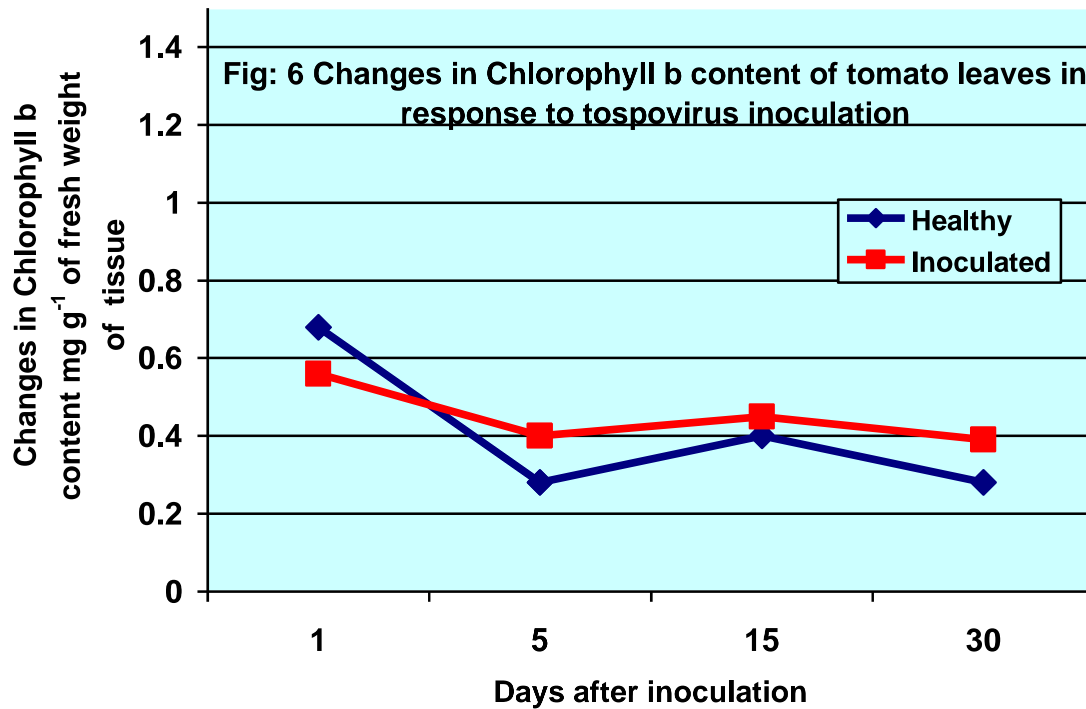


Fig: 6 Changes in Chlorophyll b content of tomato leaves in response to tospovirus inoculation
Changes in Chlorophyll b content mg g-1 of fresh weight of tissue
Days after inoculation
Healthy
Inoculated
1.4
1.2
1
0.8
0.6
0.4
0.2
0
1
5
15
30

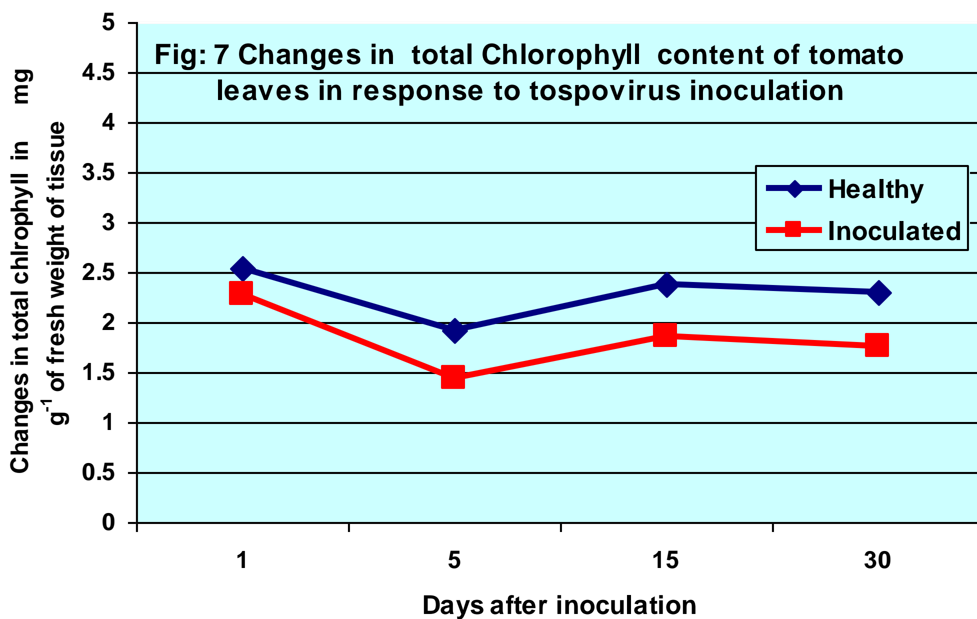


Fig: 7 Changes in total Chlorophyll content of tomato leaves in response to tospovirus inoculation
Changes in total chlrophyll in mg g-1 of fresh weight of tissue
Days after inoculation
Healthy
Inoculated
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
1
5
15
30

chlorophyll a, b and total chlorophyll at different stages of pathogenesis. Saveetha et al. (2010) reported that chlorophyll pigments a and b as well as total chlorophyll were reduced due to mottle streak virus in finger millet plants.

