DISCUSSION
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_Citrus yellow mosaic virus_ (CYMV) is widely distributed in India and is of great economic importance to the Citrus industry. The presence of the disease in commercial nurseries suggests inadvertent spread of the disease through contaminated bud wood. CYMV is capable of infecting the major commercial Citrus cultivars and rootstocks used in India.

Diseases caused by viruses and virus like organisms are the most serious in Citrus production problems. There are no effective practical treatments to cure Citrus trees in the orchards once they get infected with viruses. These diseases have caused more damage to Citrus than to any other fruits crop. Mosaic disease in Citrus is a major limiting factor for achieving quality fruit and yield of this popular crop in India. It has been established through earlier investigations that a bacilliform dsDNA virus is associated with mosaic disease of Citrus.

The present work comprises the investigations of CYMV with special reference to field survey, transmission, host range studies, purification, serological and molecular detection of the virus.

In our study, the CYMV incidence was recorded in eight districts of Andhra Pradesh with varying levels of incidence. Similarly Ahlawat _et al._ (1985) reported the incidence of mosaic disease from North-Eastern India on
Khasi-mandarins. The incidence of the mosaic disease ranged from 10 to 70% in Citrus orchards and nurseries. The reduction in yield was up to 77% in 10 years old trees (Murti, 1983). Reddy and Murti (1985) reported that the incidence of the disease was recorded up to 46% in some commercial nurseries at Kodur in Andhra Pradesh.

In the present study the CYMV was transmitted by sap inoculation. Among the two methods of sap inoculation, leaf rub method was showed highest percent transmission of CYMV. Earlier workers reported that the mosaic disease prevalent in North-Eastern states was transmitted by aphids, *M. persicae*, *A. craccivora* (Ahlawat et al., 1985), whereas, Murti (1983) reported its transmission by *Toxoptera citricida* from South India. However, the present isolate of Citrus mosaic was not transmitted by aphids like *M. persicae*, *A. craccivora* and *A. citricola* (Ahlawat et al., 1996b). Hence, this isolate of CYMV was considered different from the one reported by Ahlawat et al. (1985) and Murti (1983). This isolate was transmitted by the vector *Planococcus citri* (Reddy, 1997; Gopal et al., 2004) and also by mechanical inoculations. Mealy bugs normally do not move from tree to tree and hence natural spread through this vector is considered to be negligible and the major spread of CYMV appears to be by the use of contaminated bud wood.

The CYMV disease is graft transmissible and also experimentally transmitted by mealy bug (*Planococcus citri*) (Reddy et al., 1996). The transmission of the mosaic virus suggests that CYMV is a member of
Badnavirus group as most of these viruses are transmitted by mealy bugs. Eight of the 10 definitive members of the Badnavirus group (CSSV, ComYMV, BSV, KTSV, PYMV, ScBV, PBV, SRBV) are transmitted by mealy bugs (Lockhart and Olszewski, 1994).

In the present investigation the CYMV was best transmitted by graft inoculation. T-budding and bark patch methods were noticed highest transmission of CYMV. Similar that of present investigation graft inoculation was reported by several authors. Ahlawat et al. (1996a) reported the CYMV was graft transmissible to 13 Citrus cultivars. The occurrence of Citrus mosaic on Sathgudi (Citrus sinensis Osbeck) and its transmission by bud, leaf and bark patch inoculation was supported by reports of Naidu and Reddy (1986) and Gopal et al. (2007). Reddy and Murti (1976) reported the transmission of CYMV by bud, bark and leaf patch grafting. Reddy et al. (1985) reported that the mosaic disease was also transmitted by Dodder (Cassytha filiformis) from infected sweet orange to acid lime. Murti and Reddy (1976) noted the successful transmission of CYMV to Hibiscus rosasinensis by grafting. Naidu and Reddy (1986) reported that the yellow mosaic virus can transmit through phloem and cambium of Sathgudi sweet orange plant but not through xylem. Ahlawat et al. (1985) documented that the disease was transmitted by bud and wedge grafting. Bark patch grafting was found better in transmission of CYMV followed by ‘T’ budding (Gopal et al., 2003).
It is a known fact that, the CYMV has wide host range. Our study also reported various non herbaceous plants viz., Sorghum, Maize, Canna indica, Rice and Banana etc as hosts of CYMV which was confirmed by PCR. This is the first report of herbaceous hosts for Citrus yellow mosaic badnavirus from India (Gopal et al., 2002). This study agrees with earlier report of Bouhida et al. (1993). They found that, ScBV is infectious to banana, sorghum and rice by mechanical inoculation and it is possible that full host range is even more extensive. Tanaka and Imada (1974) reported many herbaceous plants as the host of this virus. Badnaviruses infects both monocotyledons (e.g. RTBV, ComYMV, ScBV) and dicotyledons (CSSV, PYMV, SRSV) plants (Lockhart, 1990). Graft transmission of CYMV to Hibiscus rosasinensis by grafting was reported by Murti and Reddy (1976).

