CHAPTER 2

Review of Literature

_Bhut Jolokia_, an extremely pungent chilli, is endemic to North East India. It is known by different names and _Bhut Jolokia_ is the name as it is called in Assam. It is cultivated and consumed either raw or as a spice in culinary preparations. It is popular for its pungency and aroma, peculiar to this chilli species. The hotness and pungency of chilli is due to the presence of capsaicinoids, a group of lipophilic alkaloids produced by the plants as a self defence mechanism against the invasion of predators and herbivores. Among the capsaicinoids, capsaicin and dihydrocapsaicin are responsible for 90% of pungency in most of the chilli species (Suzuki _et al._, 1980). These compounds are found only in the members of the genus _Capsicum_ and are mainly localized in the placenta and flesh of the chilli fruit. Conventionally, an organoleptic test is being used to measure the hotness of chillies. This test was first developed by Wilber Scoville in 1912 and hence the unit of measure of hotness was given as Scoville Heat Unit (SHU). However, in modern times, with the advent of sophisticated instruments, the capsaicinoid content is being estimated more accurately with the help of HPLC and Spectrophotometer. Pure capsaicin measures 150,00,000-160,00,00 SHUs. The higher the capsaicinoid content, higher is the hotness (Borgohain and Devi, 2007).

Unlike other chilli species, cultivated in the Indian sub-continent, which contain around 1% capsaicin, _Bhut jolokia_ has around 3-5% capsaicin (Mathur _et al._, 2000; Sanatombi and Sharma, 2008). When Mathur _et al._ (2000) reported hotness of _Bhut Jolokia_ to be 8,55,000 SHUs, it superseded Red Savina Habanero of Mexico
whose pungency was 5,77,000 SHUs. *Bhut jolokia* was officially recognized as the World’s Hottest Chilli in the year 2006, measuring 10,01,304 SHUs after testing its hotness by the New Mexico State University, thereby replacing Red Savina which had SHU of 2,48,566 (Guinness Book of World Records, 2006; Bosland an Baral, 2007). However, after holding its glory for five years, *Bhut Jolokia* has been superseded by other chilli species in subsequent years, as in 2011 by the Infinity Chilli, in 2012 by the Naga Viper, in 2012 by Trinidad Moruga Scorpion and in 2013 by the Carolina Reaper (Kehie *et al.*, 2016) until now. Presently, *Bhut Jolokia* holds seventh position in the list of World Hottest Chilli-2016.

The pungency in *Bhut Jolokia* is affected by the climatic condition and time of cultivation. High rainfall and humidity with moderate climate of North East India favours development of its extreme pungency. When this chilli was cultivated elsewhere, lower levels of pungency was recorded. For example, when it was grown in Gwalior, there was reduction of capsaicin and dihyrocapsaicin by 50% (Tiwari *et al.*, 2005) and when cultivated in Pithoragarh in Uttarakhand, the pungency level came down to 2,54,896 SHUs (Pandey *et al.*, 2009).

*Bhut Jolokia*, belonging to the family Solanaceae, is a self-pollinated crop, though cross-pollination occurs (10%) when the insect population is high. Under optimum condition, the plant behaves as perennials and grows to a height of 50-100 cm or sometime even taller. The leaves are ovate in shape and the size ranges from 5.5-7.5cm in width and 10-14 cm in length. Flowers are pendant having creamy white corollas. The fruit is usually oblong in shape with a rough texture and is bright red or orange in colour, which varies with soil and climate. A single fruit bears about 25-35 slightly wrinkled seeds and weighs around 5 gram (g) on an average. A single plant
produces, on an average, around 15-20 full sized fruit and 10-14 smaller ones (Borgohain and Devi, 2007).

2.1 Cultivation practices of Bhut Jolokia in North East India

The climatic condition of North East India favours natural growth of Bhut Jolokia and subsequent development of its unique extreme hotness without much care. Traditionally, it was grown as a sporadic intercrop with paddy under Jhum cultivation in the hills as well as in homestead gardens. In NE region, there are two planting seasons for this chilli, Kharif and Rabi. In the hilly states, Kharif cultivation is followed where planting is done in the month of February/ March and harvesting starts from May/ June onwards, while Rabi is followed in the plains where planting is done in August/ September and harvesting from November/ December. In the homestead garden, it is cultivated under partial shade and the plants are kept for 2-3 years, beyond which, there is reduction in fruit size and yield. The plants grow to a height of 12-13 feet at the age of 3 years. The plants are reported to be infested by aphid and mealy bugs in traditional fields. The farmers usually sprinkle sieved ash on the leaves and base of the plant as an indigenous crop management practice (Bhagowati and Changkija, 2009; Borgohain and Devi, 2007). Though no package of practices for Bhut Jolokia could be found, Singh (2009) reported highest cost: benefit ratio (1:8.7) on sowing the crop in September 15 and planting at a spacing of 75 cm x 75 cm followed by October 15 sowing (1: 7.3).

The average yield of fresh fruits of Bhut Jolokia under rainfed condition is estimated to be around 80-100 q/ha, which on dehydration comes to around 10-12
The price of per kilogram of fruit of *Bhut Jolokia* in local market is Rs.100-150, as reported by Borgohain and Devi (2007), while the current price in Tezpur region ranges from Rs. 300-350 depending upon the season.

Since the cultivation of *Bhut Jolokia* is an unorganized sector spread out in different pockets of North East India, there is no documented data available on area, production and yield loss due to diseases. As per a rough estimate, 1000 tonnes of *Bhut Jolokia* is produced per year in different regions of North East India which may increase upto five times, if environmental factors are conducive (Meghvansi *et al*., 2010).

