6. SUMMARY

The genus *Allium* comprises of over 600 species but only few of them are grown commercially in different parts of the world. The commercially cultivated *Allium* species are annuals, bi-annuals or perennials vegetables. Onion (*Allium cepa* var *cepa*) is the most important commercial vegetable world-wide. Onion is a part of both vegetarian and non-vegetarian human diet. In India, onion is the major vegetable for the poor masses and have serious impact to influence politics. After onion, garlic (*Allium sativum*) is another important commonly grown commercially and mostly used as spice and for medicinal purposes. Shallot and leek are gown in limited areas of Tamil Nadu, Karnataka and West Bengal. These are not popularly used as vegetables in India.

Virus diseases are the major constraints in production of onion and garlic throughout the world. However only limited viruses have been reported on leek and shallot from different parts of the world but none from India. The viruses in garlic, leek and shallot are propagated because of their nature of vegetative propagation. These viruses are not seed-borne and therefore seed-crop raised through onion seeds is escaped from infection initially but subsequently it is infected by aphid vectors movement from infected sources. Therefore, it was important to study the viruses infecting these crops in India, their molecular characterization and development of diagnostic systems and to find out the sources of resistance which have been reported in this dissertation.

In India, onion and garlic are grown in almost all the states of the country but the major producing states are Gujarat, Karnataka, Maharashtra, Orissa and Uttar Pradesh. During the present studies leaf and bulb samples were collected from different parts of India to determine the distribution of viruses in these crops. Initially, these samples were scanned by electron microscopy (EM) to determine the presence of virus(es) and the EM positive samples were further tested in Immunosorbant electron microscopy (ISEM) for the identification of viruses in those samples. Of the 80 samples tested in EM, 58 were found positive.
The ISEM results showed *Onion yellow dwarf virus* (OYDV) in all the onion leaf samples collected from various parts of the country, except one from NHRDF, Karnal which was later found to be positive for *Leek yellow stripe virus* (LYSV). All the leaf samples of garlic showed OYDV and *Garlic latent virus* (GarLV). The LYSV was found in all the samples of leek, one sample of garlic collected from Delhi and two samples of onion and garlic (one each) collected from NHRDF, Karnal and NHRDF, Nasik respectively and one sample of garlic leaf collected from farmer’s field at Nasik. *Shallot latent virus* (SLV) was found only in samples of garlic from NHRDF, Karnal, NHRDF, Nasik and farmer’s field at Nasik. However all the garlic bulb and garlic leaf samples were found to be positive for OYDV and GarLV, and only three sample were found to be positive with LYSV. The shallot samples also showed OYDV in all the samples and GarLV in one sample only. These results were conclusive that OYDV is widely present in *Allium* species throughout the country and other viruses are of limited occurrence.

ELISA system is more sensitive than EM and ISEM to identify the viruses. For testing more samples, Direct Antigen Coating Enzyme-linked Immunosorbant Assay (DAC-ELISA) was standardized. The antibodies used in ELISA system were specific for OYDV, LYSV, GarLV ands SLV. For DAC-ELISA, the leaf samples of onion and garlic collected from Nasik, Rajasthan and J & K while the bulb samples of garlic collected from Nasik, Karnal and Delhi were used as antigen. All these samples were found to be positive for OYDV and LYSV while the GarLV and SLV was occasionally observed. Molecular diagnostic systems “RT-PCR and Multiplex PCR” were subsequently developed for more reliable detection of the viruses infecting these crops. In addition to normal procedures of RNA isolation, a new method for RNA isolation on NCM membrane based using different buffers was developed, which was found to be much simpler and cost effective.