The present investigation also revealed similar results regarding the contents of chlorophyll a and b as well as total chlorophyll. A lower content of chlorophyll a, b and total chlorophyll was noticed. In case of healthy plants chlorophyll a content increased from 1.83 mg g^{-1} at 1 DAI to 2 mg g^{-1} at 30 DAI and chlorophyll b decreased from 0.68 mg g^{-1} at1 DAI to 0.28 mg g^{-1} at 30 DAI. Total chlorophyll was 2.54 mg g^{-1} at 1 DAI which decreased to 2.31 mg g^{-} at 30 DAI in case of uninoculated healthy plants. Whereas in case of tospo virus inoculated tomato plants, chlorophyll a content decreased from 1.6 mg g^{-1} at 1 DAI to 1.3 mg g^{-1} at 30 DAI. Chlorophyll b content decreased from 0.56 mg g^{-1} at 1 DAI to 0.39 mg g^{-1} at 30 DAI and the content of total chlorophyll was found to decrease from 2.29 mg g^{-1} at 1 DAI to 1.76 mg g^{-1} at 30 DAI. The reduction in chlorophyll is due to the destruction of chloroplast due to virus infection and reduction in the rate of photosynthesis as reported by Singh and Singh (1985) (Fig 5 – 7).

Phenol content in tomato plants inoculated with tospovirus was observed to be significantly higher than the healthy uninoculated plants. The phenol content in inoculated plants was 26.33 µg g^{-1}at 1 DAI which reduced to 12.66 µg g^{-1} at 5 DAI but later increased and reached a maximum value of 37.33 µg g^{-1} at 15 DAI. Later the phenol content was found to decline to a value of 16.83 at 30 DAI. In case of healthy tomato plants the maximum phenol content of 28.66 µg g^{-1} was observed at 15 DAI which later decreased to 10 µg g^{-1} at 30 DAI (Fig 8). The results of in chilli due to infection of gemnivirus and found that total phenol was significantly high in diseased leaf as compared to healthy leaf. Saveetha et al. (2010) also reported increased phenol contents in finger millet plants infected by mottle streak virus. Accumulation of phenol observed in virus infected plants was due to excess production of hydrogen peroxide by increased respiration or the present study are in line with many reports. Tanguy and Martin (1972) reported a higher content of total phenols in the leaves of *Nicotiana tabacum* due to tobacco mosaic virus infection. Sutha et al. (1997) found that total phenol increased in TSWV infected plants. Meena et al. (2008) investigated the changes in chilli due to infection of gemnivirus and found that total phenol was significantly high in diseased leaf as compared to healthy leaf. Saveetha et al. (2010) also reported increased phenol contents in finger millet plants infected by mottle streak virus. Accumulation of phenol observed in virus infected plants was due to excess production of hydrogen peroxide by increased respiration or

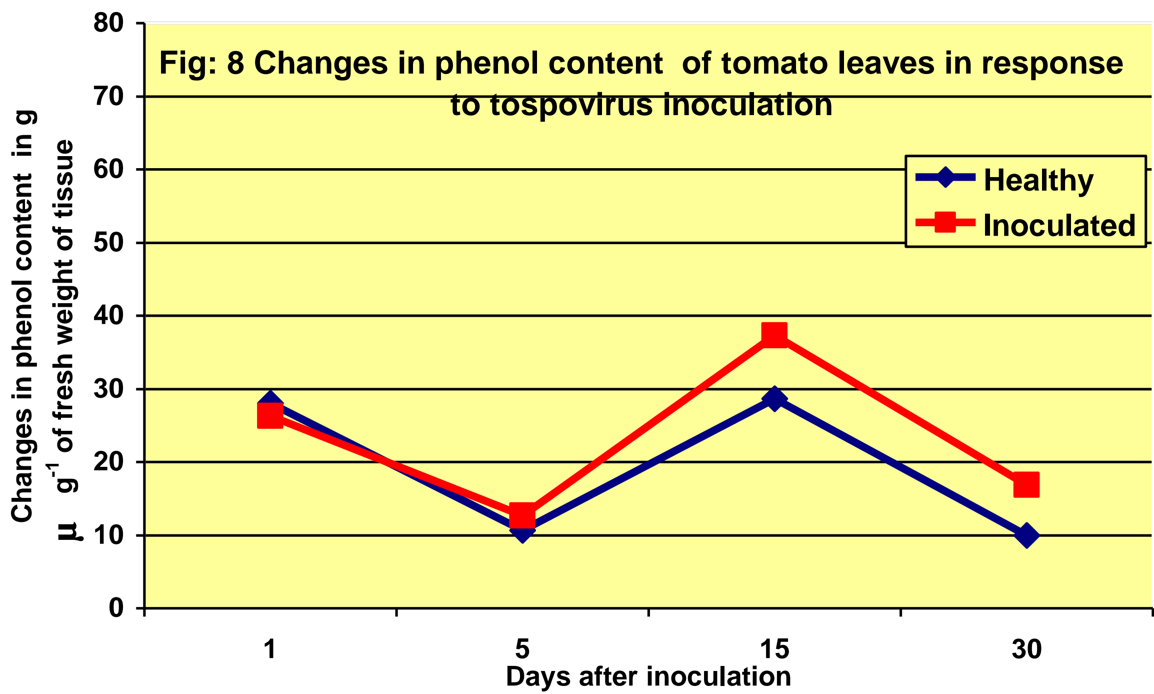


Fig: 8 Changes in phenol content of tomato leaves in response to tospovirus inoculation
Healthy
Inoculated
Changes in phenol content in g
µ g-1 of fresh weight of tissue
80
70
60
50
40
30
20
10
0
1
5
15
30
Days after inoculation