### 2.2 Prospects of *Bhut Jolokia*

Traditionally, *Bhut Jolokia* is used as spice or consumed raw due to its pleasant and palatable aroma unique to this chilli. Since time immemorial, this chilli has been used in treating myriads of human ailments such as asthma, gastrointestinal abnormalities, toothache, muscle pain, arthritis and removal of puss from boils. (Bhagowati and Changkija, 2009). This chilli has been patented under Geographical Indication Registry by the Government of Nagaland (Kehie *et al*., 2014).

Since *Bhut Jolokia* contains around 3-5% of capsaicin, unlike other chilli crops which has on an average 1% capsaicin, is the most promising species for extraction of oleoresin on commercial scale, having wide applications in pharmaceutical and defence sectors. Capsaicin is a costly product and is in high demand, both in the domestic and international market. Capsaicin has been found to have wide array of pharmacological activities such as analgesic, anti-inflammatory, anti-cancer, anti-
obesity, hepatoprotective, anti-oxidants, anti-microbial, anti-pyretic, anti-diabetic, etc. (Meghvansi et al., 2010; Roy, 2016).

Besides its pharmacological uses, *Bhut Jolokia* has tremendous potential for its use as value added products in the form of dry chilli, as whole, powdered or flakes, paste, distilled oil for flavor, pickles, sauce, etc. which needs to be explored. This chilli is gaining popularity in the western world and is a popular ingredient of several recipes of restaurants worldwide. Frontech Agritech Pvt. Ltd. in Jorhat, an agri-business company is involved in exporting of *Bhut Jolokia* to markets in Europe and US.

In recent times, *Bhut jolokia* has been explored for unusual application in preparation of chilli grenades, sprays (Singh, 2013) and as elephant repellant (Hussain, 2007). DRDO has been forefront in using oleoresins, extracted from *Bhut Jolokia*, in grenades for use as non-lethal mob dispersal agent, in lieu of tear gas which is harmful, and to flush out terrorists from their hide-outs. Defence Research Laboratory, Tezpur has developed several variants of chilli grenades which have been field tested and adopted by the army, paramilitary and various state police forces. Oleoresin from *Bhut Jolokia* has also been used in aerosol spray for use for self-defence by women and elderly, which can be sprayed onto the eyes of the assaulters. Conservationists in Assam have tried using *Bhut Jolokia* to keep away elephants from venturing into crop fields and human settlements. They either smeared it on the fences alongwith grease or use in smoke bombs. The elephants are repelled by the strong pungent smell of the chilli without causing any harm to them.
Thus, *Bhut Jolokia* or Naga King Chilli is a chilli species of North East India with high potential for commercial application. This chilli crop brought fame to this region by being the world’s hottest chilli and, if explored judiciously with proper post-harvest handling and good market network, can bring about economic upsurge of the farmers of this region. Due to the rising demand for this chilli, both in the domestic and international market, it is being cultivated on a wide scale in different regions of North East India. However, this chilli is reported to be infected with leaf curl disease (Talukdar *et al.*, 2015; Baruah *et al.*, 2016; Adluri *et al.*, 2016), caused by begomoviruses, leading to total crop loss which is a serious concern among the farmers in this region.

### 2.3 Begomoviruses

Begomoviruses are a group of viruses belonging to the genus *Begomovirus*, which is the largest and most important genus of the family Geminiviridae (Borah and Dasgupta, 2012), members of which have circular single-stranded DNA (ssDNA) genome. The name of the family has been derived from the Latin word, *Gemini*, meaning twins due to the presence of the characteristic twin virus particles. The virions measure 18-20 by 30-35 nm and consist of two quasi-icosahedral capsids with $T = 1$ symmetry. Each geminate particle contains one single-stranded (ss) DNA molecule that replicates in the nuclei of infected cells. Besides the genus *Begomovirus*, the family comprises of another three genera, namely *Mastrevirus*, *Curtovirus* and *Topocuvirus* which differ from one another in their genome organization, host range and insect vectors (Dijkstra and Khan, 2007).
Begomoviruses are transmitted by whiteflies (*Bemesia tabaci* or *Bemisia argentifolia*) in a circulative-nonpropagative manner, while few species of this genus are mechanically transmissible, for example, Bean Golden Mosaic Virus (BGMV) and Mungbean Yellow Mosaic Virus (MYMV). These viruses can be inactivated through heat treatment at temperature ranging from 40 -55°C. BGMV retains its infectivity in sap stored at 23°C for about 3 days, while others loose in 1-2 days when stored at 20°C (Dijkstra and Khan, 2007).

Geminiviruses are known to cause heavy economic damage to many important horticulture plants such as solanaceous vegetables, cucurbits, beans and cassava, mainly in the tropics and the sub-tropics (Brown, 1991; Brown and Bird, 1992). *Tomato yellow leaf curl virus* (TYLCV) cause considerable losses to tomato cultivation in the Middle East (Hassan *et al.*, 1985), while *Bean golden mosaic virus* is a major pathogen infecting bean cultivation in Brazil, Central America and the Carribean Basin (Galvez and Morales, 1989). Besides, they are also known to infect several weed species (Bird *et al.*, 1975; Brown, 1991; Costa, 1976), but not much is known about their relation with the geminiviruses infecting field crops (Rojas *et al.*, 1993).

The ICTV has recommended a system for classification and nomenclature of begomoviruses on the basis of complete nucleotide sequences of DNA-A of both mono- and bipartite types. As per this system, two viral sequences are different, if there is less than 89% similarity of the DNA sequences and, if there is more than 89% similarity, they are said to be variants of the same virus. In case of naming a new virus, the nomenclature should reflect the name of the host from which it was first isolated and its symptoms. The abbreviated form should reflect the year and place of
collection of that particular virus (Fauquet et al., 2000, 2003).

Due to the increasing instances of geminivirus infection causing economic losses to nation’s economy, it is important to characterize them in order to identify them and to develop rapid and accurate detection assays, which will be of immense help in epidemiological studies as well as in formulating breeding strategies for disease resistance and management.

