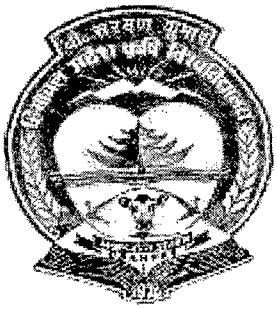
SCENARIO OF POTYVIRUS (ES) INFECTION IN LILY AND TULIP IN HIMACHAL PRADESH

THESIS

By

ANU PRIYA

Submitted to



CSK HIMACHAL PRADESH KRISHI VISHVAVIDYALAYA
PALAMPUR-176 062 (H.P.) INDIA

IN

Partial fulfillment of the requirements for the degree

OF

MASTER OF SCIENCE IN AGRICULTURE
(AGRICULTURAL BIOTECHNOLOGY)
2005

"What can be more clear and sound in explanation, than the love of a parent to his child?"

AFFECTIONATELY
DEDICATED
TO MY
INIMITABLE
PARENTS

06

ACKNOWLEDGEMENTS

The foremost and sincerest thanks are due to 'Almighty God' who bestowed me with his blessings, endowed me with strength and fortitude and directed me aright.

With an overwhelming sense of legitimate pride and genuine obligation which gives me exuberant pleasure and privilege to express my eternal gratitude to my learned and revered advisor Dr. A. A. Zaidi, Scientist & Head, Floriculture Division, Institute of Himalayan Bioresource Technology (IHBT, CSIR) Palampur and chairman of Advisory Committee for his excellent and praiseworthy guidance, constant supervision, keen interest, pertinent criticism and juvenile encouragement during the time of research work and compilation of dissertation. He is gratefully acknowledged for providing the freedom to work various facilities and creating a cordial atmosphere in lab during the course of work.

I am greatful to Dr. P. S. Ahuja, Director, IHBT for providing me the opportunities and necessary facilities to venture in to the fascinating world of biotechnology.

I also acknowledge Dr. S. R. Thakur, Programme Director, Advanced Centre for Hill Bioresource and Biotechnology CSKHPKV, Palampur for providing expert feedback and advice.

I am immensely indebted to Dr. Vipin Hallan (Scientist C) IHBT, Dr. Y. S. Paul (Plant pathology) and Dr. C. P. Awasthi (biochemistry), the members of my Advisory Committee for their valuable help, constructive criticism and inspiring guidance during the period of study and preparation of manuscript.

Heartfelt thanks are due towards Dr. S. K. Sharma (Dean, COBS) for providing excellent and praiseworthy advice, moral support and keen interest during the tenure of study.

I am grateful to Dr. Raja Ram, Dr. Neeraj Verma and Mr. A. R. Sherpa who despite of their busy schedule provided me their cordial cooperation, sincere advice and valuable suggestions during course of investigation and preparation of this manuscript.

I emphatically express my venerable thanks to the Department of Biotechnology, Government of India, New Delhi for providing financial assistance in the form of scholarship.

My thanks are due, to the Dean, Post Graduate Studies for providing me necessary facilities to complete my research work.

I am thankful to Mr. Dig Vijay for his immense help in sequencing.

Special thanks to Dr. T. R. Sharma, Dr. Kapila, Dr. R. S. Chauhan, Dr. K. D. Sharma and Dr. R. Rathour for making valuable suggestions and extending timely help and guidance during my seminars.

Every effort is motivated by an ambition and all ambitions have an inspiration behind. So I avail the blessings, affection and moral encouragement of my gracious Daddy, Mummy, Shalu and Sanju whose heartfelt blessings, constant moral encouragement and positive spirit motivated me to higher achievements and inspired me to search ahead.

It also gives me an immense pleasure in acknowledging the moral support provided by my brothers Aman and Sandeep for helping me at each and every step

Words cannot match the warmth emotion when I thank my seniors Mr. G. Raikhy, Mr. S. Kulshrestha, Mr. Kundan, Mr. A. K. Singh, Mr. L. Singh, Ms. Vaneeta, Ms. Arpana, Ms. Nuzhat, Mr. M. Kumar, Mr. B. Kumar and Mr. S. Kumar who stood by my side in the various ups and downs, celebration and confusion, during the entire duration of my research work.

I can hardly over look the cooperation, timely help and moral support extended by my friends especially Harsh, Ramawatar, Rishi, Vandana, Tanuja, Vivek, Bhupinder, Satinder, Mohit, Sanjeev, Vijay, Ankita, Arti, kanika, Sunil, Sunny and Dinesh for their remarkable help on numerous occasions that eased my way to accomplish this task.

I am also thankful to Walia Ji for giving final touch to the manuscript.

I acknowledge with cordial thanks for the help and cooperation of Mr. Ranjeet[2] and Mr. Raggu for providing their assistance in their related aspects.

Words do not match when I remember the moral and heartful support of Prachi, Shabana, kulmeet and all other friends in every walk of life

I owe entire responsibility for all errors and omissions.

Place: Palampur

Dated: 19th July, 2005

Arun Priya
19/07/05

Dr. A.A. Zaidi
Scientist & Head

Floriculture Division
Institute of Himalayan Bioresource Technology (CSIR), Palampur-176061

CERTIFICATE- I

This is to certify that the thesis entitled **"Scenario of potyvirus (es) infection in lily and tulip in Himachal Pradesh"**, submitted in partial fulfillment of the requirements for the award of the degree of **Master of Science (Agriculture)** in the subject of **Agricultural Biotechnology** of CSK Himachal Pradesh Krishi Vishvavidyalaya, Palampur is a bonafide research work carried out by **Anu Priya (A-2003-30-01)** daughter of **Sh. Dharm Singh** under my supervision and that no part of this thesis has been submitted for any other degree or diploma.

The assistance and help received during the course of this investigation have been fully acknowledged.

Place: Palampur
Dated: July, 19th 2005.

Ayaz A. Zaidi
(A.A. Zaidi)
Chairman,
Advisory Committee

CERTIFICATE- II

This is to certify that the thesis entitled, **"Scenario of potyvirus (es) infection in lily and tulip in Himachal Pradesh"**, submitted by **Anu Priya (A-2003-30-01)** daughter of **Sh. Dharm Singh** to the CSK Himachal Pradesh Krishi Vishvavidyalaya, Palampur in partial fulfillment of the requirements for the degree of **Master of Science (Agriculture)** in the subject of **Agricultural Biotechnology** has been approved by the Advisory Committee after an oral examination of the student in collaboration with an External Examiner.

(Dr. A.A. Zaidi)
Chairman,
Advisory Committee

External Examiner

(Dr. Vipin Hallan)
Member

(Dr. Nageswer Singh)
Member

(Dr. Y.S. Paul)
Dean's nominee

12.8.05

(Prof. S.R. Thakur)
Head of the Department

(Dr. B. Prasad)
Dean,
Postgraduate Studies

CONTENTS

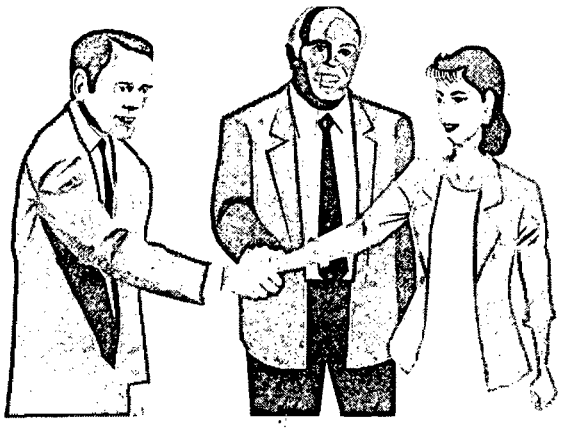
LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

ABBREVIATION USED

°C	degree Celsius
µg	microgram
µl	microlitre
ORF	Open reading frame
RNA	Ribose nucleic acid
DNA	Deoxyribonucleic acid
EDTA	Ethylenediamine tetra acetic acid
g	gram(s)
h	Hour(s)
Kb	Kilo base (pair)
M	molar (conc.)
mg	milligram
min	minute(s)
mM	millimolar
NaCl	sodium chloride
ng	nanogram
PCR	polymerase chain reaction
pH	puissance de hydrogen (ion conc.)
PBST	phosphate buffer saline tween
PVP	polyvinylpyrrolidone
s	second(s)
%	Per cent
Tris	tris (hydroxy methyl) amino methane
UV	Ultra violet
kDa	kilo dalton
HCl	Hydrochloric acid
rpm	revolution per minute
TAE	Tris acetate EDTA
H_2O	water
$CaCl_2$	Calcium chloride
CP	Coat protein
BLAST	Basic local alignment search tool



Introduction

Chapter-I

INTRODUCTION

Flowering plants originated during the Cretaceous Period, nearly 100 million years ago. Flower is the blossom of a plant. It reflects the purity, beauty, tranquility, honesty and divinity of nature. Beautification of a place depends upon its floral diversity. Although floriculture is an age-old profession, still India is in developing stage regarding the commercial cultivation of flowers. The importance of flower growing, from environment and economic point of view was not understood properly till seventies. However in the last decade it has progressed to a global industry regarding commercial production of flower.

Lily (*Lilium* spp.) and tulip (*Tulipa* spp.) belonging to family Liliaceae, are the most handsome and popular ornamental bulbous plants. The name lily is used chiefly for plants of the genus *Lilium* and related species but is applied also to plants of other families e.g. the water lily, the cala lily, and especially the numerous species of the amaryllis family, whose blossoms closely resemble the true lilies in appearance. Because of the showy blossom characteristics of the family, many species are cultivated as ornamentals. In religion and art, lily symbolizes purity and as the flower of the resurrection. It is ranked among top five cut flowers in the floriculture trade market. Because of its attractive flower colours, it is grown in beds, borders and pots. It is also used for indoor cultivation, where a large variation in broken colour of flowers along with quality degradation has been observed. Most of lilies are indigenous to Europe, Asia and North America, while some are native to the tropics at higher elevations (Beattie and White, 1993).

In India, they are commonly grown in hills and in places having mild climate but the Easter lily (*Lilium longiflorum*) can be grown with success in the plains of many parts of the country. Asiatic and Oriental hybrids are grown in Jammu and Kashmir, Uttranchal and Himachal Pradesh for commercial purpose where most of planting material has been imported from The Netherlands.