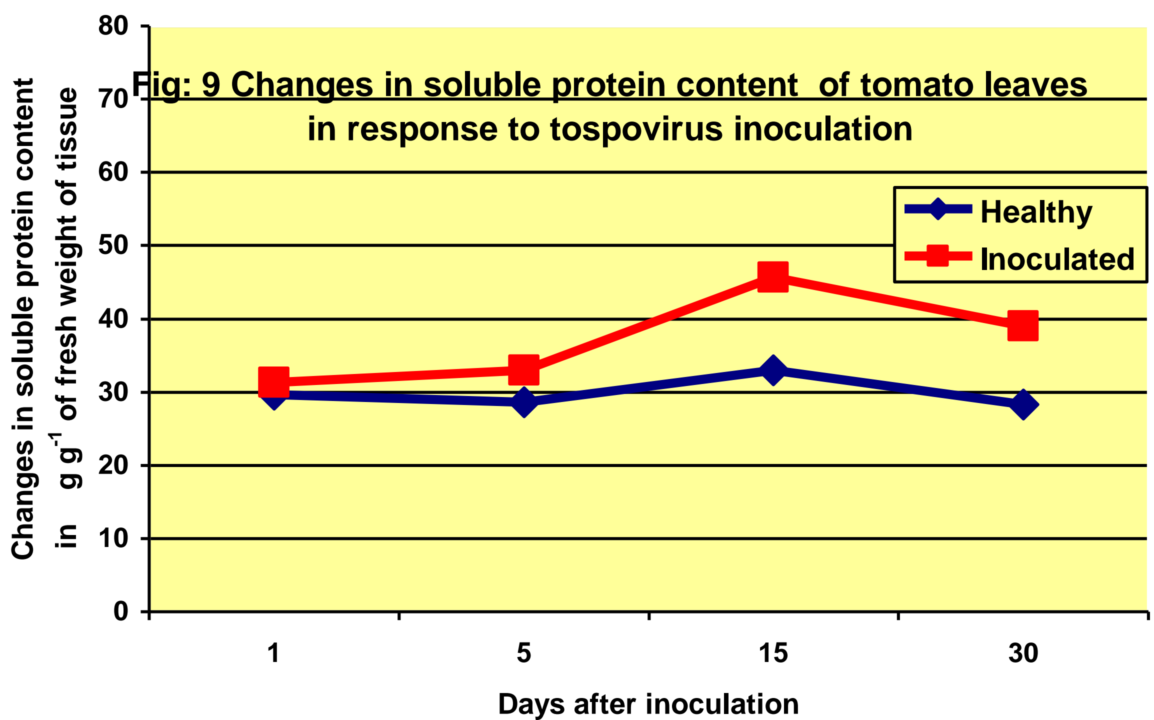


Fig: 9 Changes in soluble protein content of tomato leaves in response to tospovirus inoculation
Healthy
Inoculated
Changes in soluble protein content
in g g-1 of fresh weight of tissue
80
70
60
50
40
30
20
10
0
1
5
15
30
Days after inoculation

due to activation of HMP- shunt pathway, acetate pathway and release of bound phenolics by hydrolytic enzyme as reported by Sutha et al. (1997).

A significant difference was observed in the level of protein between healthy and tospovirus inoculated plants. The protein content in inoculated plants was 31.3 µg g^{-1} at 1 DAI which increased to 33 µg g^{-1} at 5 DAI and again increased to the highest value of 45.66 µg g^{-1} at 15 DAI. Thereafter the protein content reduced to 39 µg g^{-1} at 30 DAI. In case of healthy plants the protein content was found to be highest at 15 DAI (33 µg g^{-1}) and thereafter decreased to a value of 28.33 µg g^{-1} at 30 DAI (Fig 9). Higher protein content due to virus infection in plants has been reported by many authors (Manickam et al., 2000, Yardimci et al., 2006 and Selman et al., 2008). Ashfaq et al. (2010) reported that infection by urdbean leaf crinkle virus increased total soluble protein contents at 15 and 30 days. Sinha and Srivaastava (2010) also reported increased protein contents in mungbean plants due to infection by mungbean yellow mosaic virus. Kovalenko and Shepelevitch (2003) they found that severe virus infection induced pathogenesis associated proteins.

All these reports are in conformity with those obtained in the present investigation. The total soluble protein was found higher in case of inoculated plants. The increase in total protein content in the virus inoculated plants was due to increase in viral proteins and non-viral induced proteins as reported by Kovalenko and Shepelevitch (2003).

The present study indicated significant changes in the activity of defence related enzymes like peroxidase, polyphenol oxidase and phenylalanine ammonia-lyase in the tospovirus inoculated plants. The activity of peroxidase was found to increase initially and later was found to decrease. Peroxidase activity was the highest at 5 DAI (200 $min^{-1}g^{-1}$) in inoculated plants and later decreased to 49.66 $min^{-1}g^{-1}$ at 30 DAI. In case of uninoculated control the maximum activity was observed at 5 DAI (66.33 $min^{-1}g^{-1}$) as compared to a high value of 200 $min^{-1}g^{-1}$ in inoculated tomato plants (Fig 10).