2.4 Genomic organization of Begomovirus

The genus Begomovirus consist of 132 species (Fauquet and Stanley, 2005). The generic name Begomovirus has been derived from the name of the type species, Bean golden mosaic virus-Puerto Rico (BGMV-PR) (Hull, 1970). Based on their genome organization, they are divided into two types viz. monopartite (having single DNA component called DNA-A) and bipartite (having two DNA components called DNA-A and DNA-B). The size of both DNA-A and DNA-B component is approximately 2.6-2.8 kb. The DNA-A component consists of six Open Reading Frames (ORFs), one (AV1) or two (AV1 and AV2) in the viral sense (V-sense) and four (AC1 to AC4) in the complementary sense (C-sense) strand. DNA-B contains two ORFs, one (BV1) in the V-sense and the other (BC1) in the C-sense strand. In DNA-A, AV1 codes for coat protein (CP), AV2 for a protein of unknown function, AC1 for a replication associated protein (Rep), AC2 for a transcriptional activator (TrAP), AC3 for replication enhancer (Ren) and AC4 for suppressor of RNA silencing. In DNA-B, BV1 codes for a Nuclear shuttle protein (NSP) and BC1 for a Movement protein (MP) which are responsible for intracellular and intercellular
movement of the virus particle respectively. The non-coding region known as intergenic region (IR) contains the origin of replication (Ori), where viral Rep protein binds for initiating Rolling Circle Replication and is of approximately 500 bp in size and is conserved between the two DNA components of bipartite begomoviruses. Besides, it also contains the promoter/ regulatory elements for expression of the viral genes in both V-sense and C-sense strand (Borah and Dasgupta, 2012; Dijkstra and Khan, 2007).

Begomoviruses have been broadly classified into Old World viruses, those prevalent in eastern hemisphere as in Europe, Africa and Asia, and New World viruses, that of western hemisphere, the Americas. All indigenous New World viruses are bipartite, whereas, Old world viruses contain both bipartite and monopartite begomoviruses (Padidam et al., 1999). Monopartite begomoviruses usually contain one smaller DNA fragment, of approximately 1.4 kb in size, which are generally termed as Betasatellite and was earlier termed as DNA-β. Betasatellites contains an ORF (βC1) which codes for a multifunctional protein having RNA silencing activity, besides other unknown functions. The satellite DNA help in the replication of the virus and is also known to attenuate disease symptoms in the affected host plant (Briddon et al., 2001; Jose and Usha, 2003; Idris et al., 2011). The monopartite viruses are incapable of inducing disease symptoms in absence of their betasatellite component, when inoculated as infectious or as full length clone, as observed in case of Cotton leaf Curl Multan Virus (CLCuMV) (Briddon et al., 2001) and Ageratum yellow vein virus (AYVV) (Tan et al., 1995). These betasatellites are reported to be associated with several diseases caused by begomoviruses, as evidence from the report of isolation of Cotton leaf curl virus after the occurrence of epidemics in the central
provinces of Pakistan during 1990 (Mansoor et al., 2003).

2.5 Molecular diagnosis of Begomovirus

Accurate diagnosis of plant diseases is a prerequisite for any crop management programme. In case of viral diseases, treatment after infection is not much effective and hence, control measures should be adopted before the occurrence of infection. Viral diseases can be effectively managed through the use of disease free planting material, for which, sensitive diagnostic assays are most vital for a successful certification programme. Recent development in the field of molecular biology and biotechnology has played a significant role in the development of rapid, accurate and sensitive diagnostic assays.

Application of Enzyme-inked Immunosorbent Assay (ELISA) technique for detection of viruses was a significant step in the development of sensitive and specific assays. Development of Tissue-blot Immunoassay (TBIA), a variant of ELISA, has much simplified virus testing, through reduction of cost and ease of application in locations, where no much facility exist. Another variant of ELISA, Immuno Chromatographic Assay (ICA) expedited the process of virus detection, where results could be obtained within 10-15 minutes in comparison to TBIA which takes 2-3 hours. However, ICA is costlier than TBIA. Breakthrough occurred in virus detection with the development of nucleic acid based assays such as cDNA hybridization and Polymerase Chain Reaction (PCR). These techniques are rapid and highly specific and can also be used as confirmatory test for TBIA. These techniques can be used for detection of both DNA and RNA viruses. PCR products can be further cloned and
sequenced which is useful for identification of new viruses or strains (Makkouk and Kumari, 2006).

### 2.6 Begomoviruses infecting chilli crops in the Indian sub-continent

*Begomovirus* infection has emerged as a potential threat to cultivation of several economically important food and fibre crops such as vegetables, root crops, legumes and fibre crops (Varma and Malathi, 2003) with high yield loss and in certain cases, the extend is estimated to be 100% (Dasgupta et al., 2003). Within the last decade, a large number of *Begomovirus* infection has been reported and investigated, with more focus on the molecular characterization and interaction between begomoviruses and their host, with an aim to gain insights into molecular cross talks that might help to know novel and unknown aspects of their biology which shall help in devicing better management practices (Borah and Dasgupta, 2012).

Chilli plants infected by *Begomovirus* exhibit symptoms such as curling, crumpling and mottling of leaves, leaf mosaic, puckering, distortion of leaves, twisted petioles, shortening of internodes and petioles, crowing of leaves resulting in stunting of the plants (Khan et al., 2006; Khan et al., 2007; Senanayak et al., 2007; Sinha et al., 2013). Besides, there may be distortion of leaf shapes, reduction in leaf size and thickening of leaves and the infected plants either do not produce fruits or bears small sized fruits.

Chilli plant is reported to be infected by various begomoviruses in the Indian sub-continent. The etiology of leaf curl disease in chilli was first reported in the 1960s (Mishra et al., 1963; Dhanraj and Seth, 1968). *Chilli Leaf Curl Virus* (ChLCV) has
been lately described and reported, since leaf curl disease of chilli was earlier thought to be caused by *Tomato Leaf Curl Virus* (ToLCV). Several researchers have reported distinctiveness of ChLCV and its distribution in the sub-continent, based on molecular characterization, which shall be briefly discussed in the succeeding sections.