In HP, lilies are grown in Chamba, Kangra, Solan, Shimla, Kullu and Mandi districts; however the production is still a prospect. The main limiting factor in large scale cultivation of lily lies in its susceptibility to a number of diseases caused by viruses, fungi, bacteria and other abiotic factors.

Various viruses that affect the lily production are *Tulip breaking virus* (TBV) (synonyms *Lily mosaic virus, Lily streak virus) Cucumber mosaic virus* (CMV), *Lily symptomless virus* (LSV), *Lily mottle virus* (LMoV), *Turnip mosaic virus* (TuMV) and *Strawberry latent ringspot virus* (SLRSV).

Out of these TBV, CMV and LSV (aphid borne viruses), are some economically important viruses. These viruses affect the quality and yield of cut flower causing great economic losses (Lawson, 1981; Raju and Olsen, 1985). Virus infection produces several distinct symptoms including irregular mottling of the leaves, reduced plant growth and colour breaking in flower. Lilies infected with viruses become greatly weakened and susceptible to other diseases.

Although tulips were generally associated with Holland, both the flower and its name originated in the Middle East. However, the primary gene centre of the genus *Tulipa* L. is located in the Pamir Alai and Tien Shan mountain ranges in Central Asia (Hoog, 1973). Tulip, plant of the large genus *Tulipa*, is hardy, bulbous-rooted member of the family Liliaceae (lily family). Cultivated tulips (popular as garden and potted cut flower plants) are chiefly the varieties of *T.*

gesneriana. Tulips are excellent for rock gardening, informal bedding and for containers. Large varieties of the hybrids are now available for cultivation. Flowering occurs in mid spring season. They have deep, cup-shaped blossoms of various colours. There are around 100 species, originated from Southern Europe, North Africa, Asia (Anatolia), East Iran, North-east China and Japan.

In India, tulips are also grown in hills and places with temperate climate such as Jammu and Kashmir, Uttranchal and Himachal Pradesh for cultivation where planting material has been procured from The Netherlands. In India the important markets for their cut flowers are Mumbai, Delhi, Kolkata, Bangalore, Chennai and cities like Pune, Ahmedabad, Indore, Ludhiana and Chandigarh. In Himachal Pradesh, tulips are also popularly grown in Chamba, Solan, Shimla, Kullu and Mandi districts.

Some modern varieties display multicoloured patterns, which results from a natural change in the upper and lower layers of pigment in the tulip flower. Historical variegated varieties such as those admired in the Dutch tulipomania gained their delicately feathered patterns from viral infection. Carolus Clusius first described this phenomenon as Tulipomania in 1576, soon after the introduction of the tulip bulbs in The Netherlands as a new ornamental plant from Middle East **(Dash, 1999).**

Five viruses has been characterized from tulips on the basis of their host range, serology, polymerase chain reaction (PCR) and sequence analysis and they were concluded as distinct potyviruses. These viruses are TBV (*Tulip mosaic virus*), *Tulip band breaking virus* (TBBV), *Tulip top breaking virus* (TTBV), *Rembrendt tulip breaking virus* (RTBV) and **LMoV** (Dekker *et al.*, 1993).

It has been found that tulip plants that exhibit flower colour variegation also show weakness leading to loss of the cultivar. Virus infection also produces several distinct damaging symptoms including irregular mottling and flecking of leaves, reduced plant size, distorted and twisted plant growth, colour breaking in flower and concentric brown ring patterns on bulbs scales.

Preferably for disease free production of lilies and tulips, these should be grown under insect-proof conditions to avoid insect vectors. However, lilies can also be grown well under outdoor conditions provided that there is no damage from frost. Mostly, lilies are propagated vegetatively so various result reports of trials on the possibility of spreading TBV by cutting flowers with a knife and by beheading plants with a machine have been found. Aphid is considered to be the most important insect vector responsible for the transmission of LMoV and TBV within lilies and tulips, respectively. There are no reports on transmission through seed or dodder.

During a survey, some of the plants of lily and tulip growing at the experimental fields of Institute of Himalayan Bioresource and Technology, Palampur were found exhibiting mosaic and colour breaking symptoms. To characterize the viruses causing these diseases serological (Enzyme linked immunosorbent assay, ELISA) and molecular approaches such as RT-PCR, cloning and sequencing of amplified genes and hybridization studies were carried out with the following objectives -

1. Amplification, cloning and sequencing of the CP gene of Potyvirus (es) from various isolates.
2. Understanding variability in CP gene.

Review of Literature

Chapter-II

REVIEW OF LITERATURE

Lily (*Lilium* spp.) and tulip (*Tulipa* spp.), belonging to the family Liliaceae, are popular ornamental bulbous plants. Both are vegetatively propagated plants. Large number of bulbs of tulip and lily have been introduced from time to time in various regions of India especially in Himachal Pradesh. But there is always a decline in the vigour of varieties and bulb production due to various viral diseases which has slower down their production. These viruses cause stunting of the plant, produces mosaic and necrotic symptoms on the leaves and reduce the quality of flower of tulip and lily.

The various viruses found infecting tulips are *Cucumber mosaic virus* (CMV), *Lily symptomless virus* (LSV), *Lily mottle virus* (LMoV), *Turnip mosaic virus* (TuMV), *Tulip breaking virus* (TBV) and *Strawberry latent ringspot virus* (SLRSV) (Cohen *et al.*, 1985). Of these TBV threatened bulb and flower production of tulip seriously. TBV was first reported in *Tulipa* spp. from Surrey (England) by Cayley (1932).

Asjes (1978) reported detailed information on *Tulip mosaic virus*, particularly the symptoms and their relative importance in inspecting tulips for disease.

Through sequence analysis tulip breaking complex has been elucidated and found to include Tulip breaking virus (TBV) *Tulip top breaking virus* (TTBV), *Tulip band breaking virus* (TBBV), *Rembrandt tulip breaking virus* (RTBV) and LMoV, of which TTBV was found to be strain related to TuMV (Dekker *et al.*, 1993).

Potyviruses can be classified on the basis of various molecular parameters, such as gene sequences data, nucleic acid hybridization, coat protein sequence data or high performance liquid chromatography, peptide profiles and phenotypic characterisistics, such as particle morphology, host range, symptomology, cross-protection, cytoplasmic inclusion, particle morphology and serology (Ward *and* Shukla, 1991). Analysis of coat protein sequences of LMoV isolates revealed two subgroups, corresponding to the earlier 'TBV-lily strain' and 'TBBV' isolates. (Zheng *et al.*, 2003).

2.1 *Tulip breaking virus* (TBV)

2.1.1 Natural infection

A potyvirus Wa-TV isolated from tulip and several infected herbaceous species including *Nicotiana banthamiana* was serologically related to *Bean yellow mosaic virus* (BYMV) as well as to the TuMV, TBV and TCBV (Hammond and Chastagner, 1988).

Eikelboon *et al.* (1992) reported *Tulipa fosteriana* cultivars Cantata and Princeps had a high degree of resistance to TBV. Aarnes (1994) gave brief description of TBV related to its identification, transmission, symptoms produced, varietal resistance and control.

Straathof *et al.* (1997) reported that all cultivars of *T. gesneriana* tested were susceptible to TBV, however partial and absolute resistance has been found in *T. fosteriana* cultivars. To introduce resistance from *T. fosteriana* in the *T. gesneriana* assortment interspecific crosses were made. To select TBV resistant hybrids efficiently, a screening test applicable at individual seedlings was described. To test individual seedling, an incomplete diallel was made between the susceptible *T. gesneriana* cultivars 'Christmas Marvel', 'Kees Nelis' and 'Lustige Witwe', the

partial resistant *T. fosteriana* cultivars 'Juan' and 'Madame Lefeber' and the absolute resistant *T fosteriana* cultivars 'Cantata' and 'Princeps'. Individual seedlings were inoculated using viruliferous aphids in the first, third or fifth year after sowing. Six weeks after inoculation, leaves were tested for TBV by ELISA. All seedlings were grown till maturity and the occurrence of breaking symptoms in the flower were monitored.

Villemson (2001) detected new disease on white cultivars of *Lilium* such as Siberia and Dame Blanche whose bulbs originated in The Netherlands. The causal agent was identified as TBBV.

Se and Kanematsu (2002) reported a virus isolate from tulip with flower breaking symptoms in cultivar Toyama prefecture. Although it was presumed to be LMoV after detection by LMoV specific RT-PCR but the coat protein gene of this virus (825 nt) showed 96.4% amino acid sequence identity with TBBV.

Sharma (2002) reported the occurrence of TBV in HP on the basis of its particle morphology, serology, nucleic acid, protein composition and partial coat protein sequence (EMBL Acc. number AJ 549932).

2.1.2 Symptoms

Van Slogteren and de Vos (1966) reported that tulip infected with CMV shows a flower break which might be confused with that caused by TBV but is usually distinctive because it is confined to the margins of the petals.

Van Slogteren and Asjes (1970) reported that white and yellow varieties of tulip are incapable of breaking because they lack anthocyanin. Their colour being determined by colourless or yellow plastids in the mesophyll layer. Infected plants generally show leaf mottling.

Muramatsu (1971) reported that tulip varieties Feu Brilliant and Red Pitt showed only dark coloured flowers on plants inoculated with TuMV in the previous season while on William Pitt, breaking was either dark coloured or faded. When the sap from latter leaves was used as inoculum, flowers from the bulbs of the inoculated plants developed almost the same type of breaking as those of the corresponding inoculum source plant.

Hoog (1973) reported that during early 1637, Dutch growers produced new varieties of tulip with flower breaking patterns by grafting tulip bulbs with broken flower to bulbs with uniformly coloured flowers.

'Enchantment' lilies, when inoculated with either the full or the self-break isolates of TBV, developed symptoms on leaves and on the bulb scales which were characteristics features of diseases called brown ring formation (Asjes *et al.*, 1973).

Asjes (1978) reported details about TBV infection, particularly symptoms produced and their relative importance in inspecting tulip.

Among various viruses that had been identified in lilies grown in Israel, TBV induced local lesion on *Chenopodium amaranticolor* (Cohen *et al.*, 1985).

According to Christie and Edwardson (1985) plant virus inclusions are the intracellular structures resulting from virus infection. These structures may contain scattered and aggregated virus particles as well as other virus related nucleic acids. Certain types of inclusions (cylindrical inclusion of potyvirus group) may consist almost entirely of virus induced non capsid protein. The inclusion differed from the surrounding cytoplasm and organelles in their staining reaction and structural appearance as visualized by light and electron microscopy.