The primers used for the detection of different viruses were either taken from published domain or were designed and synthesized during the study. The OYDV was amplified from onion leaf samples using primer sets OYDV 3 & OYDV 4, OYDV 5 & OYDV 6 and OYDV 5 & OYDV 7 and an amplicon of ~400 bp, 500bp and 1.1 kb respectively was obtained. The primer set OYDV 3 & OYDV 4 and OYDV 5 & OYDV 6 for OYDV could amplify only the part of CP gene while the primer set OYDV 5 & OYDV 7 were designed to amplify the part of RNA dependent RNA polymerase gene (NIB), full CP and a part of 3’ UTR region. The primer set OYDV 5 & OYDV 7 was designed to study the variability
among the Indian isolates of OYDV as well as their relationship with the isolates from
different countries. The garlic leaf and bulb samples were tested using primer set OYDV 5 &
OYDV 7, GLV6 & GLV 4 resulting in an amplicon of ~1.1kb and 300 bp respectively.
The amplicon of around 200bp was obtained using primer set Allex1 & Allex2 which
confirmed the presence of Aleexivirus in garlic and shallot bulb samples. This is being
reported for the first time in garlic and shallot in India. Leek samples were tested using
primer set OYDV 5 & OYDV 7 and an amplicon of around 1.1 kb was obtained indicating
the presence of OYDV in leek samples too.

Once the PCR system was developed it was thought desirable that these viruses should be
molecularly characterized. The cloning of the PCR product was therefore done and
recombinant clones were confirmed by colony PCR and restriction analysis. The positive
clones for OYDV 3 & OYDV4, GarLV 6 & GarLV 4 and OYDV 5 & OYDV 7 were sent
for sequencing to Technoconcept India Pvt. Ltd., New Delhi (Microsynth, Switzerland).

One clone with OYDV 3 & OYDV 4 was cloned and sequenced earlier to check whether
the amplification obtained by this set of primer is specific to OYDV. Later on five OYDV
clones with primer set OYDV 5 & OYDV 7 were sequenced from both sense and anti-
sense direction i.e. T7 and SP6 while only one clone of GarLV was sequenced from one
direction only i.e. T7. Both the sequences for OYDV clones were assembled and aligned
using BIOEDIT software to remove the overlapping regions. After removing the
overlapping region from one of the sequences, both the sequences were joined together to
get the full sequence of the clones. All the sequences of OYDV isolates contained part of
NIB gene, full CP and part of 3' UTR region. The nucleotide sequence data have been
submitted to GenBank and the Accession numbers for four garlic cultivars have been
received. The accession numbers are DQ519034 for Delhi isolate, EU04556 for Rajasthan
isolate, EU04557 for J & K isolate and EU04558 for Karnal isolate. The sequenced region
of OYDV isolates of onion and garlic contained 129 to 149 nucleotide in NIB gene
(depending on the isolate), 771 nucleotides in CP gene translated to 257 amino acids and
211 nucleotides in 3' UTR region. The sequenced region of GarLV isolate of garlic from
Delhi contained 308 nucleotides translated to 102 amino acids.

All the sequences obtained for OYDV isolates were compared using Bioedit software with
already existing sequences of OYDV from China, Japan, Brazil, Israel and Netherlands.
The sequence identity matrices were generated using Bioedit software and phylogenetic trees were constructed using TREECONW software. The results revealed that all the Indian isolates varied among themselves within a range of 80.4% to 85.4% in respect to their nucleotide sequences and 78.3% to 90.6% in respect to their aminoacid sequences. However, when the sequence identity matrix was generated comparing with the CP gene of available gene sequences of OYDV isolates from other countries, the Indian isolates showed nucleotide identity in the range of 81.9% to 100% among themselves, 79.3% to 99.8% with garlic type Japanese isolates, 70.4% to 75.1% with wakgej type Japanese isolates, 78.0% to 89.6% with Chinese isolates, 81.3% to 88.7% with Brazilian isolate, 79.3% to 82.8% with Isrealian isolate and 80.2% to 82.7% with Netherland isolates. While on the other hand when CP gene amino acid sequences were analysed, the Indian isolates showed nucleotide identity in the range of 86.7% to 100% among themselves, 84.8% to 94.9% with garlic type Japanese isolates, 70.6% to 75.2% with wakgej type Japanese isolates, 84.7% to 93.3% with Chinese isolates, 89.8% to 96.1% with Brazilian isolate, 85.2% to 91.4% with Isrealian isolate and 84.8% to 90.2% with Netherland isolates. Similarly the Indian isolates were found to be varying diversely with isolates of different countries when NIB nucleotide and aminoacid sequences and nucleotide sequences of 3' UTR region were analysed.