### 2.7 PCR based detection of *Begomovirus*

PCR with all its variants is a basic tool for disease diagnosis either alone or in supplement to other techniques. The efficiency of PCR based diagnostic techniques greatly depends on the specificity of the primer, polymerase type, buffer composition and stability, purity and concentration of dNTPs, cycling parameters and the quality of the starting template (Lopez *et al.*, 2009). Host range and disease symptoms of both Tomato leaf curl and Chilli leaf curl diseases are same and hence, it is difficult to determine the virus based on symptoms. PCR based assays are very useful for rapid and accurate detection of virus type.

Chilli (*Capsicum annum*) and Tomato (*Lycopersicon esculentum*) crops in the Indian sub-continent suffer economic losses due to leaf curl disease caused by begomoviruses. With the help of modern molecular biology techniques, attempts have been made by various researchers to identify the virus causing leaf curl disease and their etiology, which shall help in devising better disease management strategies. Geminiviruses, comprising of viruses causing leaf curl disease, are suitable for detection and characterization with the help of PCR based assay since they replicate by means of double-stranded circular DNA intermediate, which is the replicative form and can act as a template for PCR amplification (Stanley, 1991).
Shih *et al.* (2003) tried to enumerate the virus infecting tomato and chilli in the Punjab Province of Pakistan. They amplified the virus by PCR using the degenerate primers PAL1v1978 and PAR1c715. Expected PCR product size of 1.4 kb was obtained in case of the tomato virus and further sequencing of DNA-A was done using specific primers. Two primer sets viz. DNABLC1/ DNABLV2 and DNABLC2/DNABLV2 were used to amplify DNA-B. The size of the two DNA genomes, DNA-A and DNA-B of the tomato leaf curl isolate was found to be 2,739 and 2,728 nucleotides respectively. The DNA-A of the chilli leaf curl virus consisted of 2,754 nucleotides which shared less than 75% nucleotide identity with the tomato isolate from Pakistan and shared 87% identity with *Tomato leaf curl Bangladesh virus*. DNA-Beta of 1.3 kbp was detected using Beta01/ Beta02 primers, while no presence of DNA-B component was reported when tested with DNA-B specific primers. Thus, the *Chilli Leaf Curl Virus* (ChiLCuV) was found to be a new monopartite *Begomovirus* infecting chilli in Pakistan.

In the Punjab province in Pakistan, chilli cultivation often overlaps with cotton and tomato which results in infection of chilli plants by *Begomovirus* causing leaf curl disease in both cotton and tomato. Cotton leaf curl disease (CLCuD) is an important disorder for cotton cultivation caused by *Begomovirus*. During a survey conducted in 2002-2003 in the cotton growing zones in Pakistan, chilli plants exhibiting leaf curling and vein thickening were collected. The total DNA was isolated which was resolved in agarose, blotted onto nylon membrane and hybridized using a non-radioactive probe derived from *Cotton leaf curl Multan virus* (CLCuMV). Positive results were obtained in all the samples, which were further validated through PCR using primer pairs CLCV1 (5’-CCGTGCTGCTGCCCATTTGTCCGCAC-3’) and CLCV2
(5’-CTGCCACAACCATGGATTACGCACAGGG-3’) designed to amplify conserved sequences of begomoviruses isolated from cotton and okra. Presence of DNA- β was determined using a set of universal primer. PCR products of expected size were obtained in both the cases, thereby, confirming CLCuD infection in chilli, which is found to be an alternate host for this Begomovirus (Hussain et al., 2003). Besides, the chilli crop in Pakistan is also infected by Tomato Leaf Curl New Delhi Virus (ToLCNDV) which was confirmed through Southern Blotting using a radioactive probe and further validation with PCR using degenerate Begomovirus DNA-A primers (Hussain et al., 2004).

Khan et al. (2006) for the first time reported infection of Tomato leaf curl New Delhi virus (ToLCNDV) in chilli in India. The leaf curl virus, from infected chilli plants growing around Lucknow, was artificially transmitted to healthy seedlings of chilli and tomato with the help of whiteflies (Bemisia tabaci). The chilli plants exhibited symptoms similar to that observed in the field, while, the tomato plants exhibited severe leaf curling, as those caused by ToLCNDV. Begomovirus infection was further confirmed through PCR with the help of primers designed to amplify coat protein region of ToLCNDV. PCR product of ~800 bp, as expected, was obtained which was cloned and sequenced. On comparing the sequence with the available database at NCBI, it showed 83-93% similarity with various isolates of ToLCNDV (X78956, AY428769, TLU15016 and AF448058). However, the isolate was found to be distantly related to Pepper leaf curl Bangladesh virus (AF314531) and Chilli leaf curl Multan virus (AF336806), showing 86 and 81% identity respectively. Based on the sequence identity, the virus was described as an isolate of ToLCNDV and this was the first report of ToLCNDV affecting chilli in India. Considering the earlier report of
infection of chili by ToLCNDV in Pakistan, chilli crop is considered a major alternative host of ToLCNDV in the Indian sub-continent.