In inoculated plants polyphenol oxidase was found to be highest at 1 DAI (5.16 $min^{-1}g^{-1}$) as against a lower value of 1.4 $min^{-1}g^{-1}$ in healthy plants. The polyphenol

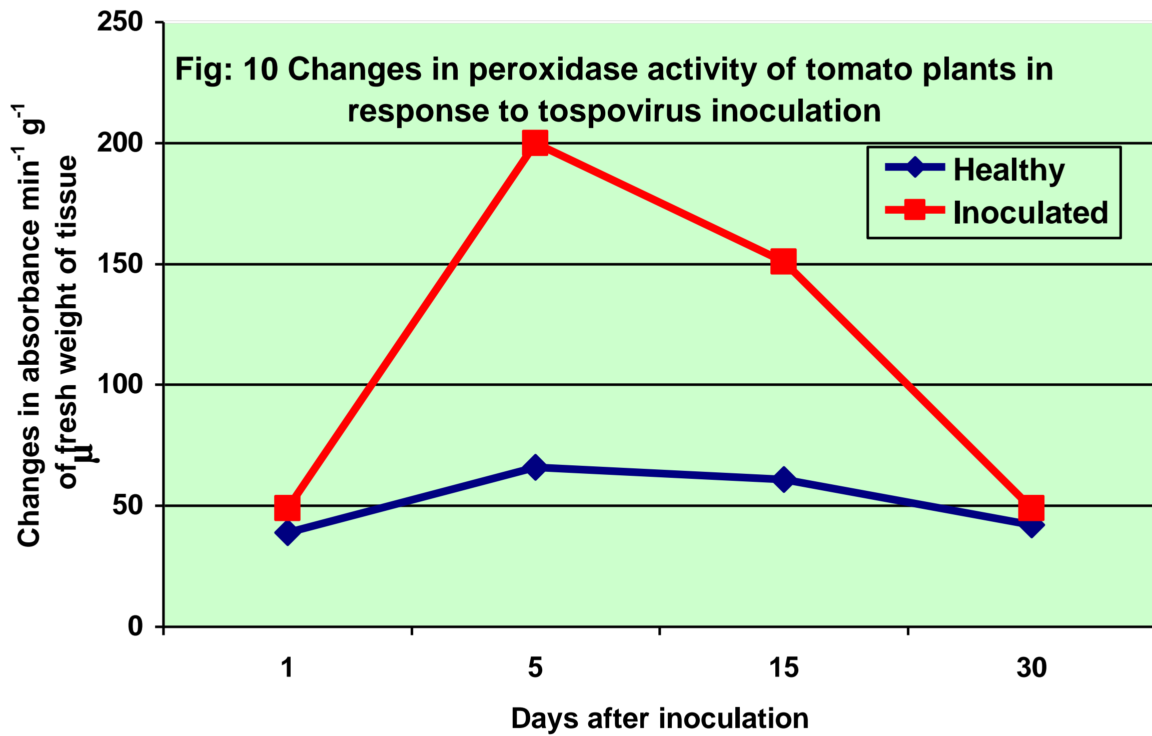


Fig: 10 Changes in peroxidase activity of tomato plants in response to tospovirus inoculation
Healthy
Inoculated
250
200
150
100
50
0
Changes in absorbance min-1 g-1 of fresh weight of tissue
1
5
15
30
Days after inoculation