However, different isolates of OYDV showed phylogenetic relationship with OYDV isolates from other countries on the basis of their nucleotide sequences. The garlic Delhi (OYDVDEL) and Rajasthan (OYDVGRAJ) isolate showed closer relationship to Brazilian and Chinese isolates respectively. The garlic J & K (OYDVGI&K) and onion Nasik (OYDVON) isolate were grouped with isolates of Japan while the garlic Karnal (OYDVGKAR) isolate showed relationship with both Chinese and Japanese isolates. Similar result was obtained in case of OYDVDEL, OYDVGKAR and OYDVGI&K and OYDVON isolates, while OYDVGRAJ isolate grouped with OYDVDEL, OYDVGKAR and Brazilian isolates when aminoacid sequences were used for phylogenetic analysis. When NIB nucleotide sequences were used for phylogenetic analysis, it was found that, OYDVON isolate was grouped with OYDVDEL, OYDVGKAR and OYDVGRAJ which grouped together and further showed relationship with Chinese and Japanese isolates. The OYDVGI&K isolate did not group with isolates of OYDVDEL, OYDVGKAR and OYDVGRAJ but showed relationship with Chinese and Japanese isolates. Similar result was found when NIB amino acid sequences were used for
phylogenetic analysis except that OYDVON isolate and OYDVGJ&K isolate were grouped with Japanese isolates. On the basis of phylogenetic tree analysis of nucleotide sequences of UTR region of OYDV isolates, OYDVGDEL, OYDVGKAR and OYDVGRAJ isolates grouped with Chinese, Japanese and Netherland isolates. While OYDVGJ&K isolate showed relationship to Chinese and Japanese isolates and OYDVON isolate showed relationship only with Japanese isolates. This indicated that OYDV isolates from India had relationship with isolates from other countries indicating their diverse origin. It appeared from the relationship studies that probably the viruses of these crops moved from other countries through planting material.

The CP nucleotide sequence identity matrix and amino acid sequence identity matrix for GarLV isolates showed that the Indian isolate of GarLV had maximum similarity with Japanese isolates.

Four viruses have been investigated in Allium species in India and their PCR diagnosis has been worked out. PCR detection of individual viruses is not only expensive but time consuming too and therefore the multiplex PCR system was developed to detect commonly occurring OYDV and GarLV simultaneously in the same PCR reaction, which reduced the cost of PCR diagnosis. To confirm the amplified product in Multiplex PCR, the amplicons produced for OYDV and GarLV were directly sent for PCR product sequencing from one direction i.e. T7. Both the sequences obtained for OYDV and GarLV matched with already existing sequences of OYDV and GarLV respectively which confirmed that the products of multiplex PCR were of OYDV and GarLV.

Six cultivars of onion and 23 collections of garlic were tested in ELISA and ISEM to identify sources of resistance in onion and garlic. In ELISA, all the onion cultivars and garlic collections showed positive reaction with OYDV. However in addition to OYDV, GarLV was found in 17 garlic collections, LYSV was found in 12 collections and SLV was found only in three garlic collections. On the other hand, the ISEM results indicated that all the onion cultivars and garlic collections carried OYDV, GarLV was detected in 15 garlic collections, LYSV was detected in 6 collections. The results of the screening clearly showed that OYDV is widely prevalent in all tested commercial onion cultivars and garlic clones. However, an Allexivirus in shallot and garlic was observed for the first time in India.
The results obtained during this research work clearly showed that some very important informations have been generated such as OYDV is the major viral infection in *Allium* crops, OYDV was detected in leek and shallot for the first time in India and Allexivirus has been detected for the first time in Indian garlic and shallot. Diagnostic systems such as EM, ISEM, RT-PCR, Multiplex PCR have been standardized and validated to detect viruses in identified crops which will be useful for diagnosis of these viruses. Molecular characterization including cloning and sequencing of OYDV and GarLV have been done for the first time in India. The Indian isolates of OYDV were found to be of diverse origin on the basis of sequence identity and phylogenetic analysis and a simplified and cost-effective method of RNA isolation has been developed. However, no sources of resistances could be identified in the limited varietal screening which maybe taken up on large scale in future using the diagnostics developed under this programme.