In Ludhiana in Punjab state of India, chilli crop is reported to be infected by *Tomato Leaf Curl Joydebpur Virus* (ToLCJV). Chilli plants were found to exhibit severe curling, distortions of leaves, blistering and stunting which are characteristic symptoms of *Begomovirus* infection. In order to ascertain the viral strain, DNA of three diseased plants was extracted followed by PCR assay using previously reported primers. All the three samples gave positive amplification of 1.4 kbp and 1.3 kbp of DNA-A and DNA-Beta fragment of *Begomovirus* respectively. But no amplification was observed for DNA-B genome. Further sequencing of the entire genome of DNA-A, based on the sequence of 1.4 kbp fragment, was done which consisted of 2767 nucleotides (DQ673859) and contains the geminiviral conserved nanosequence TAATATTAC in the intergenic region. On blasting this sequence at NCBI database and further processing using MegaAlign software (DNASTAR), highest identity of 90.8% was observed with a *Begomovirus* infecting tomato in India (DQ673859) followed by 90.3% with *Tomato leaf curl Joydevpur virus* (ToLCJV, AJ875159) reported from Bangladesh and showed low sequence identities (73-80%) with other partial sequences of Indian chilli Begmoviruses reported from Lucknow, Bahraihi and Kanpur (DQ119573, DQ431845, AY883570, DQ431844, DQ431846 and DQ141676) which are basically different isolates of *Tomato leaf curl New Delhi virus*. Based on the sequence identity percentage, the isolate of chilli virus from Punjab is reported to be a strain of ToLCJV and is thus, the first report of natural infestation of ToLCJV in chilli in India (Shih *et al.*, 2007).
Senanayake et al. (2007) for the first time reported infection of *Chilli Leaf Curl Virus* (ChiLCuV) in chilli crop in India. High incidence (100%) of leaf curl disease was observed in farmer's field in Narwa and Tinwari villages in Jodhpur district of Rajasthan. The virus from the diseased plants of Narwa village was experimentally transmitted to healthy plants of chilli by means of whiteflies which developed clearing and curling of leaves leading to stunting of the test plants. Microscopic examination of the field infested chilli plants with the help of electron microscope revealed presence of few typical geminate particles. In order to confirm *Begomovirus* infection, PCR was conducted using degenerate primers AVF28 (5’-GCCCATYGTCTTTYCCNGT-3’ and AV29R (5’-GGCTTTYCRTACATRGG-3’), which gave a product of size ~1.0 kbp. It was cloned and sequenced which yielded a 995 bp sequence (DQ445255). On blasting the sequence at NCBI, it showed close proximity with the intergenic region (IR) and part of the replication initiator protein, AV1 and AV2 genes of ChiLCuV reported from Pakistan by Shih et al., 2003 and shared 96.5% sequence identity with it.

Rai et al. (2010) reported emergence of a new variant of chilli leaf curl virus in North India. Leaf curl disease infected chilli plants were collected from Varanasi, Mirzapur, Gorakhpur and Mahrajganj districts of Uttar Pradesh. DNA was extracted from the collected samples and the highly conserved coat protein region was amplified and sequenced using coat protein gene (CP) specific primers. The sequences were compared with 25 similar *Begomovirus* sequences available at NCBI. The CP sequence identity of the Varanasi isolate was found to be 86% with the Gorakhpur and Mirzapur isolates and 87% with the Mahrajganj isolate. Based on the CP gene sequence identity, phylogenetic analysis was conducted which grouped 29 sequences
into three major clusters. The Gorakhpur, Mahrajganj and Mirzapur isolates showed 99% sequence identity and clustered with *Tomato leaf curl Joydebpur virus*- [Kalyani], while the Varanasi isolate clustered with *Chilli leaf curl virus*- [Amritsar:Papaya] with 88% sequence identity. The Varanasi isolate differed from the other three isolates and thus, can be considered as a different variant of *Begomovirus* infecting chilli in North India.

Sivalingam *et al.* (2012) conducted survey of chilli leaf curl diseases occurring in the major chilli growing hot arid regions of Bikaner, Nagur, Jodhpur and Jalore districts of Rajasthan. The survey revealed that highest disease incidence was recorded in Jodhpur followed by Jalore district. Based on the available sequences of the conserved region of begomoviruses, primers were designed for PCR amplification of the *Begomovirus* infected chilli leaf samples, followed by cloning and sequencing of PCR amplified products. Results confirmed *Begomovirus* infection in the sampled regions with association of a satellite DNA β component.

Another new *Begomovirus* was reported to infect Chilli (*Capsicum annum*) cultivation in the Velland region of Kerala, which caused 100% crop loss. In order to confirm the presence of *Begomovirus* in the symptomatic plants, the total DNA was extracted and used as template for PCR amplification of the *Begomovirus*-specific fragments using universal primer pairs PAL1v1978/ PAR1c496 and Beta01/ Beta02 for DNA-A and DNA-β components respectively. PCR products of 1.5 kbp of DNA-A and 1.4 kbp of DNA-β were obtained which confirmed presence of *Begomovirus* infection. The extracted DNA was subjected to Rolling Circle Amplification (RCA) using φ29 DNA polymerase followed by digestion with *KpnI* which yielded a fragment of ~2.8 kbp. The fragment was eluted and cloned into pTZ57R/T. Similarly,
DNA-β component was also cloned into pTZ57R/T. The positive clones were subjected to restriction digestion of the plasmids which showed presence of a single molecule. Sequencing of the clones revealed viral genome of 2788 nucleotides with seven predicted ORFs (AV1, AV2, AC1, AC2, AC3, AC4 and AC5). On comparing this sequence with the known Begomovirus sequence available at NCBI database, it showed sequence identity of 77% with a Pakistan strain of Pepper leaf curl Bangladesh virus (AF314531), and 64-76% with other begomoviruses reported from the Indian sub-continent. This virus formed a separate clade in the phylogenetic tree and as per ICTV guidelines, it was considered as a new Begomovirus, which was named as Chilli leaf curl Vellanad virus (ChiLVeV). The associated betasattelite components (JN663876 and JN663877), each containing a single ORF C1, shared maximum identity of 95.6% and 96.9% with Radish leaf curl betasattelite and Tomato leaf curl Bangladesh betasattelite respectively and thus they can be considered as isolates of satellite DNAs reported earlier (Kumar, et al., 2012).