In this study, coat protein analysis of the CYMV isolates was done by SDS-PAGE and confirmed by western blot with previously produced antiserum. The virus preparations when electrophoresis showed one major band of capsid protein with relative molecular mass of about 44 kDa and one minor band of 14 kDa. The minor band was considered to be the degraded product of 44 kda protein. The protein bands within the range and it is the characteristic feature of Badnaviruses. The present results are in agreement with the reports of Francki (1972) who suggested that infectivity assay, electron microscopy, analytical centrifugation, U.V-absorption and serological tests as the criteria to judge the quality of virus purification procedure. Lockhart (1986) reported $A_{260/280}$ ratio
was determined as 1.6 which is in the range of most *Badnaviruses*. The purified preparation of the virus showed non-enveloped bacilliform virus particles measuring 130×30 nm. Similar morphology of the virions has been reported by earlier workers (Ahlawat *et al.*, 1996a and 1996b).

Uhde *et al.* (1993) reported similar results with *Cacao swollen shoot virus* (CSSV) where the major capsid protein was 43 kDa but two degraded products were in the range of 37 to 33 kDa. The coat protein of *Commelina yellow mottle virus* (ComYMV) was 39 and 37 kDa (Lockhart and Khaless, 1988), whereas capsid coat protein of *Rice tungro bacilliform* (RTBV) was 37 kDa with one minor band of 33 kDa which was considered as a degraded product of 37 kDa (Qu *et al.*, 1991). Therefore, the results of coat protein analysis of the present virus were similar as have been reported for other *Badnaviruses*.

In DAC-ELISA, test samples in the present study reacted positively with the previously produced polyclonal antiserum. Earlier studies observed that 1:1000 was found suitable for the detection of this virus. In the present study also, it was same. Further, it was observed that all the plant parts tested except fruit-rag and fruit-juice could give positive reaction to the antiserum. This is the first report indicating the absence of CYMV in fruit rag. Earlier this technique was also reported for the detection of *Badnaviruses* by Ahlawat *et al.* (1996a). The use of sero-diagnostic technique for the indexing and production of virus free Citrus plants have already been reported by Baranwal
et al. (2003). A polyclonal antiserum has been developed and used for detection of the virus in Enzyme-Linked immunosorbent assay (ELISA) and Immuno-sorbent electron microscopy (ISEM) by Pant et al. (1997). Harrison et al. (1997) studied that the diagnostic purposes, it may be useful to select monoclonal antibodies (MAbs) that react with a common antigenic determinant on several strains of the virus. Hobbs et al. (1987) reported the indirect ELISA method (Direct antigen coating, DAC-ELISA) antigen is detected using antigen specific antibody. Hansen and Wick (1993) reported the ELISA has been widely used in large scale diagnosis of plant viruses especially in perennial, vegetatively propagated crops such as tuber potatoes, bulbs and fruit trees. Martin (1998) reported the ELISA has also been used successfully to detect plant viruses in insect vectors and seeds.

It has been noticed in this study that the dot blot ELISA was effective in detection of CYMV in infected leaf samples of Sweet orange, Acid lime, Rangpurlime and Pummelo pink. Similar observations were made by several authors. Detection of Badnavirus is difficult by ELISA because of the low concentration of virus (Lockhart and Olszewski, 1994). Lin et al. (1990) reported that the ELISAs, in the form of multi well ELISA, dot-blot ELISA and tissue print ELISA have been developed for numerous viruses. Abad and Moyer (1992) reported the sensitivity of tissue print ELISA is atleast as high as dot- blot ELISA and low volumes of highly diluted antiserum are used.
DAC-ELISA and Dot blot ELISA were found to be sensitive, requires very low quantity of reagents. Dot blot ELISA was more effective than conventional ELISAs because it was very sparing in their use of antigen (0.05mm yields a 0.3mm blot) and it may be used with a roster of different antigens simultaneously to detect and characterize a range of antibodies of interest. They have been used to detect many plant viruses using either polyclonal or monoclonal antibodies (Banttari and Goodwin, 1985; Hibi and Saito, 1985). These two methods can be used to test large number of samples at a time.