In *Tulipa* spp. especially in Darwin hybrids, colour breaking of flowers and chlorosis on leaves had been observed while leaf chlorosis and degeneration symptoms persist in *Lilium* spp. and Mid-Centuary hybrids (Phillips, 1986).

Lee *et al.* (1998) reported that symptoms in 1038 tulip plants under investigation in June 1997 in the Korean republic were 80.5%, 8.3% and 5.2% of yellow mottle, stripes and mosaic respectively of which yellow mottle and stripes symptoms revealed 7.5 Kbp and 10 Kbp dsRNA bands respectively while *Lilium* with mosaic symptoms had ds RNA profiles of 3.4, 3.0, 2.2 and 1.0 Kb.

Kim *et al.* (1998) reported that in 1996 the incidence rate of viral diseases in tulip cultivar Casablanca was 52.7% in Korean Republic. The major symptoms observed were mosaic, stunting, yellowing along with 30% reduction in flower number/plant.

Lesnaw and Ghabrial, (2000) reported that TBV induces on the petals of its host tulip, beautiful variegated coloured patterns known as tulip breaking, which were responsible for the 'Tulipmania' of 17th century Europe.

2.1.3 Transmission

Hughes (1931) reported that *Yezabura tulipa* transmits TBV between stored tulip bulbs, but this has not been confirmed by Brierley and Smith, (1944). TBV has been reported to be transmitted by insect vectors such as *Myzus persicae*, *M. euphorbiae, Aphis gossypii, A. fabae,* and *Dysaphis tulipae* (family Aphididae), by mechanical inoculation or by grafting but neither by contact between plants nor by pollen or seed (Phillips, 1986).

By means of aphid (*M. persicae*) each of three isolates of TBV, causing full, self and average break symptoms in the petals of infected 'Rosa Copland' tulips were transmitted to separate virus-free plants of *Lilium* mid century hybrids 'Enchantment' grown under glass at 20°C (Derks, 1992).

Asjes *et al.* (1996) had also reported that speed of spread of vectors was generally differentially rapid over the seasons, while a range of about 25 aphid species were able to transmit TBV.

2.1.4 Host range studies

Brierley and Smith (1944) inoculated 79 dicotyledenous species from 66 genera and 22 families with TBV and back tested by inoculation to *Lilium formosanum*. All tests were negative, but the species were not named and in a further survey, 39 monocotyledonous species also proved to be insusceptible.

Van Slogteren (1971) reported that two genera, *Tulipa* and *Lilium*, both in Liliaceae got infected with TBV. Various other experimentally susceptible species to virus are *Calochortus, Fritillaria pudica, Lilium* (cvr Concorde, Sterling star), *Lilium formosanum*, *Lilium longiflorum Ornithogalum thyrsoides, Tulipa hybrids, Zigadenus fermantii, Nicotiana* spp, *Chenopodioum quinoa* and *Fetragonium expanse.*

Phillips (1986) reported that experimentally few families are susceptible hosts of TBV, which include Liliaceae, Hycenthaceae, Calochortaceae and Melanthiaceaea. Romanow *et al.* (1991) studied the resistance of seven tulip cultivars to TBV, after mechanical inoculation by determining the number of infected plants and the level of virus in these plants over a period of one and a half years. Three cultivars (Apeldoorn, Kees Nelis and Lustigue Witwe) appeared to be susceptible. Four cultivars (Cantata, Juan, Madame Lefeber and Princeps) showed a high degree of resistance. Virus could not be detected in theses plants after storage.

Derks *et al.* (1997) distinguished three LMoV isolates from each other by a host range consisting of lilies and herbaceous test plants.

Lisa *et al.* (2002) reported LMoV as an important virus of lily that also cause flower breaking in tulips, is considered to have a natural host range restricted to family Liliaceae.

2.1.5 Biological properties

Brierley and Smith (1944) reported that infectivity in lily sap is retained for 4-6 days at 18°C while McWhorter and Brierley (1963) reported that the infectivity in tulip sap is lost after 10 min at 65-70°C (TIP) and after dilution beyond 10^{-5}..

2.1.6 Purification

Derks *et al.* (1982) purified virus by homogenization in 0.1M Tris buffer, clarification with Triton x-100 and differential centrifugation. TBV suspension was absorbed with an antihost protein antiserum.

Franssen and Hulst (1986) produced hybridomas to purify TBV. The cell lines were selected by ELISA by a direct coating of the purified virus to the plate. Although attempts to obtain highly purified virus preparation had failed because of small amount of virus in leaf extract and aggregation of virus particle.

Sharma (2002) reported the effect of different buffers, pH, molarities, additives and organic solvents for purification of TBV.

2.1.7 Morphology

TBV is a nucleocapsid filamentous virion, not enveloped, usually flexuous with a clear modal length between 680-900 nm and 14 nm wide with axial canal and basic helix obscure (Brandes and Bercks, 1965).

2.1.8 Genome

Genome size is approximately 10kb (Hill and Benner, 1976; Hari *et al.*, 1974). Only one sedimenting component in purified preparation had been reported with sedimentation coefficient 153S at purity ratio A260/280=1.03-1.21. The genome consists of single stranded, linear RNA (Langeveld, 1991). Zheng *et al.* (2003) reported that sequence of LMoV was 9644 nt long and encoded a polyprotein of 3095 amino acid with a calculated molecular weight of 351.0 KDa that had only 45.1-54.4% identity to other completely sequenced potyviruses. They also found that two partial LMoV sequenced from different cultivars were identical to one another and very similar (98.3% identical nucleotides) to the corresponding region of the complete sequence. Analysis of the coat protein sequences of LMoV isolates revealed two subgroup corresponding to earlier 'TBV-lily strain' and 'TBBV'isolates. It was the first report of complete sequence of LMoV.

2.1.9 Detection techniques

2.1.9.1 Serology

According to Van Schadewijk and Eggink (1980), enzyme-linked immunosorbent assay (ELISA) proved to be a sensitive test for detection of TBV. Special precautions are necessary for the preparation of a TBV antiserum such as serological purification of the TBV suspension by the complete removal of contaminations, i.e. *Lily symptomless virus* (LSV) and fraction-1-proteins, with anti-LSV and anti-FI sera. During the growing season, TBV was detected in tulip leaves and bulbs by ELISA. During storage, TBV can be detected in bulbs from primarily infected tulips.

Romanow *et al.* (1986) reported that virus detection techniques such as enzyme-linked immunosorbent assay (ELISA) can measure virus establishment,

replication and movement rates. According to him prevention or reduction of TBV spread can be based on a number of resistance mechanisms. Franssen and Hulst (1986) reported that with purified TBV, all monoclonal antibodies reacted better with a direct coating of the virus than with an indirect coating using polyclonal antibodies. However a coating with monoclonal antibodies derived from one cell lines hardly gave any ELISA value, neither for purified virus nor for diseased materials.

Van Schadewijk (1986) reported that ELISA could readily detect primary infection of TBV in scales. Bulb testing shortly after lifting the bulbs should be avoided. Both ELISA reading and reliability of the test improved during three weeks storage period of the bulbs.

ELISA proved to be a sensitive test for detection of viruses. Hsu *et al.* (1988) selected mouse monoclonal antibodies for cryptotypes of TBV on antigen coated plants. In double antibodies sandwich enzyme linked immunosorbant assay (DAS-ELISA) lower A_{405} reading was obtained with TBV polyclonal antibody coated plates.

Hammond and Chastagner (1989) reported that during testing of many tulip cultivars for TBV using an indirect antigen coated form of ELISA with a panel of monoclonal (MAbs) and polyclonal antibodies of TBV, TCBV and WaTV, several plants showed a spectrum of reactivity with the MAbs and polyclonal sera that differ from typical TBV infection.

Boonekamp *et al.* (1990) reported that over 7, 00,000 samples of tulip, lilies and irises were tested annually by bulbs inspection centre using polyclonal antisera however less results are obtained in case different serotypes of a virus exist, virus is difficult to detect in specific cultivar.

Chang *et al.* (1998) reported production of antibodies against CMV, LMoV and TBV. Polymerase chain reaction protocols and number of serological tests viz., ELISA, SDS immunodiffusion and immunoblotting has been developed for the identification of viral disease of *Lilium*. A standard virus indexing protocol has also been developed for indexing of *Lilium* bulbs.

2.1.9.2 Molecular techniques

2.1.9.2.1 RT PCR

Corpet (1988) had presented an algorithm for the multiple alignments of sequences, either proteins or nucleic acids, which is both accurate and easy to use on microcomputers.

Langeveld (1991) reported that local areas of conserved amino acids sequences in the replicase and coat protein of potyvirus were used to select nucleotides sequence for the construction of sets of degenerate oligonucleotides primers for amplification of DNA fragments of potyvirus specific template in a combined assay of reverse transcription-polymerase chain reaction (RT-PCR).

Ohira *et al.* (1994) reported cDNA synthesis of TBV from tulip and cloned in *E.coli* (one clone containing a 4.5 Kb insert was identified by restriction enzyme analysis, dot immunobinding assay and partial sequencing). Subsequent sequencing of 1479 nucleotides of the 3' terminus revealed that the sequence contains one open reading frame (ORF), followed by an untranslated region of 225 nucleotides and a poly (A) tail, the deduced amino acid sequences include C-terminus of the predicted RNA dependent RNA polymerase and the coat protein. A glutamine-alanine dipeptide was identified as a putative NIa protease cleavage site at the N-terminus of the coat protein.

2.1.9.2.2 cDNA-Probes

Derks *et al.* (1994) reported that LMoV isolates can be distinguished from TBV either with antisera prepared against potyvirus inducing colour breaking in tulips or with cDNA probes. Although LMoV could not be differentiated serologically from RTBV, RTBV was not detectable with an LMoV cDNA probes.

2.2 *Lily mottle virus* (LMoV)

Brierley and Smith (1944) and Yamaguchi (1963) reported that in *Lilium formosanum,* mottle or streak symptoms in the leaf tips were observed when seedling were mechanically inoculated with extracts from petals, leaves or bulbs scales of infected tulips. Leaves and flower produced later were distorted.