Sinha et al. (2013) reported infection of chillies, grown in the experimental farm of IVRI at Varanasi by Raddish Leaf Curl Virus (RaLCV). For characterization and confirmation of Begomovirus infection, DNA was isolated and PCR amplification was carried out using Begomovirus coat protein (CP) specific forward and reverse primers 5’-AGAATTATGTCCAAGCGACCA-3’ and 5’-AAGCGTTGGGATACACAAA-3’. An amplicon of 750 bp was obtained which was cloned and sequenced. Phylogenetic analysis of the CP sequence revealed close relationship of the isolate with the Indian and other Asiatic isolates, exhibiting 100% sequence identity with RaLCV and ToLCNDV.
From the above reports, it was observed that chilli plants in the Indian subcontinent are being infected by several \textit{Begomovirus} species \textit{viz.} \textit{Chilli Leaf Curl Virus} (ChiLCuV), \textit{Cotton leaf curl Multan virus} (CLCuMV), \textit{Tomato Leaf Curl New Delhi Virus} (ToLCNDV), \textit{Tomato Leaf Curl Joydebpur Virus} (ToLCJV), \textit{Raddish Leaf Curl Virus} (RaLCV) and \textit{Chilli leaf curl Vellanad virus} (ChiLVeV). Thus, this aspect should be taken into consideration while devising disease management strategies or in developing resistant varieties against Chilli leaf curl disease.

2.8 \textbf{Role of different genome components of \textit{Begomovirus} in disease etiology}

The role of different components of \textit{Begomovirus} is not fully known, though few reports are available on their role in disease development and severity in Chilli.

Chattopadhay \textit{et al.} (2008) studied the role of DNA Beta complex in disease severity during study of chilli leaf curl disease incidence in the experimental fields of Indian Institute of Vegetable Research (IVRI), Varanasi, Uttar Pradesh. They cloned complete DNA-A genome alongwith its cognate component, DNA betasatellite, after isolation of the total DNA from the diseased leaves. They initially verified the presence of \textit{Begomovirus} through PCR using primer pairs PAL1v1978 and PAR1c496. The viral genome was then digested with restriction endonucleases (\textit{BamHI, EcoRI, HindIII, PstI}) which was then cloned into pUC18 that was previously linearized with the same enzymes. Similarly, the presence of DNA betasatellite was confirmed using earlier reported primer pairs and a product size of 1.3 kbp was amplified which was cloned in pTZ57R vector. No DNA-B component was amplified even after repeated attempts. In order to assess infectivity of the various components
of the virus, DNA-A and DNA-β genomes were sub-cloned as partial tandem repeats in the binary vector pCAMBIA23301. In case of DNA-A, the full length BamHI fragment was inserted to produce a 1.5 mer tandem repeat named ‘pTChVA’, while for the DNA-β component, KpnI (1,111)- BamHI (1,148) fragment was cloned to produce a 1.9 mer tandem repeat named ‘pTChVβ’. Clones of partial tandem repeats were artificially inoculated into chilli, tobacco and tomato plants and it was observed that the viral genome could induce symptoms of leaf curl disease, while the DNA-β could not alone induce any disease symptoms. On co-inoculation of the test plants with both the clones pTChVA and pTChVβ, it was observed that infectivity and symptom severity increased. Thus, when the viral genome was inoculated alone, the symptoms were milder and took longer time to develop as compared to those produced when co-inoculated with DNA-β. The presence of the viral genome and DNA-β was tested through PCR and the whole genome sequencing of both the viral genome and DNA-β satellite were done by dieoxynucleotide chain termination method using ABI automated sequencer with primers specific to either cloning vectors or established viral sequences. Sequence similarity searches were conducted by comparing sequences of other Begomovirus sequences available at the GeneBank databases. The results indicated that the viral genome shared greater proximity with the begomoviruses reported from Asia and was most closely related to ChiLCV-PK [PK: Mul:98], followed by ChiLCV-Kha [PK:Kha:04] and ChiLCV-IN [IN:PRM:Tom:05] with 95, 88 and 87 percentage identity respectively. The IR region of the viral genome showed highest identity of 90% with ChiLCV-PK[PK:Mil:98] and is thus considered as a strain of ChiLCV-PK and is named ChiLCV-PK[IN:Var:06]. From the phylogenetic studies, it was observed that this strain forms clusters with
begomoviruses infecting chillies in the Indian sub-continent.

Shafiq et al. (2010) studied the role of DNA-B genome of bipartite *Begomovirus* in disease development in chilli plants. Leaf samples were collected for detection of the *Begomovirus* through PCR using degenerate primers BegomoF/BegomoR (Shahid et al., 2007) to test presence of *Begomovirus*. A PCR product of approximately 2.8 kb was obtained confirming association of *Begomovirus* with the symptomatic chilli plants. The authors have previously characterized the virus infecting chilli plants which they reported to be ChLCB [Pk:Fai69:04, Acc. No.: AM279673] (Hussain et al., 2009). Lather, full length *Begomovirus* and betasatellite was amplified through PCR using universal primers *viz.* BegomoF/ BegomoR and Beta01/ Beta02 respectively (Shahid et al., 2007; Briddon et al., 2002). The primer pair BC1F/BC1R (Hussain et al., 2004) was used for amplification of the DNA-B component. PCR products of expected size were eluted and cloned into pTZ57R/T vector and a single clone positive for presence of full length *Begomovirus*, named PGL1, was selected for epidemiological studies. The clone, on sequencing, was found to contain 2747 nucleotides (Acc. No.-AM691745) which showed highest sequence identity of 99% and 89% with PepLCLV-[PK:Lah:0404](AM404179) and PepLCuBDV-PK[Pk:Kha:04] (D116881) respectively. Thus, the clone PGL1 is an isolate of PepLCLV for which the authors proposed the isolate descriptor PepLCLV-[PK:Fai:04]. The infectivity of the clone PGL1 was tested through *Agrobacterium* mediated inoculation, either alone or in combination with the DNA-B component of ToLCNDV or *Chilli Leaf Curl Betasatellite* and *Cotton Leaf Curl Multan Betasatellite* in *Nicotiana benthamiana*, *N. tabacum* cv. Samsun and *Capsicum annum*. Plants of *N. benthamiana* inoculated with PGL1 exhibited low infectivity with very mild disease
symptoms, and low titre of the viral DNA was detected by PCR. Inoculation of *N. tabacum* and chillies, either alone or in combination with betasattelite did not induce any symptoms of leaf curling. However, co-inoculation of PepLCLV with the DNA-B component of ToLCNDV induced disease symptoms with severe leaf curling in *N. benthamiana*, *N. tabacum* and chillies with higher accumulation of the viral components as compared to those infected with PepLCLV alone. It has been observed that PepLCLV has been mobilized into chillies upon interaction with the DNA-B component of ToLNDV. Interestingly, the putative rep-binding interons found in PepLCLV (GGGGAC) differs from that of ToLCNDV (GGTGTC) at two base positions. Thus, the phenomenon of component capture is leading to the emerging diversity of the Begomoviruses infecting chillies in the Indian sub-continent.