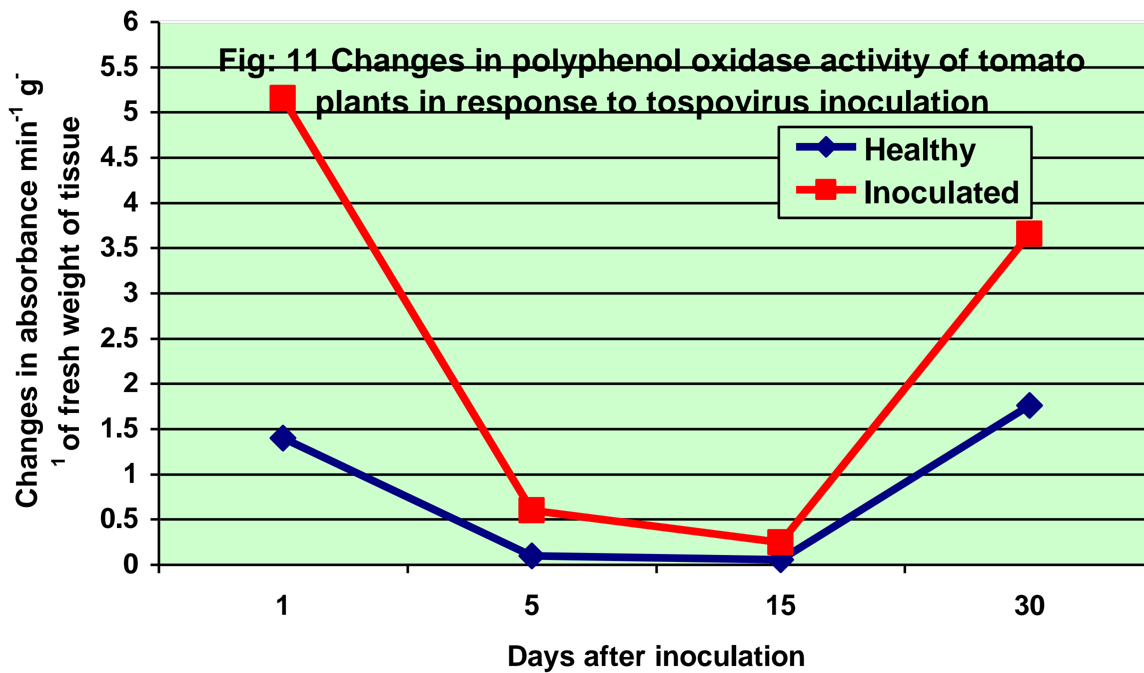


Fig: 11 Changes in polyphenol oxidase activity of tomato plants in response to tospovirus inoculation
Healthy
Inoculated
6
5.5
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
Changes in absorbance min-1 g-1 of fresh weight of tissue
1
5
15
30
Days after inoculation

oxidase activity was found to decrease in tospo virus inoculated plants to 0.25 $min^{-1}g^{-1}$ at 15 DAI and 0.06 $min^{-1}g^{-1}$ in healthy plants (Fig 11).

Phenylalanine ammonia- lyase activity was higher in inoculated plants and the highest value of 3.07 $min^{-1}g^{-1}$ was obtained at 15 DAI and 0.8 $min^{-1}g^{-1}$ at 15 DAI in healthy plants. Then the activity of the enzyme was found to decrease in both cases (Fig 12).

Higher activity of defence related enzymes in virus infected plants have been reported by many workers. Devanathan et al. (2005) observed high peroxidase (PO) activity in bunchy top banana virus infected cultivars of banana. Vera Quecini et al. (2007) found increased peroxidase activity in petunia hybrids and *Nicotiana tabacum* due to TSWV infection. Enhanced peroxidase activity and polyphenol oxidase activities was found to occur in diseased leaves as compared to healthy leaves of chilly infected with gemnivirus (Meena et al., 2008). Clarke et al. (2002) and Karthikeyan et al. (2007) also observed significant increase in peroxidase activity in *Phaseolus vulgaris* and *Vigna mungo* plants after inoculation with white clover mosaic virus (WCIMV) and urdbean leaf crinkle virus (ULCV) respectively. Fritig et al. (1973) reported higher PAL activity due to TMV infection prior to the actual development of local necrotic lesions. Ashfaq et al. (2010) reported that higher peroxidase activity was observed in healthy leaves of susceptible genotype than in resistant genotypes of urdbean inoculated with urdbean leaf crinkle virus (ULCV). 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 in the present study is due to high susceptibility of the plant to the disease.

Viral infection leads to the appearance of specific soluble proteins. The analysis of the protein profile of bud necrosis infected tomato plants revealed three extra bands with molecular weights of 28, 15 and 12 kDa. These three novel proteins were not observed in healthy control. De Avila et al. (1993) analysed the protein composition of a number of tospovirus isolates and reported that the migration rates of different isolates correspond with the molecular mass of 29 kDa and 28 kDa. Satyanarayana et al. (1996) performed PAGE of purified nucleocapsid protein of peanut yellow spot virus (PYSV), a distinct Tospovirus species and reported a protein with a molecular mass of 29 kDa.

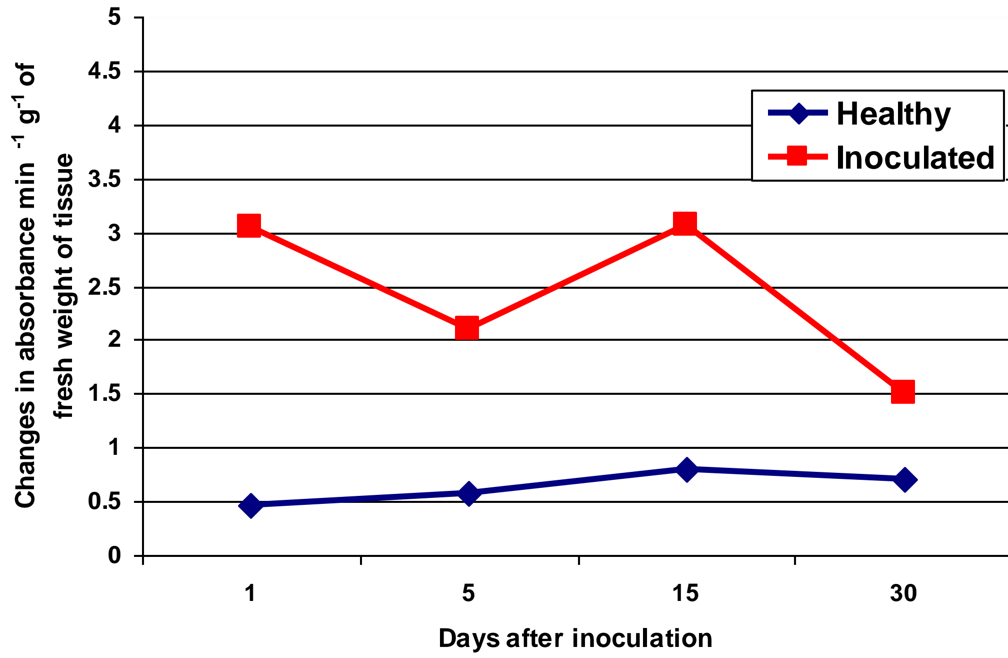


Fig: 12 Changes in phenylalanine ammonia- lyse activity of tomato plants in response to tospovirus inoculation
Changes in absorbance min -1 g-1 of fresh weight of tissue
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
1
5
15
30
Days after inoculation
Healthy
Inoculated