Allen (1975) reported three viruses associated with most viral diseases of lily; LSV, TBV and CMV. It was observed that most lilies contained LSV. Vegetative propagation of plants on tissue culture media freed lilies from viruses.

Two types of particles (640 and 740 nm long) were observed by electron microscopy and identified by immune-electron microscopy as LSV and TBV respectively in *Lilium* hybrids Pink Perfection by Bertaccini and Marani (1982).

Derks *et al.* (1994) reported that LMoV isolates were distinguishable from TBV with antisera prepared against potyviruses inducing colour breaking in tulip and with cDNA probes.

Derks *et al.* (1997) reported that LMoV can be detected reliably with ELISA in bulbs of Asiatic hybrids stored at 0-2°C for atleast 2-3 weeks after lifting prior to testing. But same protocol failed to detect LMoV in *L. longiflorum* and oriental hybrids.

In case of LMoV, symptoms are mosaic, yellowing and stunting of plant along with 30 % reduction in flower number /plant (Kim *et al.*, 1998).

Niimi *et al.* (1999) reported that dot immunobinding assay (DIBA) and indirect ELISA can be used to detect TBV-L and CMV in the scale segments of *Lilium**Enchantment. The frequencies of TBV-L and CMV occurring alone in the population were 1.6% and 0.9% respectively.

Singh (1999) studied the identification of CMV on lily and produced virus free plants through clonal propagation.

Jeong *et al.* (2000) reported viral symptoms on lily in the highland areas which were differentiated into mosaic (43.8%), crinkle (9.2%), mottle (0.9%) and stripe or line patterns. Viral infection rate of *Lilium* oriental hybrids was 2-4 times higher than that of *Llium* Asiatic hybrids.

Bouwen and Van der Vlugt (2000) reported during a survey for a European Union funded project on viruses of *Alstroemeria*, that *A. brasiliensis* plants were found expressing virus like symptoms, including leaf chlorosis with deep green oval spots and flower colour breaking. In Enzyme-linked immunosorbent assay (ELISA), no positive reaction was obtained with antisera to *Alstroemeria mosaic virus, Cucumber mosaic virus, Freesia mosaic virus, Tobacco rattle virus* or Potyvirus specific monoclonal antibodies. ELISA reactions were positive with antisera to LMoV and RTBV. In electron microscopy preparation of *A. brasiliensis*, potyvirus like particles were observed. By sap inoculation, this virus was transferred to a range of host species. *C. quinoa* and *N. occidentalis* expressed local lesions; *N. clevelandii* expressed local and systemic mottle and *N. benthamiana* expressed local lesion, systemic vein yellowing and leaf crinkling. Isolated total RNA from infected *N. benthamiana* was used for initial cDNA

synthesis and polymerase chain reaction amplification with a potyvirus specific primer set. On sequencing it shows 92% homology with corresponding region of LMoV RNA. This was the first report of natural infection of *A. brasiliensis* by LMoV.

Kim *et al.* (2001) reported that virus detection on lily by one step RT-PCR was more rapid and reliable than by the conventional RT-PCR methods.

Bellardi and Bertaccini (2001) reported that in the Lazio region (Southern Italy) some lilies of pink cultivar showing flower breaking were infected by *Turnip mosaic virus* (genus potyvirus). This observation was confirmed by immunosorbent electron microscopy (ISEM) and gold labeling antibody decoration (GLAD) using antisera to *Tulip top breaking virus* (TTBV).

Asjes *et al.* (2002) reported the seasonal detection of DAS-ELISA of LMoV in primary and secondary infected plants in the yearly growth cycle and effects of level of virus source incidence and virus spread on it. The inefficiency of detection of LMoV in bulbs of Oriental hybrids was confirmed. It was also found that LMoV was not reliably traceable in the young plants for one to two months after emergence from soil.

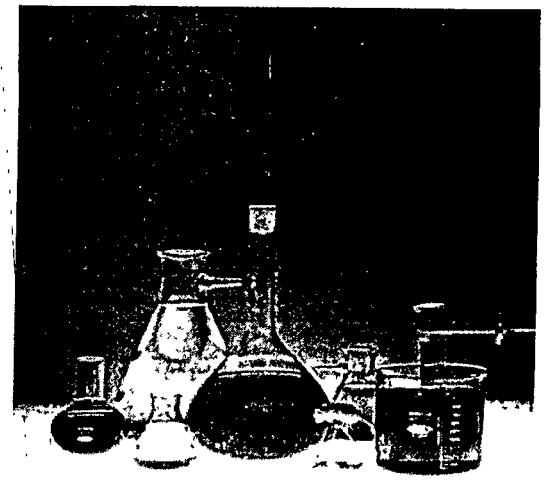
Chen *et al.* (2002) used conserved potyvirus primers in RT-PCR to amplify virus fragments from bulb crops such as garlic and lily in China and the fragments were subsequently sequenced and compared in phylogenetic analyses. LMoV was amplified from dragon-teeth lily (*Lilium brownii* var. viridulum).

Bellardi *et al.* (2002) reported that during 1999 cultivation in Lazio-region, some lilies of pink colour showing colour breaking on petals were infected by a potyvirus isolate, which was serologically related to TuMV. The new potyvirus associated with flower colour breaking was similar to TTBV, which is closely related to TuMV.

Niimi *et al.* (2003) reported that RT-PCR is the more sensitive methods than ELISA to determine the presence of viruses in various *Lilium* spp.

Park *et al.* (2003) reported that a study was performed to investigate the viral infection status of imported *Lilium* oriental hybrids bulbs through direct negative staining methods in virus infected leaves of lily cultivar Casablanca. By ELISA and direct tissue blotting immune assay an unknown virus in *Lilium* was observed, which was filamentous particle of 2000 nm length. They also reported that sugar content in leaves and scales were higher in healthy plants than in virus infected.

Zheng *et al.* (2003) gave first report of complete sequence for LMoV and LSV.



Materials and Methods

Chapter-III

MATERIALS AND METHODS

3.1. Enzyme Linked Immunosorbent Assay (ELISA)

The use of ELISA for the detection of plant viruses is well documented. A modified procedure Double Antibody Sandwich Enzyme Linked Immunosorbent Assay (DAS-ELISA) (Clark and Adams, 1977) was performed for the detection of LMoV due to its specificity and simplicity for routine virus detection. LMoV is strongly immunogenic, so it can be detected satisfactorily by serological methods. Antibodies were procured from BioRad, USA and used as per manufacturer's instructions. This method was followed to detect LMoV in lily.

DAS-ELISA Protocol

1. LMoV specific antibody 100 µl procured from BioRad (USA) (1:500 dilutions with coating buffer) was coated in wells including blank.
2. Plate was incubated at 37°C for 2 h.
3. The plate was washed 3-4 with PBST buffer to remove unbound antibodies.
4. Leaf sap (100 µl) (extracted in general extraction buffer with 1:10 dilution) was added in wells except blank, in which 100 µl PBST buffer was added and incubated at 4°C overnight.
5. After incubation, plate was washed 3-4 times with PBST buffer to remove unbound antigens (virion particles).
6. Alkaline phosphatase conjugated antibody (100 µl) (1:500 dilution in ECI buffer) was added including blank followed by incubation at 37°C for 2 h.
7. The plate was washed 3-4 times with PBST buffer.

8. Substrate (p-nitro phenyl phosphate) (100 µl) (1mg/ml) was added in each well followed by incubation at 37°C for 10 - 20 min.
9. Absorbance was taken at 405 nm in ELISA reader (Labsystems, Finland).

3.2 Nucleic acid based detection of *Tulip breaking virus* (TBV) and *Lily mottle virus* (LMoV)

3.2.1 Isolation of total RNA from plants

The total RNA was extracted from virus-infected tulip and lily plants using the two methods viz. RNA aqueous and RNA wiz™ (Ambion, USA).

Total RNA isolation using RNA Wiz™ (Ambion, USA)

All steps were carried out at room temperature unless specified.

a) The infected leaves were crushed to fine powder in liquid nitrogen using baked mortar and pestle.
b) The tissue powder (100 mg) was immediately transferred to an eppendorf containing 500 µl of RNA Wiz solution and mixed properly.
c) The homogenate was incubated for 5 min.
d) Chloroform (100 µl) was added to homogenate; tubes were covered with aluminum foil and incubated for 10 min.
e) The mixture was centrifuged at 10,000g for 15 min at 4°C. The mixture separated into 3 phases:
   (i) Upper aqueous phase containing RNA
   (ii) Inter phase containing DNA
   (iii) Lower (organic) phase containing protein

f) Without disturbing interphase, the aqueous phase was carefully transferred to a new eppendorf and 0.5x starting volume of RNAse free water was added and mixed well.

g) Isopropanol (one starting volume) was added, mixed well and incubated for 10 min. The mixture was centrifuged at 10,000g for 15 min at 4°C to pellet down RNA.

h) The supernatant was decanted and the pellet was washed with at least one starting volume of cold 75% ethanol by vortexing and centrifuged at 10,000g for 5 min at 4°C. Supernatant was discarded.

i) Pellet was air dried for about 10 min and RNA was resuspended in 50 µl of RNAse free sterile water.

Total RNA isolation by RNA aqueous (Ambion, USA)

A. For RNA isolation lysate was prepared by crushing the sample thoroughly in liquid nitrogen to powder form and put in eppendorff which already contain lysis solution of about 700 µl.

B. The mixture was vortexed and centrifuged at top speed i.e.14000g for 5 min to remove debris. (Solution should not be too viscous).

C. Equal volume of 64% ethanol was added to lysate taken into another eppendorff and mixed thoroughly by vortexing or inverting the tube several times.

D. Filter cartridge was inserted into other collection tube to prepare binding column.

E. Lysate and ethanol mixture was pipetted into filter cartridge (700 µl at one time)

F. Filter cartridge was centrifuged for 30 sec to 1 min and flow through was discarded so that the collection tube does not overflow.

G. Step was repeated with wash solution by applying wash solution-I i.e. 700 µl to this aqueous filter cartridge and it was centrifuged till all wash solution was through the filter and later was discarded.

H. Now step was repeated with wash solution -2&3.

I. Discarded the eluted wash solution.

J. Filter cartridge was inserted into a fresh collection tube and elution solution (preheated to 70-80°C) was applied as earlier.