Akhter *et al.* (2014) conducted infectivity assays of *Tomato Leaf Curl New Delhi Virus* (ToLCNDV) and *Chili Leaf Curl Betasattelite* (ChLCB). For this study, full length viral genomic DNAs were produced by means of restriction digestion of their respective clones reported previously by Akhter *et al.* (2009). Restriction enzymes *MluI*, *XbaI* and *KpnI* were used for digestion of clones of DNA-A, DNA-B and ChLCB respectively. The viral components were inoculated into *Nicotiana benthamiana* through biolistic gun method. Viral load was detected through PCR using primer specific for each component. Plants inoculated with DNA-A and DNA-B components of ToLCNDV exhibited disease symptoms 18 days post inoculation (dpi), while symptoms in plants inoculated with both components of ToLCNDV and ChLCB appeared 15 dpi with severe leaf curling and yellow mosaic which increased with the passage of time. This confirms that, association of betasattelite with begomoviruses, both monpartite as well as bipartite, results in enhanced disease severity and is an
indications of rapid changes occurring in *Begomovirus* complexes infecting chilli and other major crops in the Indian sub-continent.

### 2.9 *Begomovirus* infection in *Bhut Jolokia* in Assam

*Bhut Jolokia*, due to its high capsaicinoid content and unique hotness, is becoming popular for its uses in pharmaceutical industry, defence and as value added products. In recent times, oleoresins produced from it are being used in pain killers, capsi spray, grenades and as elephant repellants. With its growing demand for commercial applications, this chilli is being cultivated in large scale, more particularly in the state of Assam. But this chilli is being infested by leaf curl disease causing serious economic losses amounting to total crop loss. Based on symptomological studies and ELISA assay, *Bhut Jolokia* has been reported to be infected by *Chilli Leaf Curl Virus* (Talukdar et al., 2015; Baruah et al., 2016) but they have not characterized the *Begomovirus* infecting the chilli crop. Adluri et al. (2016) did partial characterization of *Chilli leaf curl virus* infecting *Bhut Jolokia* with an aim to device a rapid PCR based assay for screening of germplasms for their susceptibility to leaf curl disease.

Talukdar et al. (2015) conducted field surveys of viral, fungal and bacterial diseases of *Bhut Jolokia* in Sivsagar, Jorhat and Golaghat, which are the major *Bhut Jolokia* growing districts of Assam and recorded percentage of disease incidence and variable nature of symptoms. It was observed that the incidence of viral diseases (60%) was more than the fungal (10%) and bacterial (3%) diseases in the farmer’s field. Among the viral diseases, *Potato Virus Y* (PVY), *Cucumber Mosaic Virus* (CMV), *Tomato Spotted Wilt Virus* (TSWV) and *Chilli Leaf Curl Virus* (ChLCV) was reported based on symptomatology, transmission, host range and serological assays.
The ChLCV isolate was experimentally transmitted to chilli, tomato and tobacco seedlings through grafting and whiteflies (*Bemisia tabacci*) which produced symptoms of upward curling and rolling of leaves, vein thickening, crinkling of leaf surface, short side branches resulting in bushy appearance of the diseased plants. Based on these typical disease symptoms on the test plants, the viral agent was identified as *Chilli Leaf Curl Virus*.

Baruah *et al.* (2016) studied incidence of viral disease complex in *Bhut Jolokia* and its detection and management in Assam. Double antibody sandwich ELISA (DAS-ELISA) and PCR assays were employed to detect four viruses *viz.* *Cucumber Mosaic Virus* (CMV), *Potato Virus Y* (PVY), *Groundnut Bud Necrosis Virus* (GBNV) and *Chilli Leaf Curl Virus* (ChLCV). They recorded disease incidence of 55%, 36%, 44.9% and 42.5% by CMV, PVY, GBNV and ChLCV respectively. The *Bhut Jolokia* plants were found to be infected by one or more than one virus together forming the disease complex. The typical symptoms of ChLCV infection in the field included upward and downward curling of leaves, reduction in leaf size, puckering and dwarfing of severely infected plants with no or little fruit.

Adluri *et al.* (2016) developed a PCR based assay for detection of chilli leaf curl virus infecting *Bhut Jolokia* using two pairs of specific primers ChLCVF1-ChLCVR1 and ChLCVF2-ChLCVR2 which yielded PCR products of size 550 and 568 bp respectively. Further, the primer pair ChLCVF2/ ChLCVR2 was used to screen different genotypes of *Bhut Jolokia*, collected from various locations of Assam, Nagaland and Manipur, for susceptibility to leaf curl disease and it was found that 4 were highly susceptible, 11 susceptible, 14 moderately susceptible and 1 symptomless genotypes. Sequence similarity search of *Bhut Jolokia* ChLCV isolates at NCBI
database, exhibited 87 to 90% homology with other isolates of Indian ChLCV. The Jorhat isolate is thus considered as distinct from the other Indian isolates for which the name ChLCV-\textit{Bhut Jolokia Jorhat} (ChLCV-BJ-JRT) was proposed. In this study, no attempts were made to test the presence of DNA-B and DNA-\(\beta\) genomes of the Jorhat isolate of ChLCV which is essential for complete characterization of the virus.