Cortes *et al*. (1998) reported that the N protein of TSWV was about 28.8 kDa which is less than IYSV (30 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 is the N-protein of tospovirus causing the bud necrosis disease as reported by De Avila et al. (1993), Satyanarayana et al. (1996) and Cortes et al. (1998).

Isozyme analysis of defence related enzyme polyphenol oxidase was performed which indicated that there is no distinct polymorphism in PPO between inoculated and uninoculated plants. Same banding pattern was observed for both healthy and infected tomato plants. Two isoforms with relative mobility (Rm) values of 0.60 and 0.77 were observed in both healthy and infected plants. The activity of the two isoforms were observed to be higher in bud necrosis infected plants compared to the healthy plants. Arpita and Subrata (2008) reported that PPO bands were found to be hyperactive in diseased plants in comparison with control plants. Novacky and Hampton (1967) separated isozymes from TMV infected tobacco and CMV and reported only quantitative changes in both host plants. Sindhu (2001) conducted electrophoretic analysis of isozyme from cowpea aphid borne mosaic virus (CABMV) susceptible cowpea varieties and found five isoforms of polyphenol oxidase.

5.6 IMMUNOLOGICAL AND MOLECULAR DIAGNOSIS

ELISA is one of the reliable techniques to detect and characterize a virus. ELISA was conducted to identify the virus causing the bud necrosis disease of tomato, using polyclonal antibodies of TSWV and WSMV. The result indicated high reactivity of the virus isolate to WSMV. The present finding confirmed that the bud necrosis virus infecting tomato is a tospovirus, serologically more related to WSMV. The average absorbance value recorded for diseased sample (0.81) was more than that of healthy sample (0.016). The virus causing bud necrosis disease of tomato in India failed to react with the antisera of TSWV in ELISA. Umamaheswaran et al. (2003) reported that, tospovirus isolated from tomato reacted positively to polyclonal antisera of WSMV and GBNV. 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 showed that the bud necrosis virus infecting tomato is not TSWV but is serologically related to WSMV as positive reaction was obtained only with antisera to WSMV and not to TSWV, which is in accordance with reports by Bhat et al. (2001) and Umamaheswaran et al. (2003).

Serological detection using dot immunobinding assay (DIBA) showed a positive reaction of the tospovirus isolate from tomato with antisera specific to WSMV. This result also confirmed that the bud necrosis disease of tomato is caused by a tospovirus. Takaaki et al. (2003) reported successful identification of tospovirus from the leaves of tomato using DIBA. Anjaneya et al. (2008) also reported that necrosis affected tomato plants showed positive reaction with GBNV polyclonal antiserum in DIBA.

Detection of the tospovirus infecting tomato was performed using polymerase chain reaction which is a sensitive and specific method used for detecting many viruses. The presence of tospovirus in the virus isolates obtained from tomato plants showing characteristic symptoms of the bud necrosis disease was confirmed. Primer specific to tospovirus resulted in an amplified product of size 800 bp. Umamaheswaran et al. (2003) reported that when RT-PCR of tospovirus isolates from tomato was performed using primer pair derived from the nucleocapsid protein gene sequence of GBNV and WSMV, an amplicon of 800 bp was visualized on analysis using PCR . Hemalatha et al. (2008) reported that viral RNA from tospovirus infected tomato on amplification by RT-PCR resulted in a product of 831 bp. Both these reports are in conformity with the results obtained in the present study. Hence it is confirmed that the bud necrosis disease of tomato is caused by a tospovirus serologically related to WSMV and GBNV.

5.7 ELIMINATION OF VIRUS THROUGH MERISTEM CULTURE

Meristem culture was done to find out the possibility of eliminating the bud necrosis virus infecting tomato from the infected planting material. Meristem culture proved highly successful in eliminating the virus. The meristem from the infected tomato plants were regenerated into plantlets and were tested for the presence of the virus by subjecting it to DAC- ELISA. The absorbance of healthy and the plantlet regenerated from healthy and infected meristem were found to be 0.13 and 0.12

respectively which was on par with the healthy field sample but lower than that of the infected field sample which was used as the positive control. It recorded an absorbance of 0.81. Thus the result indicated that meristem culture is a highly useful technique to eliminate the tospovirus and produce virus free plantlets. Mirghis et al. (1995) reported efficient plantlet regeneration in tomato from meristems. Grout (1999) reported that meristem culture allows plants to be freed from viruses. Alam et al. (2004) reported elimination of viral diseases through meristem culture in tomato. Kumar et al. (2009) achieved elimination of CMV and tomato aspermy virus through meristem culture.

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Summary

6. SUMMARY

The study entitled "Characterization of bud necrosis disease of tomato" was conducted at College of Agriculture, Vellayani, Thiruvananthapuram.

