5.1 SUMMARY

A survey was done to check the incidence and distribution of tospoviruses in bell pepper plants from various districts viz. Hamirpur, Kangra, Bilaspur, Sirmaur and Solan of Himachal Pradesh. Epidemiological study on disease incidence of tospovirus was carried out in relation to various meteorological parameters using AZ instrument. Parameters included in studies were temperature (°C), Relative humidity (%), altitude (m) and pressure (bar). All the meteorological parameters recorded from various districts were further analyzed using simple correlation studies using statistical software SPSS version 20. Two types of isolates were selected for study i.e. open field and protected (poly house) bell pepper plants. It has been observed that the symptoms like chlorotic and necrotic ring symptoms, concentric ring pattern, curling of leaves and stunted growth of plants were typical of tospoviruses. Symptoms observed on bell pepper plants have given the indication of presence of tospoviruses. A total of 30 villages were surveyed during study period for two years and severity of disease was found to be maximum in poly house grown plants as compare to open fields. Disease incidence was found to maximum 80% in most of the villages of Himachal Pradesh.

After morphological identitification, tospoviruses were identified using serological reactions. A total of 60 bell pepper plants were taken for this confirmation. Samples were confirmed by using both Direct Antigen Coating- Enzyme linked immunosorbent assay (DAC-ELISA) and Double Antibody Sandwich-Enzyme linked immunosorbent assay (DAS-ELISA). Therefore, DAS-ELISA was performed by using Group specific antiserum (Bioreba) to check the presence or absence of tospovirus in selected samples from five districts of Himachal Pradesh. Out of 60 bell pepper isolates, 54 were found to be positive for tospovirus infection. Further, type of tospovirus present in all positive isolates from different districts was confirmed using DAS-ELISA with monoclonal antibodies specific for TSWV as well as GRSV and DAC-ELISA using GBNV/CaCV specific polyclonal antiserum. All the 60 samples were found negative for TSWV and GRSV, thus ruled out the possibility of these two viruses from Himachal Pradesh and also supported earlier findings. To get the more authentic results molecular identification was performed to confirm the type of tospovirus in infected bell pepper samples. Two samples were taken from each district (except district Kangra) representing a pool
of both open filed and poly house grown samples. From district Kangra, three samples were selected from polyhouse conditions for molecular identification. Therefore, a total of 11 symptomatic bell pepper isolates which were positive consistently with serological tests were used for molecular identification. RT-PCR was performed in three stages just because of mimicking of typical ring pattern like symptoms by three tospoviruses namely *Tomato spotted wilt virus* (TSWV), *Groundnut bud necrosis virus* (GBNV) and *Capsicum chlorosis virus* (CaCV). Primers were designed for coat protein (based on N gene or coat protein specific primers available at NCBI database) for all these three tospoviruses and also for partial S and M segments and finally full S segment of GBNV for performing RT-PCR. Results of initial RT-PCR of 11 isolates were found to be negative for TSWV thus ruled out the possibility of this virus and supported the results of serological identification. Second stage RT-PCR was done in two stages, first with partial S and M segments primers for GBNV with only one sample i.e. C1 from district Sirmaur because of its prominent symptoms. Surprisingly, C1 sample when amplified with partial S segment primer showed amplification of ~ 500bp and BLAST identity with CaCV and M segment primer showed amplification of ~ 600 bp showed identity with GBNV. This result given a indication of mixed infection of GBNV/CaCV in C1 isolate. All the 11 isolates were further amplified using GBNVCP specific primers, all isolates except one from Badripur village (which was positive) of district Sirmaur were found negative. BLAST analysis of this isolate showed 95% identity with Indian isolate. In third stage RT-PCR, all the isolates were found positive with CaCV CP primers. Therefore, during these findings a mixed infection for GBNV/CaCV in only one isolate (C1) of district Sirmaur was identified. All the fragments from 11 isolates were purified and sequenced and were found to be 99% identical to CaCV isolates reported from Chilli (South India). In phylogenetic analysis, all the 11 isolates were clustered together and showed identity with already reported Indian CaCV isolates. Similarly, GBNV isolate from C1 isolate have showed identity with GBNV isolates reported from India. C1 isolate of Sirmaur district was positive for both GBNV and CaCV. Therefore, this sample was further taken for amplification using GBNV full S segment primer. Sample was amplified and showed amplification of ~2200bp. Amplified fragment was purified and cloned in 2.1 vector. Recombinant plasmid was purified from positive colony and send for sequencing which further confirmed its presence as GBNV.
Last objective was performed to develop rapid and more sensitive technique for the detection of tospoviruses in infected samples of bell pepper plants. Amperometric biosensor was developed and compared to DAC-ELISA to check the sensitivity for the identification of typical tospovirus specially CaCV. DAC-ELISA was performed using various dilutions but was found to be sensitive till 1:1000 dilutions whereas immunosensor was found sensitive up to 1,640,000 dilution. Cyclic Voltammetry studies was performed which showed that Amperometric biosensor (Immunosensor) was approximately 500 times more sensitive as compare to DAC-ELISA. Serological identification and PCR based methods have their own limitations for the detection of viruses in plants. In plant isolates, where virus titer is very less to be detected by ELISA this technology will be fruitful. In future also, this technique will be highly important in quarantine procedure while importing the important experimental material or other plants from various countries.
5.2 CONCLUSIONS

- First of its kind survey in different districts of Himachal Pradesh for identification of tospoviruses in bell pepper plants.
- Higher disease incidence (80%) was recorded from polyhouse grown bell pepper plants.
- Out of 60, 53 symptomatic samples were found to be positive with serological test using tospovirus group specific antibody.
- *Tomato spotted wilt virus* (TSWV) and *Ground nut ring spot virus* (GRSV) were not observed in Himachal Pradesh.
- CaCV was found to be prevalent tospovirus in bell pepper as compare to GBNV.
- First report of CaCV in its natural reservoir i.e. *Amaranthus* sp.
- First report of mixed infection of GBNV and CaCV from Badripur village of district Sirmaur.
- 10 isolates of CaCV representing all the districts were sequenced, showed 99% similarity with already reported Indian isolate from chilli.
- Immunosensor based detection method was developed for the detection of CaCV.
- In future, for important epidemiological studies, many other factors need to be included.
- Various studies are required for thrips species present in Himachal Pradesh for better conclusion about tospoviruses transmission.
- Tospoviruses status needs to be established in future for other cash crops of Himachal Pradesh.