In the above reviews, it is observed that chilli plants in the Indian sub-continent are infected by more than one type of Begomovirus. Therefore, this aspect should be taken into consideration for the development of an effective management strategies and resistance breeding. With regards to leaf curl disease of \textit{Bhut Jolokia} in Assam, no reports are available on complete characterization of the \textit{Begomovirus} causing the disease.

### 2.10 Development of real time PCR assays for detection of plant viruses

PCR has been considered as a gold standard for detection of a variety of templates in a wide range of scientific applications including virology (Mackay, 2002). However, conventional PCR has few demerits such as laborious post-PCR applications and does not provide information about the pathogen load in the sample. Traditionally, the amplified DNA is detected by means of gel electrophoresis containing ethidium bromide and visualized after irradiation by ultraviolet light. Southern blot requires hybridization of the amplicon with a probe which is again a time consuming process and involves several post PCR handling steps due to which there is risk of contamination of the working environment (Holand \textit{et al.}, 1991). Although use of PCR for detection of phytopathogens has been reported decades back, few of the plant disease diagnostics laboratories uses PCR for routine disease
diagnosis, which may be due to the long time period required for establishment of the
causal agent. The presence of a single band of PCR product in the gel electrophoresis
does not alone proves the identity of the pathogen which needs further validation
through Southern blot technique (Schaad and Frederick, 2002). However, these
limitations are overcome with the invention of real-time PCR technology (Livak et al.,
1995).

The use of real-time PCR for determination of pathogen load in a diseased
plant has increased in recent years (Winton et al., 2002). Several studies stated the
advantages of using real-time PCR in plant viral disease diagnosis over the
conventional PCR, hybridization or immunological techniques (Mumford et al., 2000;
Korimbocus et al., 2002; Boonham et al., 2004). The major advantage of real-time
PCR is visualization of amplicon as amplification progresses, which has been made
possible through labeling of PCR products, probes or primers with fluorogenic
molecules, which avoids the use of radiogenic probes (Mathews and Kricka, 1988).
Other advantages include the requirement of fewer chemicals and shorter reaction
time, besides the provision for performing additional analysis during the process of
detection (Lopez et al., 2009). The fast rate of real-time PCR may be attributed to
reduced cell cycle, elimination of post–PCR processing and the use of fluorogenic
probes and method for their emission while the process is in progress (Wiitwer et al.,
1990; 1997). There is no need for post-PCR evaluation of the amplicon, for which,
these assays are also called closed or homogenous systems. These systems reduces the
time required for result turn-over, minimizes chances of cross contamination and
allows to closely monitor the performance or sensitivity of the assay (Higuchi et al.,
1993). Real-time PCR has proven to be cost-effective in laboratories with high
throughput applications (Martell et al., 1999), since the initial cost of the system is high. In real-time PCR, there are three fluorescence methods generally employed for detection of target amplicons *viz.* TaqMan probes, molecular beacons and fluorescent resonance transfer (FRET) probes (Mackay, 2002). This assay needs a fluorescent probe or primers which need to be designed from sequences within the amplicon generated by the conventional PCR (Schaad and Frederick, 2002).

Most of the assays employed for detection of viruses are qualitative in nature indicating the presence or absence of the viral pathogen. The advent of real-time PCR has revolutionized virus diagnosis through quantification of the viral load (Mackay, 2002). The determination of template concentration can be done in terms of either absolute or relative quantitation. Absolute quantitation provides the exact copy number of the target gene in a sample, while relative quantitation indicates changes in the concentration of a target gene in comparison to a related matrix (Freeman et al., 1999). Using a standard curve, generated by plotting the threshold cycle (C\textsubscript{t}) versus the logarithm (log) of the concentration of the known samples, the copy number of any unknown samples can be determined (Heid et al., 1996).

Real-time PCR assay was used for the first time in plant viral diagnosis for detection of *Potato leaf roll virus* (PLRV) in dormant potato tubers using TaqMan probes. This assay helped in the reduction of time needed for inspection of potato tubers for PLRV to 1 day from 05 weeks (Schoen et al., 1996). TaqMan assays were also employed for detection of *Tomato spotted wilt virus* (TSWV) in plants (Roberts et al., 2000) as well as in insects (Boonham et al., 2002). Real-time PCR assays has also been reported for detection of *Cymbidium potyvirus* (CymMV) and *Odontoglossum ringspot tobamovirus* (ORSV) infecting orchids (Eun et al., 2000), *Tobacco rattle*
virus and Potato mop-top virus in potato tubers (Mumford et al., 2000).

Real-time PCR assay was used for detection of Sugarcane yellow leaf curl virus which helped in screening of healthy planting material. The assay was reported to be highly sensitive as evidence from the fact that it could detect the virus even in samples that were considered healthy based on conventional assays such as RT-PCR, TBIA and ELISA (Korimbocus et al., 2002). Mason et al. (2008) used real-time PCR assays for detection of Tomato leaf curl Sardinia virus in tomato plants and its vector Bemisia tabaci and found that it was 2200-fold more sensitive than the membrane hybridization which could detect the virus upto 10 copies in a sample.

Though not much fluorescent probes or primers are available for real time PCR detection of Begomovirus, many will be developed in the near future. Since conventional PCR assays are available for several begomoviruses, adapting them to real-time format will be relatively easier. Moreover, sequences of several plant virus genomes are being deposited in the public domain at a high rate, which will be available for designing of primers and probes for real-time assays.