The symptoms were observed as small necrotic ring spots, which later turned into necrotic patches leading to the death of the infected leaves. The growing tips and buds were observed to be severely affected with systemic necrosis. Dark brown streaks were observed on the stem of infected plants. On mature fruits the symptoms were seen as yellow rings and also orange red discoloration in a concentric pattern. Stunting of growth was also noticed.

The virus was found to have its host range confined to the members of the families Chenopodiaceae, Solanaceae, Fabaceae and Cucurbitaceae. It produced systemic symptoms on *Vigna unguiculata* cv Pusa komal, *Vigna radiata Cucumis melo* var acidulous, *Benincasa hispida, Canavalia gladiata and Lycopersicon esculentum*. Distinct chlorotic local lesions were produced by the virus on *Chenopodium amaranticolor* and *Chenopodium quinoa*.

The virus was found to be very efficiently transmitted by mechanical means using 0.01 M phosphate buffer (pH 7.2) containing 0.1 per cent 2-mercaptoethanol with carborundum powder as abrasive to the local lesion host, *Chenopodium amaranticolor*. No seed transmission was recorded but the virus was successfully transmitted through grafting of infected scion on healthy root stock.

Physical properties of the virus were studied. The virus recorded a dilution end point (DEP) in the range of 10^{-3} to 10^{-4}, thermal inactivation point (TIP) of 50^0C to 55^0C, and longevity *in vitro* (LIV) of 8 h at room temperature (28 ± 2^0C) and 24 h at 8^0C.

The biochemical changes due to virus infection were studied in healthy control and virus inoculated tomato plants. The carbohydrate levels in inoculated plants were less compared to the uninoculated tomato plants. The content of chlorophyll a, b and total chlorophyll was less in inoculated tomato plants as compared to the healthy plants. The phenol content was found to be more in inoculated plants. There was an increase in protein contents in inoculated plants compared to the healthy plants. In case of

inoculated plants the activity of the defence related enzymes were higher than uninoculated plants and was found to increase initially but decrease later.

SDS - PAGE was carried out to analyse the protein profile of tomato plants under inoculated and uninoculated conditions and to investigate the presence of virus induced proteins. Protein profile of healthy samples showed six proteins whereas the virus infected samples produced eight proteins. Out of the eight proteins six proteins were identical to that of the healthy and three extra bands were obtained. The three extra novel proteins in the diseased samples were with molecular weight 28, 15 and 12 kDa respectively.

Native polyacrylamide gel electrophoresis was performed for the isozyme analysis of polyphenol oxidase (PPO). Two isoforms in both healthy and inoculated plants were observed. These isoforms were with relative mobility (Rm) values of 0.60 and 0.77 respectively. The activity of the two isoforms were more in the inoculated plants compared to the uninoculated plants.

The virus causing bud necrosis disease in tomato was confirmed as tospo virus by serological analysis such as ELISA and DIBA. The virus isolate showed close relationship with WSMV. The Virus was also detected using PCR and an amplicon of size 800 bp was obtained using primer specific to tospovirus.

Meristem culture was done for eliminating the bud necrosis virus infecting tomato from the infected plants. The meristem from the infected tomato plants were regenerated into plantlets and were tested for the presence of the virus by subjecting it to DAC- ELISA. The absorbance of the plantlet regenerated from healthy and infected meristem were found to be 0.13 and 0.12 respectively which was on par with the healthy field sample but lower than that of the infected field sample which was used as the positive control which recorded an absorbance of 0.81. Thus the result indicated that meristem culture is a highly useful technique to eliminate the bud necrosis virus and produce virus free plantlets.

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Appendices

APPENDIX-I

Buffers for Sap extraction

1. A: 0.2M Solution of monobasic sodium phosphate (27.8 g in 1000 ml)
   B: 0.2M Solution of dibasic Sodium phosphate (53.65g of Na_2HPO_4. $12H_2O$ in 1000 ml)
   39.0 ml of A is mixed with 61.0 ml of B diluted to a total of 200 ml.

2. 0.1M Tris buffer (PH 7.2)
   A: 0.2M solution of Tris (24.2g in 1000ml)
   B: 0.2M HC1
   50 ml of A is mixed with 44.2 ml B diluted to a total of 200 ml.

3. 0.01M phosphate Mercaptoethanol buffer:- (P^H 7.2)
   KH_2PO_4 - 800 mg
   K_2HPO_4 - 2.2 g
   Mercaptoethanol -100 µl
   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) - Add 0.5ml/L of Tween-20 to PBS

.

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

APPENDIX IV

Buffers for biochemical analysis

1. 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 $C_2H_3O_2Na$ or 27.2 g of $C_2H_3O_2Na$ $3H_2O$ in 1000 ml).

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

2. 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.

APPENDIX V

Stock solutions for isozyme analysis

1. Tris-glycine electrode buffer stock solution, pH 8.3

   Tris - 6.0 g

   Glycine - 28.8 g

   Distilled water - 1000 ml

2. Electrode buffer

   Dilute the Tris- glycine electrodebuffer stock solution with distilled water in a 1: 9 ratio.