K. Stored the eluted product containing RNA at –80°C.

3.2.2 Detection of TBV and LMoV by Reverse transcription-Polymerase chain reaction (RT-PCR)

PCR is an efficient means of *in vitro* amplification of specific DNA sequence by using a pair of oligonucleotides binding to the opposite strands of DNA. PCR was devised and named by Mullis *et al.* (1986). In general, PCR is a very useful tool in the detection and diagnosis of viruses and other pathogens specifically.

To detect TBV and LMbV in plant samples, RT-PCR was carried out using TBV coat protein (CP) gene and potyvirus group specific primers. The primers used were as follows

TBV CP gene specific primers (23mer)-

TBV Forward (U): 5'-GCATTGAGCAACACGTTCGAGCA-3'

TBV Reverse (D): 5'-TTAAATACCGCGCGCTCCCATGA-3'

Potyvirus group specific primers (25mer)-

Up primer- 5'-TGAGGATCCTGGTGTATHGARAAYG-3'

Down primer-5'-GCGGATCCTTTTTTTTTTTTTTTT-3'

Standardization of RT-PCR

T_m of the primer was calculated using above formulae, annealing temperature was calculated i.e. annealing temperature (°C) =[T_m (°C) -5]. Based on this several annealing temperatureswere tested for amplification.

3.2.2.1 Reverse transcription (RT)

Reverse transcription is the process by which cDNA is synthesized from RNA. This was done with the help of reverse transcriptase enzyme, which is RNA dependent DNA polymerase and it catalyses the conversion of RNA into cDNA.

Composition of RT-PCR reaction

Contents	**quantity**
RT buffer (5X)	5 µl
dNTP mix (40 mM)	1.5 µl
Primer (D) (200 ng/µl)	1.0 µl
RNase inhibitor (28 u/µl)	0.25 µl
RNA sample	6.0 µl
Reverse transcriptase (MLV-RT, 200 u/µl)	0.5 µl
H_2O	9.75 µl
Total	25 µl

The mixture was then mixed by pipetting up and down and centrifuged briefly to collect the mixture at the bottom of the tube (0.2 ml). RT mixture was same for both gene and group specific PCR except for primers used. cDNA synthesis was carried out at 37°C for 1.15 h followed by denaturation at 70°C for 5 min. The tubes were then immediately placed on ice.

3.2.2.2.1 Amplification of c DNA using TBV specific primers

PCR reaction mix prepared was as follows:

Contents	**Quantity**
Taq buffer (10X)	5 µl
dNTP mix (10 mM)	3 µl
Primer (U, 200 ng/µl)	1 µl
Primer (D, 200 ng/µl)	1 µl
cDNA	10 µl
Taq DNA polymerase (1.5 u/µl)	0.3 µl
H_2O	29.7µl
Total	50 µl

The reaction was carried out under following temperature conditions:

Steps	**Temp (°C)**	**Time (min)**
1. Initial denaturation	94	3
2. Denaturation	94	0.30
3. Annealing	52	2
4. Elongation	72	2

Steps 2-4 were repeated 30 times and a final elongation was carried out at 72°C for 10 min.

3.2.2.2.2 Amplification of coat protein gene of LMoV using group specific primers

Condition for polymerase chain reaction was same as above except for the primer pair along with the annealing and elongation temperatures, which were standardized at 54°C for 2 min and 72°C for 2 min.

3.2.3 Agarose gel electrophoresis of PCR product

After completion of amplification, the PCR product (10 µl) mixed with 4µl of gel loading dye was loaded on 1% agarose gel and electrophoresed in TAE buffer at 80 V for 1-2 h. DNA marker (100 bp or 250 bp) was loaded in one well for size comparison. After run, the gel was stained in ethidium bromide (0.5 mg/ml) and viewed under UV transiluminator and photographed in AlphaDigidoc™ (Alpha Innotech Corporation).

3.2.4 Elution of DNA from agarose gel

The PCR amplified product was eluted from the gel using Perfectprep® Gel Cleanup (Eppendorf AG, Germany) as per the following procedure.

Protocol:

A. The band was exised from the agarose gel and trimmed to minimize the extra gel weight.

B. Gel was weighted in colourless tarred tube.

C. Three volumes of gel solubilization solution was added to the gel and vortexed for every 2-3 min during incubation at 50-60 °C for 10 min.

D. Then placed GenElute Binding column in a new 2 ml collection tube and 500 µl of column preparation solution was added to it.

E. Binding column was centrifuged for 1 min at 12000g-16000g and flow through fluid was discarded.

F. Then one gel volume of 100% isopropanol was added to the gel solution and mixed homogenously.

G. This mix of gel was poured to the binding column assembled in 2 ml collection tube (700 µl at a time) and centrifuged for 1 min at 12000g-16000g.

H. Flow through fluid was discarded.

I. Added 700 µl of wash solution (diluted) to binding column and centrifuged for 1min at 12000-16000 g and discard the flow through fluid.

J. Centrifuged the binding column without any additional wash for 1 min at 12000-16000 g to remove all the wash solution.

K. Binding column was transferred to the fresh collection tube.

L. Elution solution (50 µl) or Millipore water was added to the centre of column (preheat elution solution to 65 °C).

M. Incubated for 1min and centrifuged for 1min.

N. Stored the eluted product in -20 °C for future use.

3.2.4 Cloning and Transformation

Ligation is an energy dependent process by which the foreign DNA is sealed *in vitro*. Ligase enzyme is generally used in this process, which seals the foreign DNA fragment (having cohesive or blunt ends) to the cloning vector.

The eluted RT-PCR amplified products of TBV CP gene (~700 bp) and LMoV CP gene (~700) were cloned in pGEM-T Easy vector (Promega, USA). The protocol mentioned in brief is as follows:

3.2.4.1 Ligation of eluted DNA using pGEM-T Easy vector (Promega, USA)

The pGEM-T Easy vector has a unique 3' terminal thymidine to both ends preventing the re-circularization of the vector and providing complete overhangs for PCR product, which has 5' adenine to both ends (Fig.3.1). PCR amplified partial TBV and LMoV CP gene (~700 bp) were ligated in pGEM-T Easy vector system.

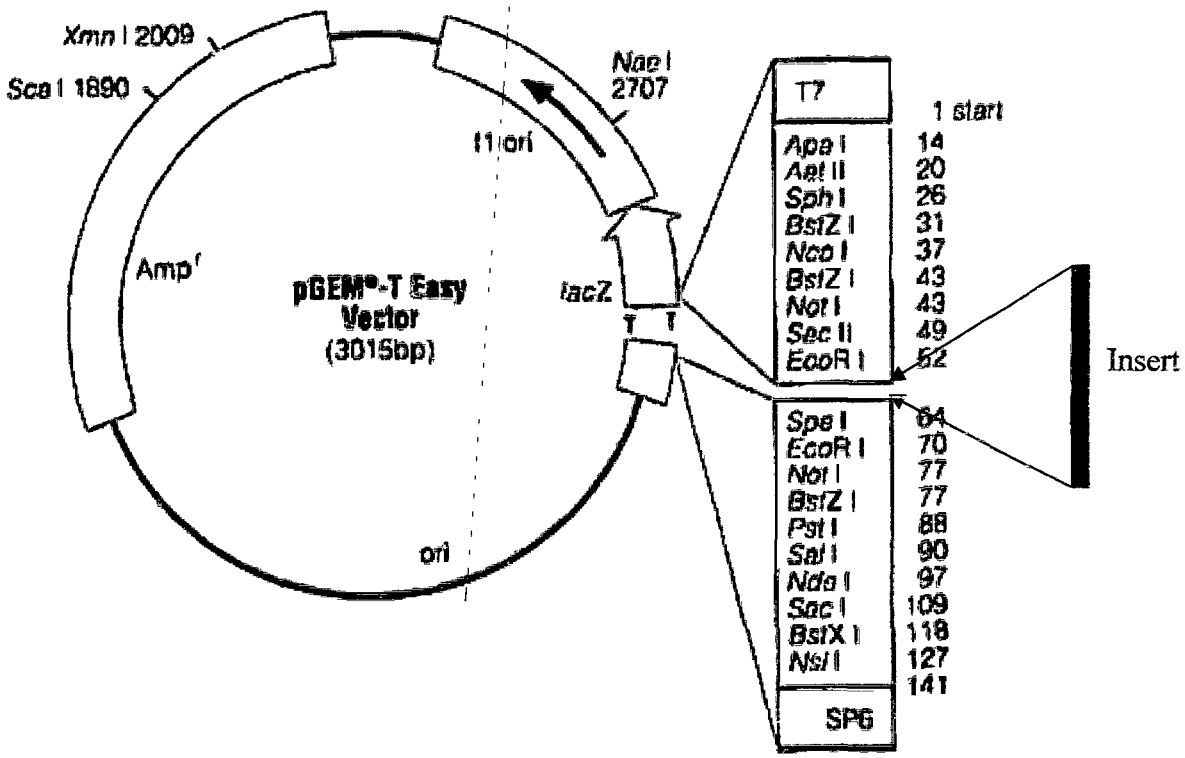


Fig. 3.1 Diagrammatic representation of pGEM-T Easy vector (Promega, USA), showing restriction sites in multiple cloning site and a unique 3' T overhang which is compatible to 5' A overhang in PCR products. Inserts in this vector can be sequenced by T_7 and SP_6 primers.

Contents	Volume
2x rapid ligation buffer	5 µl
pGEM-T Easy vector(50ng)	1 µl
Eluted DNA (insert)	3 µl
T4 DNA ligase (3 Weiss units)	1 µl
Total	10 µl

The reaction mixture was mixed by pipetting, centrifuged briefly and incubated overnight at 4°C in water bath.