In the present study, we found that the sodium sulphite method was best for detection of CYMV by PCR when compared to the CTAB method. Based on the available literature, the investigation was the first in terms of CYMV detected in plant parts of Sathgudi sweet orange except in fruit rag. Similar studies of detection of CYMV in Citrus leaves were reported by Baranwal et al. (2003) who has not reported absence of CYMV in fruit-rag. Baranwal et al. (2005) indicated that Citrus yellow mosaic virus was associated with mosaic disease in Rangpurlime and were successful in detection of CYMV by PCR. Rapid, sensitive and reliable detection of different Badnaviruses has been demonstrated by earlier workers by PCR using the primers from the conserved domain of the virus genome (Ahlawat et al., 1996a; Thomson et al., 1996; Harper et al., 1999; Muller et al., 2001). In the present study also the primers used were from the conserved domain of the virus genome and were successful in detection of the virus in Citrus samples.
In the present investigation, the polyprotein of CYMV isolates were cloned in pTZ57R/T cloning vector. The recombinant colonies were confirmed by colony PCR and restriction enzymes. Based on the sequence data new primers were designed for detection of CYMV isolates in PCR. It is in agreement with reports of Huang and Hartung (2001), who reported that the sequenced amplicon was 638 bp and showed 89% sequence identity with other Indian isolates of CYMV in Reverse-Transcriptase and RNase H domains of ORF III polyprotein. They cloned and sequenced part of inter-generic region and ORF III from other Indian isolate of CYMV infecting sweet orange but showed variability in inter-generic region.

In our study, nucleotide sequence analysis of the three isolates (CYMV-1, CYMV-2 and CYMV-4) was carried out with the previously reported CYMV isolates and other Badnaviruses. It was noticed that around 87.0-92.9% nucleotide homology and 94.7-98.6% amino acid homology was observed within our isolates. The ≈10% nucleotide sequence variation among the three isolates may be attributed to their origin as acid lime (Citrus aurantifolia (L.) Swingle), sweet orange (Citrus sinensis Osbeck) and pummelo pink (Citrus grandis Osbeck) are three different species of Citrus.

Comparative analysis of ORF III nucleotide sequence of different CYMV isolates shared 87.0-98.4% sequence identity. Sequence analysis of CYMV isolates with various Badnaviruses on other crops shared 32.2-38.9% homology at nucleotide level. Further, comparative analysis of ORF III at
amino acid level was also studied which showed 94.6-98.6% sequence identity among the different CYMV isolates. Sequence analysis of CYMV isolates with other Badnaviruses from other crops shared less homology (3.0-4.5%) at amino acid level. The cloning and sequence analysis of CYMV genome has been done (Huang and Hartung, 2001). Its genomic DNA is 7559 bp in length and contains six putative open reading frames (ORFs), all on the plus-strand of the genome and each capable of encoding proteins with a molecular mass of greater than 10 kDa. ORF III, the largest ORF, encodes a putative polyprotein for functions involved in virus movement, assembly and replication. Gupta et al. (2009) reported genome sequencing, comparison and phylogenetic analysis of Citrus yellow mosaic virus isolates originating from different Citrus species in India. Geering et al. (2000) reported genetic diversity among Banana streak virus isolates from Australia.

Our phylogenetic analysis of ORF III region of CYMV isolates revealed that CYMV-4 isolate closely related to CYMV-SO-USA (AF347695). However, CYMV isolates were also related to various other Badnaviruses like TaBV (AF357836) and ComYMV (X52938). Previously, Ahlawat et al. (1996a) reported that CYMV was related to CSSV, BSV, ComYMV, DBV and ScBV, but had the closest relationship to ScBV. Baranwal et al. (2003) reported that the CYMV has been found to be related to Banana streak virus (BSV), Cacao swollen shoot virus (CSSV), Commelina yellow mottle virus (ComYMV), Kalanchoe top-spotting virus.
(KTSV), *Sugarcane bacilliform virus* (ScBV) and *Taro bacilliform virus* (TaBV) in ISEM tests and with BSV and ComYMV in PCR using degenerate primers. Huang and Hartung (2001) reported that detection of the target CYMV sequence by PCR in leaves of sweet orange inoculated with *Agrobacterium tumifaciens* carrying pBICYMV. Similarly Muller and Sackey (2005) calculated the nucleotide and amino acid sequence identities between five *Cacao swollen shoot virus* isolates and generated a phylogenetic analysis of the isolates including other pararetroviruses. Geijskes *et al.* (2002) reported the amino acid sequence similarity between three Australian isolate of *Sugarcane bacilliform badnavirus*. Phylogenetic analysis of SCBV isolates were showed more closely related to each other than to other Badnaviruses.

Presently, mother trees of sweet orange cv. Sathgudi are being indexed for CYMV by using PCR and ELISA. The trees free from CYMV are being used for collection of bud wood for large scale production of disease free budlings of sweet orange cv. Sathgudi budded on Rangpurlime and are being supplied to the growers in South India. Further, disease free buds of sweet orange cv. Sathgudi are also being supplied to the nurserymen in Andhra Pradesh.