3. Tris- chloride buffer stock solution, pH 8.9

   HCl, 1N - 48.00 ml
   Tris - 36.6 g
   TEMED - 0.23 ml
   Distilled water -100.00 ml

4. Tris- chloride buffer stock, pH 6.7

   HCl, 1N - 48.00 ml

   Tris - 5.98 g

   TEMED - 0.46 ml

   Distilled water - 100.00 ml

5. Resolving gel acrylamide stock solution

   Acrylamide – 28.00 g

   Bis-acrylamide – 0.74 g

   Double distilled water – 100.0 ml

   Store in dark bottle at 4^0C for upto 2 weeks.

6. Ammonium persulphate solution

   Ammonium persulphate - 0.1 g

   Dissolve in 1 ml distilled water. Prepared freshly before use

7. Bromophenol blue solution

   Bromophenol - 25 mg

Make upto to 10 ml with Tris- chloride buffer solution, pH 6.7

8. Resolving gel solution (for one 1.5 mm gel, 7.5 %)

Tris- chloride buffer - 5 ml

Solution, pH 8.9

Resolving gel acrylamide solution - 10 ml

Distilled water - 25 ml

Ammonium persulphate solution - 300 μl

9. Stacking gel solution (for one 1.5 mm gel, 4%)

Tris- chloride buffer stock - 2.5 ml

Solution, pH 6.7

Resolving gel acrylamide solution - 10 ml

Distilled water - 25 ml

Ammonium persulphate solution - 300 μl

APPENDIX VI

1. Stock solutions for MS basal medium

Sl. No.	Constituents	Amount of chemical taken (mg)	Amount mg g^{-1}	Solution ml l^{-1}
1.	Stock solution I (250 ml 10 X)			
	$MgSO_4 . 7 H_2O$	3700	370	
	KH_2PO_4	1700	170	25 ml
	KNO_3	19000	1900	
	NH_4NO_3	16500	1650	
2.	Stock solution II (100 ml, 20 X)			
	$CaCl_2 . 2 H_2O$	8800	440	5 ml
3.	Stock solution III (100 ml, 100 X)			
	H_3BO_3	620	6.2	
	$MnSO_4 .4 H_2O$	1690	16.9	
	$ZnSO_4 . 2H_2O$	860	8.6	1 ml
	$Na_2MoO_4 . 2H_2O$	25	0.25	
	KI	83	0.83	
4.	*Stock solution IV (100 ml, 20 X)			
	$FeSO_4 . 7 H_2O$	556	27.8	5 ml
	Na_2 EDTA. 2 H_2O	746	37.3	

5.	Stock solution V (250 ml-500 X) $CuSO_4 . 5 H_2O$ $CoCl_2 . 6 H_2O$	 12.5 12.5	 0.025 0.025	 .5 ml
6.	Stock solution VI (100 ml- 100 X) Thiamine HCl Pyridoxin HCl Nicotinic acid Glycine Myoinositol – 100 mg Sucrose – 30 g Agar – 7.5 g	 10 50 50 200	 0.1 0.5 0.5 2	 1 ml

*Dissolve $FeSO_4 . 7 H_2O$ and Na_2 EDTA. 2 H_2O separately in 45 ml distilled water by heating and constant stirring. Mix the two solutions and adjust the pH to 5.5 and add distilled water to make up the final volume to 100 ml.

CHARACTERIZATION OF BUD NECROSIS VIRUS INFECTING TOMATO

S. SIMI.

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ABSTRACT

Studies were conducted to characterize the tospovirus causing the bud necrosis disease of tomato in Kerala. The characteristic symptoms observed were, necrotic ring spots on leaves and severe necrosis, death of the emerging buds, stem necrosis and concentric yellow colored rings on fruits. Host range studies were conducted and the virus was found to infect members of family Chenopodiaceae, Solanaceae, Fabaceae, and Cucurbitaceae. The virus was efficiently transmitted by mechanical means using 0.01 M phosphate buffer (pH 7.2) containing 0.1 per cent 2-mercaptoethanol. No seed transmission was recorded. However successful graft transmission was observed. The virus recorded a DEP in the range of 10^{-3} to 10^{-4}, TIP of 50 °C to 55 °C, and LIV of 8 h at room temperature (28±2°C) and 24 h at 8°C.

The carbohydrate levels in inoculated plants were lower compared to the uninoculated tomato plants. Similarly the content of chlorophyll a, b and total chlorophyll were also lower in inoculated tomato plants. The phenol content was found to be more in inoculated plants. There was an increase in protein content in inoculated plants compared to the healthy plants. In case of inoculated plants the activity of the defense related enzymes were higher than the control plants. Protein profile of tospovirus infected tomato plants using SDS - PAGE showed three extra novel proteins with molecular weights of 28, 15 and 12 kDa respectively. Isozyme analysis of PPO produced three isoforms in both healthy and inoculated plants with relative mobility (Rm) values of 0.60 and 0.77. The activity of the two isoforms were more in the inoculated plants. The virus causing bud necrosis disease in tomato was confirmed as tospo virus by serological analysis such as ELISA and DIBA. The virus isolate showed close relationship with WSMV. The virus was also detected using PCR and an amplicon of size 800 bp was obtained using primer specific to tospovirus. The meristem from the infected tomato plants were regenerated into plantlets and were tested for the presence of the virus by subjecting it to DAC- ELISA. The absorbance of the plantlet regenerated from healthy and infected meristem were found to be 0.13 and 0.12 respectively which was on par with the healthy field sample but much lower than that of the infected field sample which was used as the positive control which recorded an absorbance of 0.81.