3.2.4.2 Preparation of competent cells

Competent cells can be prepared either freshly for each use or can be premade and stored at –70°C for the transformation experiments. *E.coli* strain DH 5α was used for transformation.

a) Procedure for preparing competent cells, which can be stored at -70°C

a. Seed culture (5 ml) was grown using single colony and incubated overnight at 37°C in an incubator shaker.
b. Overnight culture (1 ml) was transferred into 250 ml production culture medium and incubated at 37°C till OD at 600 nm was around 0.5.
c. Culture was cooled on ice for 15 min and centrifuged at 6,000 rpm for 5 min at 4°C.
d. Supernatant was discarded carefully and the pellet was suspended in 70 ml of ice cold, sterile 0.1 M $CaCl_2$ and incubated on ice for 30 min.
e. The cells were collected by centrifugation at 6,000 rpm for 5 min at 4°C.
f. Supernatant was discarded and pellet was resuspended in 70 ml cold and sterile 0.1 M $MgCl_2$ and incubated on ice for 30 min.
g. Centrifuged at 6,000 rpm for 5 min at 4°C

h. The cells were finally recovered and suspended in 10 ml of 0.1M $CaCl_2$ containing 10% glycerol.

i. Cell suspension was divided into 200 µl aliquots and was immediately stored at –70°C.

b) Competent cells which can be used fresh each time

1. LB (50 ml) was inoculated with 50 µl overnight from culture of *E. coli* DH5α and *allowed to grow at 37°C on incubatory shaker till OD at 600nm was around 0.5.*
2. Culture was cooled on ice and centrifuging at 5,000 rpm for 5 min at 4°C pelleted cells down.
3. Supernatant was removed. The pellet was resuspended in 10 ml ice cold 0.1M $CaCl_2$ and incubated on ice for 15 min.
4. Cells were recovered by centrifugation at 5,000rpm for 5 min at 4°C.
5. Cell pellet was resuspended in 2 ml of ice cold 0.1M $CaCl_2$ and aliquots of 200 µl each were prepared to be used for single transformation reaction.
6. These competent cells can be stored on ice for not more than 12-16 h.

3.2.5 Transformation of competent cells (*E. coli* strain DH 5α) with ligated products

Transformation is the process of uptake of foreign DNA by bacterial cells (*E. coli*). From the medium this uptake and retention of foreign DNA molecules is generally detected by examining the expression of the gene carried by the foreign DNA (Brown, 1995). For example, if foreign DNA contains the gene for resistance against certain antibiotics, to which normal bacterial cells are sensitive, the transformed cells become resistant to those antibiotics. These cells are checked for the expression of the antibiotic resistance by growth on a selective medium

containing the antibiotics. The cells, which are not transformed, perish in such medium.

Protocol

a. The competent cells were taken and allowed to thaw on ice for 10 min. Ligated product (10 µl) was added and incubated on ice for 30 min.
b. Heat shock was given at 42°C for 90 sec and immediately transferred to ice for 5 min.
c. Fresh Luria broth (LB) (800 µl) without antibiotics was added to the mixture and tube contents were incubated at 37°C for 1 h with continuous shaking of the tube.
d. The tube contents were centrifuged at 12,000 rpm for 30 sec and supernatant was discarded leaving approximately 100 µl of culture in the tube.
e. The pellet was resuspended by pipetting broth and plated on LB agar plates containing ampicillin (150 µg/µl) using glass beads.
f. The plates were then incubated at 37°C for overnight for development of transformed colonies.

3.2.6 Checking of transformed colonies

About 10 colonies, growing on the LB agar plates were picked up, cultured separately on fresh LB agar plates as well as in tubes and incubated at 37° C for overnight. The plasmid DNA containing the gene of interest was purified from the bacterial cells cultured in tubes.

3.2.6.1 Plasmid DNA isolation by Boiling Miniprep method (Holmes and Quingley, 1981)

Overnight grown culture (1.5 ml) was pelleted down in a microfuge tube. Supernatant was discarded and cells were resuspended in 110 µl of STET buffer.

Freshly prepared lysozyme (in 10 mM Tris HCl, pH 8.0) was added to a final concentration of 0.5 mg/ml. The tubes were incubated at room temperature for 5 min. These were then incubated in boiling water bath for 40-45 sec. The suspension was centrifuged at full speed in a microfuge for 20 min. Pellet containing the cell debris was removed with the help of a sterile tooth prick. DNA in the supernatant was precipitated by one volume of isopropanol. Precipitated DNA was collected immediately by centrifugation for 20 min at full speed. Supernatant was removed completely and the pellet was dried to remove traces of isopropanol. DNA pellet was dissolved in sterile water and used as such for further manipulation.

3.2.6.2 Checking of recombinant clones

Several methods are used to check the recombinant clones viz. restriction digestion, insertional inactivation, colony hybridization, colony PCR, and antibiotic selection. Among these we used restriction digestion method for checking of TBV and LMoV partial CP gene clones.

Restriction digestion method for detection of TBV and LMoV

a) The following reaction mixture was prepared in microfuge tube on ice as shown in table to check the insertion of gene of insert isolated in the plasmid vector in transformed *E. coli* cells.

Restriction digestion mixture

Reaction mix components	**For pGEM-T Easy vector**
Eco R1(10 u/µl)	0.5 µl
Y^{+}Tango Buffer(10X)	10 µl
Plasmid DNA	10 µl
Water	29.5 µl
Total	50µl

b) Mixture was incubated at 37°C for 2-3 h.

c) Absolute alcohol (2.5 volumes) was added to mixture and incubated at –20° C for overnight or at –80° C for 30-60 min.

d) After incubation it was centrifuged at 14,000 g for 20 min at 4°C.

e) The pellet was washed twice with 75% alcohol and dried. The pellet was dissolved in 15 µl of H_2O and stored at –20°C for further use.

f) Agarose gel (0.1%) was prepared and 15 µl of DNA mixed with 4 µl of gel loading dye (1x) was loaded. Electrophoresis was carried out at 80 V for 2-3 h in TAE buffer.

g) The gel was stained in ethidium bromide (0.5 mg/ml) for 2 min and visualized in UV transiluminator.

3.2.7 Purification of plasmid DNA for sequencing

1. One of the colonies containing the gene of interest ligated with vector was chosen for further downstream reaction. Plasmid DNA was isolated and purified for sequencing as the procedure given below using FastPlasmid™ Mini (Eppendorf AG, Germany).
2. Transformed *E.coli* grown overnight culture (0.5-4 ml) was centrifuged at 12000-13000 rpm for 1 min.
3. Supernatant was discarded and pellet was resuspended in 400 µl of ice-cold Complete lysis solution.
4. Mixed and vortexed at highest setting for full 30 sec (This step is critical for obtaining maximum yield).
5. Incubated the mixture for 3 min at room temperature.

6. Lysate was transferred to the Spin column assembly and centrifuged at 14,000 rpm for 30-60 sec.
7. Flowthrough was discarded and 400 µl diluted wash buffer was added on to the filter and centrifuged at 14,000 g for 1 min.
8. Flowthrough was discarded and centrifuged for additional one-minute.
9. The filter was placed in a fresh microfuge tube and elution buffer (50 µl) was added on to the centre of the filter, incubated at room temperature for 1 min and centrifuged at 14,000 g for 1 min to collect DNA in the tube.
10. The eluted DNA will be purified plasmid DNA. It was carried to concentrate eluted DNA and to remove impurities.
11. DNA (2 µl) was loaded onto 1% agarose gel for checking of concentration of DNA.

3.2.8 Quantification of purified DNA

Absorbance in spectrophotometer helps to quantify of purified DNA. Absorbance of DNA was recorded in UV range from 230-300 nm. Values of A_{max}, A_{min} (A_{260}/A_{280}) were calculated to know the appropriate concentration of DNA.

3.2.9 Sequencing

3.2.9.1 Automated sequencing

Sequencing reaction was carried out in an automated sequencer (ABI Prism Model 310, version 3.4.1) using Big Dye terminator cycle sequencing kit V3 (Applied Biosystems, USA), containing 3' fluorescent dye labelled dideoxynucleotide triphosphate. The primer T7 (forward) and SP6 (reverse) were used in sequencing of clones in pGEM-T Easy vector. PCR was carried out to amplify DNA for extension through the primers in a 0.2 ml PCR tubes.

PCR reaction was setup as follows

Big dye terminator (reaction mix)	4.0 µl
Primer (1.6 pmole) forward/reverse	1.0 µl
Purified plasmid DNA (0.3 µg/µl)	2.0 µl
Water	3.0 µl
Total	10.0 µl

The reaction mixture was mixed properly, centrifuged briefly to collect the mixture at the bottom of the tube and incubated in thermocycler (Gene amp 9700). The cycling condition of reaction was as follows:

(i) 95°C for 10 sec	Denaturation
(ii) 50°C for 40 sec	Annealing
(iii) 60°C for 4 min	Extension

These steps were repeated for 25 times.

3.2.9.2 Preparation of PCR amplified DNA for sequencing

The volume of PCR extension reaction was raised to 100 µl by adding water and DNA was precipitated by adding 10 µl of 3 M sodium acetate (pH 4.6) and 250 µl of absolute alcohol.

a. The reaction contents were centrifuged at 12,000 g for 20 min at room temperature.
b. Supernatant was removed carefully and pellet was washed twice with 250 µl 70% alcohol at room temperature.
c. After washing the pellet was air-dried.
d. The pellet was resuspended in 15 µl of Template Suppression Reagent (TSR).
e. DNA was denatured at 95°C in GeneAmp (PCR system 9700) for 5 min.

f. After denaturation, the sample was loaded in automated sequencer ABI Prism 310.

g. After run, electropheratogram was obtained and analyzed.

3.2.10 Computer analysis of the sequencing data

Sequencing data obtained was analyzed with the help of tools like

(i) BLAST (Basic Local Alignment Search Tool) (www.ncbi.nlm.nih.gov) for homology search.

(ii) Multialin (www.prodes.toulouse.inra.fr/multalin/multalign.html) for multialignment. ClustalW (www. ebi.ac.uk/clustalW/index.html) for multialignment

(iii) Expasy (www. Expasy.ch.tools.dna.html) for translation.

3.3 Detection of LMoV by Northern hybridization in lily samples

Northern hybridization is an efficient means of detecting viruses based on RNA-DNA hybridization. It can detect virus concentration in pg so is more sensitive method than ELISA. To detect LMoV in lily cultivars, we used partially amplified LMoV CP gene (~700 bp) as probe in radioactive hybridization detection reaction. The procedure followed is described below:

Slot-blot preparation

3.3.1 Sample collection and RNA isolation

Various lily samples such as Corida, Brumrllio, Nerone, White Mero Star, Medi, Nove canto, Romana, Star gazer Max, London, Cavi, Expression, Adelina, Parati, Pollyanna and *L. longiflorm* were collected from various parts of H.P. Total RNA was extracted by earlier described methods for preparing radiolabelled probe.

3.3.2 Sample preparation for blot

Leaf tissue of 0.5-1g was cut into small pieces and frozen in liquid nitrogen in prechilled mortar and pestle and ground to a fine powder. Fine powdered leaf was placed in eppendorf and 300 µl TNE buffer (10Mm Tris HCl, 100mM NaCl, 1mM EDTA, pH-8.0) was added spin for 1 min at 12000 g, 10 µl supernatant was diluted with 90 µl of Millipore and it was denatured at 65°C for 1 min.

Nitrocellulose membrane was cut according to the size of the slot blot apparatus and pre wetted in distilled water for 5 min. Then the membrane was equilibrated in 20xSSC buffer for 5 min. It was briefly air dried after saturation and denatured samples were added into the slot of the apparatus and the vacuum of 70 psi pressure was applied for 3-4 h. Then the membrane was UV cross linked for 2 min at 1200 J energy.

3.3.3 Preparation of Probes

The clone was restriction digested with *Eco* R I and was labeled with P^{32} dATP isotope and it was used as probes for the detection of LMoV. The restriction digested DNA was gel electrophorsed and the insert was eluted and denatured at 98°C for 10 min. The reaction mixture contains

Material	Quantity
Insert DNA	200-500 ng
Random hexamers	100 ng
10X Klenow buffer	3 µl
dNTPs (10 mM)	4.5 µl
P^{32}dATP	1µl
Klenow enzymes (5u/ µl)	1 µl
Water	20.2 µl
Total	30 µl

Whole mixture was mixed properly by pipetting and it was incubated for 1h at 37°C after brief centrifugation. After incubation it was chilled in ice and was kept in 0-20°C for future use just before use, incubating in boiling water for 7 min denature the probe.

3.3.4 Northern hybridization using radioactively labeled cDNA probes

Northern hybridization was carried out by a modification of the method described in an ExpressHyb (BD Biosciences, USA). ExpressHyb solution was warmed at 68°C and stirred well to completely dissolve any precipitate. 10x10cm nylon membrane was prehybridised in a minimum total volume of 5-10 ml of ExpressyHyb solution with continuous shaking at 68°C for 30 min. Equal volume of buffer A (500Mm Tris HCL, 500Mm NaCl, 5mM EDTA, 5%SDS) was added to the radioactively labeled DNA probes and denatured at 95-100°C for 2-5 min. Then it was quickly chilled on ice and to this added 5-10 ml of fresh ExpressHyb solution. ExpressHyb solution was replaced with fresh solution containing the radiolabelled DNA probe. All air bubbles were removed from the container, and the Express Hyb solution was evenly distributed over the entire blot. It was incubated with continuous shaking at 68°C for 1 h. Rinsed the blot in wash solution-I at room temperature for 30-40 min with continuous agitation with few change of fresh wash solution-I. The blot in wash solution-II was washed with continuous shaking at 50°C for 40 min with one change of fresh solution. The blot was removed with forceps and excess of wash solution was drained off. Immediately the blot was put on Whatmann paper (3 mm), and exposed to x-ray film at -70°C with two intensifying screen and then film was developed after overnight incubation.

3.4 Cleaved amplified polymorphism (CAP) analysis for detection of homology between different isolates

This technique begins with the amplification of cloned cDNA, digestion of that cDNA with diagnostic-restriction enzymes (4-bp cutters) such as Hae-III, Alu-I, Taq-I and fractionation of the restricted DNA by agarose gel electrophoresis to check the homology between different isolates.

Reaction mixture was prepared differently for different restriction enzymes in microfuge tube on ice shown in tables below:

1) Hae III:

Reaction mix components	**Quantity**
Hae III (8u/µl)	0.5 µl
Buffer M(10X)	10 µl
DNA	20 µl
Water	19.5 µl
Total	50µl

2) Taq I:

Reaction mix components	**Quantity**
Taq I (10u/µl)	0.5 µl
Taq Buffer (10X)	10 µl
DNA	20 µl
Water	19.5 µl
Total	50µl

3) Alu 3A:

Reaction mix components	**Quantity**
Alu 3A (8u/µl)	0.5 µl
Buffer M(10X)	10 µl
DNA	20 µl
Water	19.5 µl
Total	50µl

Temprature profile of these enzymes

Restriction enzymes	**Temp. (°C)**
Hae III (8u/µl)	37
Taq I (10u/µl)	65
Alu 3A (8u/µl)	37

Protocol

a) Mixture was incubated at suitable temperature as given above for 2-3 h.

b) Absolute alcohol (2.5 volumes) was added to mixture and incubated at –20°C for overnight or at –80°C for 30-60 min.

c) After incubation it was centrifuged at 14,000g for 20 min at 4°C.

d) The pellet was washed twice with 75% alcohol and dried. The pellet was dissolved in 15 µl of water and stored at –20°C for further use.

e) Agarose gel (1%) was prepared and 15 µl of DNA mixed with 4 µl of gel loading dye (1x) was loaded. Electrophoresis was carried out at 80V for 2-3 h in TAE buffer.

f) The gel was stained in ethidium bromide (0.5 mg/ml) for 2 min and visualized in UV transiluminator.

Results

Chapter-IV

RESULTS

4.1 *Tulip breaking virus* (TBV)

4.1.1 Natural symptoms

Infected tulips in the experimental field of Floriculture Division (IHBT, Palampur) found to exhibit mosaic, mottling and flecking of leaves, reduced plant size, colour breaking in flower, distorted and twisted growth, (Plate 4.1). Morphological studies of TBV by electron microscopy revealed its particle size about 750 nm. While ultrathin section of TBV-infected leaf of tulip revealed the presence of pinwheel inclusion bodies in the cytoplasm (Plate 4.2) of the virus-infected cells.

4.1.2 Nucleic acid based detection of Potyviruses

4.1.2.1 Detection and amplification of coat protein (CP) gene of TBV

CP gene of TBV was amplified from the total RNA isolated from different tulip isolates using TBV specific primers (TBVU and TBVD) (Table 4.1). An amplicon of ~700 bp was observed on agarose gel in TBV infected plants (Fig. 4.1).

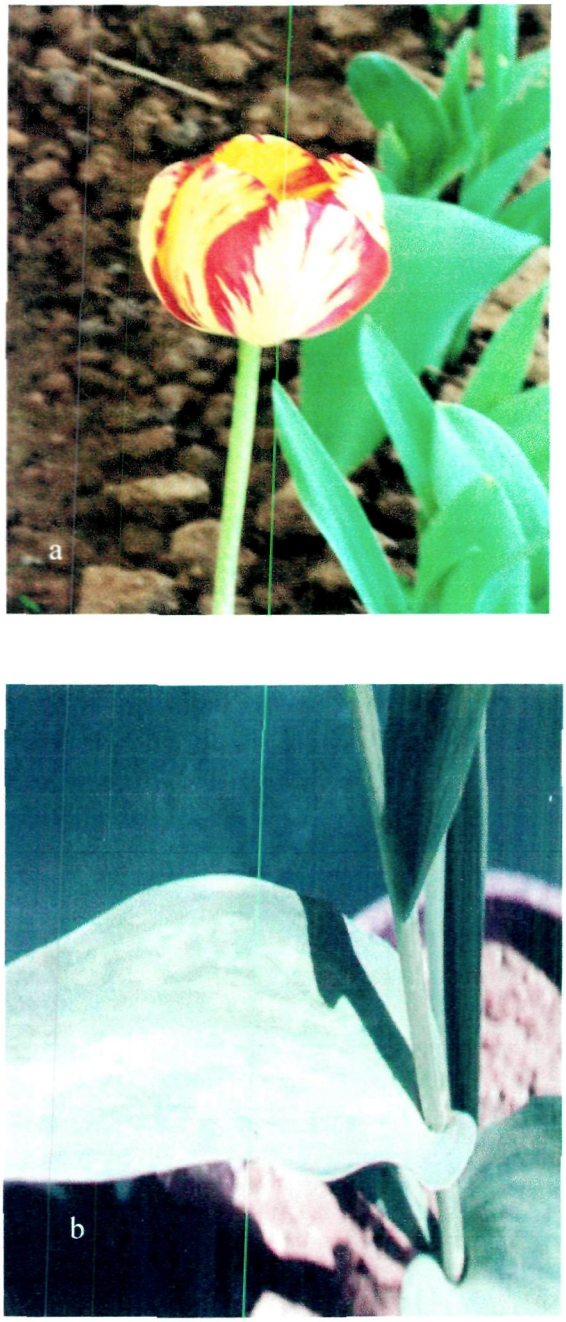


Plate 4.1 Colour breaking pattern on flower (a) and mosaic pattern on leaves (b) of tulip

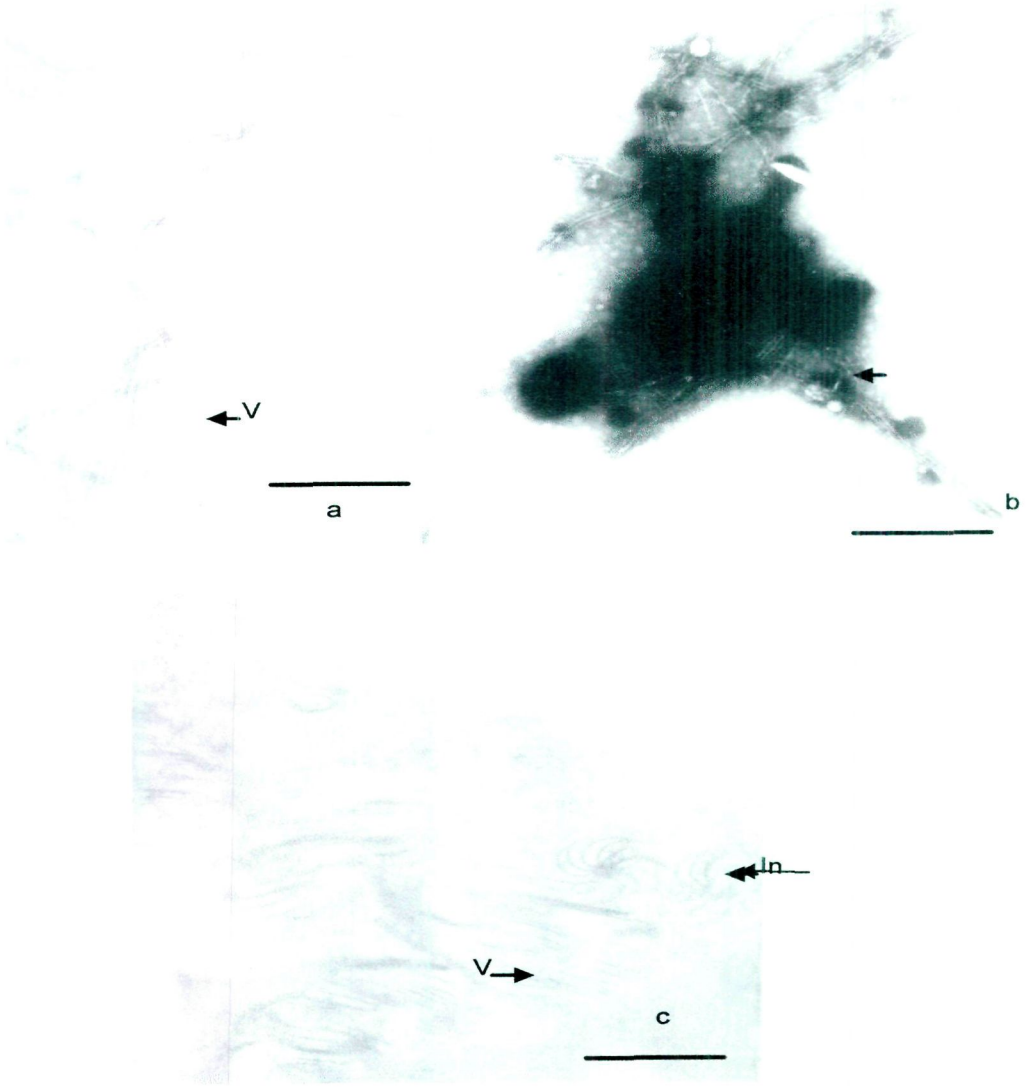


Plate 4.2 Electron microscopy of TBV virus showing (a) Particle of size 750 nm, Clumping of virion (b) with TBV antiserum (c) Virus aggregation and pinwheel inclusion in cytoplasm

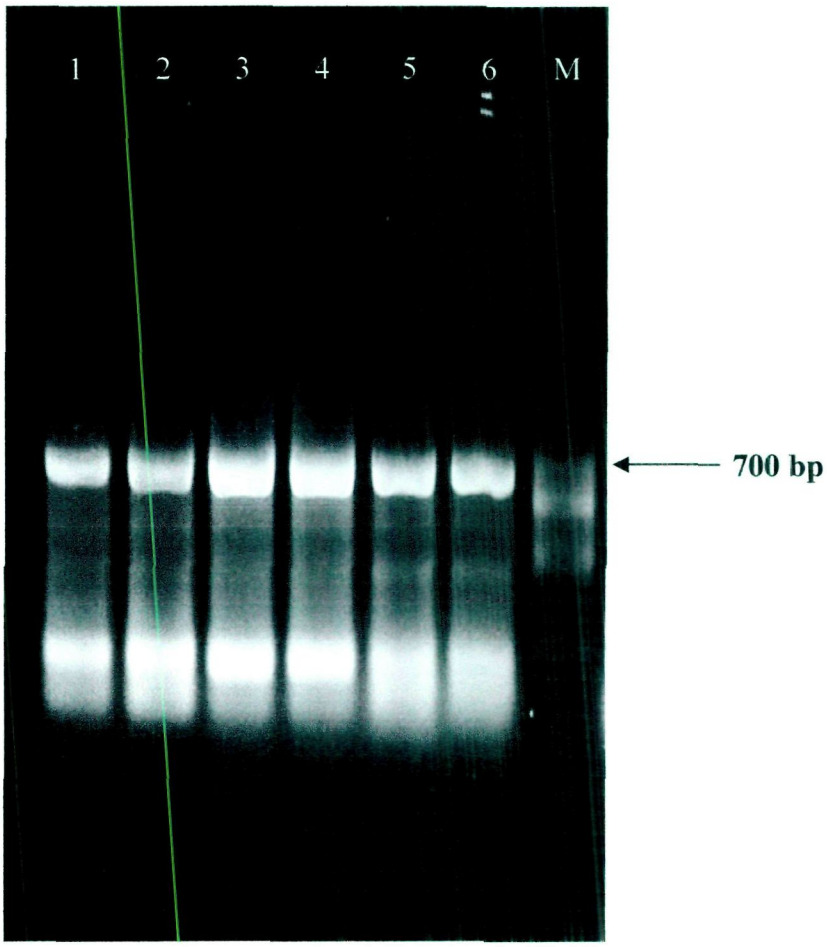


Fig. 4.1 **Agarose gel electrophoresis (1%) showing RT-PCR product of TBV CP gene. Lane M: DNA marker (100 base pair ladder). Lane 1-6: Amplified product from infected tulip leaf samples. Primers used were TBV CP gene specific. The approximately 700 bp band in lane 1-6 indicative of TBV infection.**

Table 4.1.Detection of TBV in tulip samples from various locations by RT-PCR

Location of samples	TBV
IHBT, Palampur	+
Solan	+
Darang	-
Chamba	+
Sundernagar	+
Chail	-

4.1.2.2 Cloning of RT-PCR amplified products

Amplified products of TBV CP gene obtained from tulip collected from different geographical location of HP such as Chamba, Sundernagar, Solan, and Palampur (~700bp) were cloned in pGEM-T Easy vector (Promega, USA). Clones were selected on LB agar plates supplemented with ampicillin. The clones were checked by restriction digestion with *Eco* RI to identify the recombinant plasmid containing the CP gene. Fig. 4.2 shows photograph of the gel showing *Eco* RI digested recombinant plasmid DNA. It gave fragment of approximately 700 bp in case of partial TBV CP gene as showed in lane 1-4.

4.1.3 Sequencing

The cloned DNA (isolate Chamba) was sequenced using T7 and SP6 primer in automated sequencer (ABI prism 310).

Sequence analysis

The nucleotide and amino acid sequences of partial inclusion body protein and partial CP gene of TBV from Chamba were used for multiple sequence alignment with other similar sequences from database. Partial sequence of CP

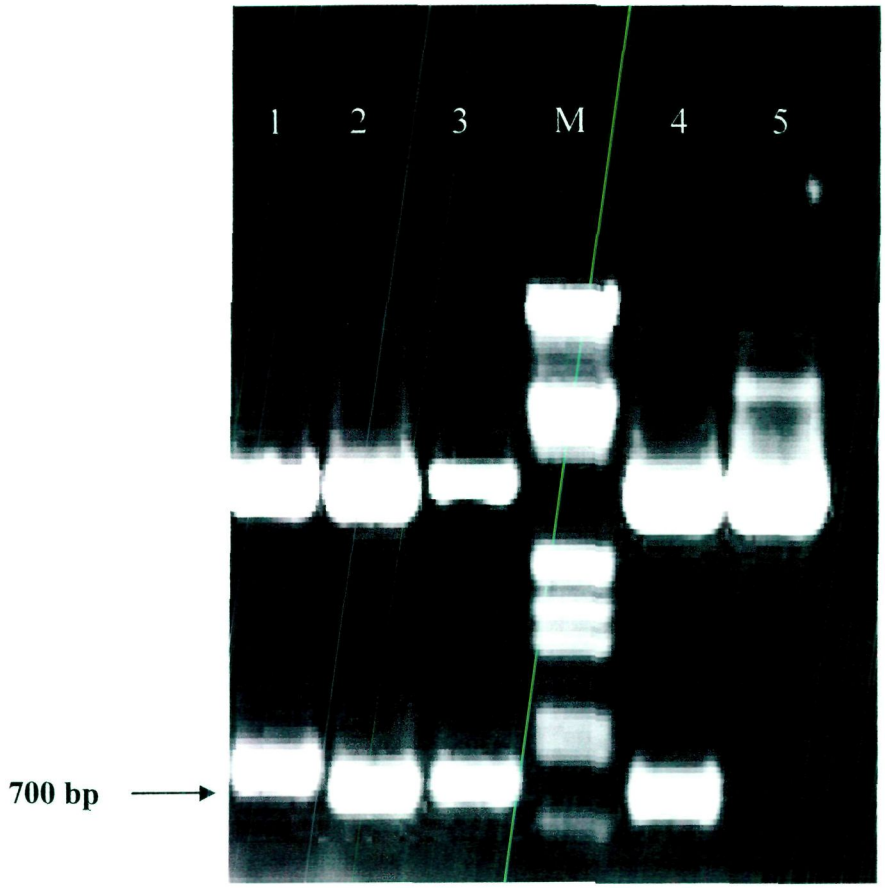


Fig. 4.2 Agarose gel electrophoresis (1.0%) showing restriction digestion product of clones of TBV to check the identity of clones. Lane 1-4: Clones contain the TBV insert. Lane 5: Clones without TBV insert, Lane M: DNA marker (λ DNA). The approximately 700 bp band in lane 1-4 after restriction digestion reaction confirms presence of TBV insert in clones

gene along with inclusion body protein gene of TBV was submitted to GenBank with accession number AM048878 (Fig. 4.3). In BLAST search the sequence of inclusion body of TBV showed 99% homology (Fig 4.4) while partial CP gene showed 100% homology (Fig 4.5) with those of other available sequence of TBV viz. accession number X63630. The amino acid sequence of inclusion body protein and CP gene showed 98% homology with similar TBV amino acid sequence.

4.1.4 Cleaved amplified polymorphism (CAP) analysis for differential study of TBV isolates

To check the scenario of TBV in tulip cultivars grown in HP, cleaved amplified polymorphism analysis was used to differentiate various TBV isolates procured from Chamba, Solan, Sundernagar and Institute of Himalayan Bioresource and Technology, Palampur. TBV CP gene was amplified and cloned. (Fig. 4.6) shows restriction digestion pattern with three 4 bp cutters (such as Hae-III, Taq-I and Alu-I). One restriction enzyme analysis may not be productive enough e.g. restriction fragments with Hae-III do not show any variation in all the isolates so two other enzymes were also used. Restriction pattern with these enzymes such as Taq-I and Alu-I was able to differentiate various isolates of TBV depending upon their digestion pattern. Digestion with Taq-I enzyme was giving maximum variability so that we put the different isolates in four groups on the basis of digestion pattern.

Fig. 4.3 Nucleotide and amino acid sequences of partial inclusion body protein gene and CP gene of *Tulip breaking virus* (TBV)

A. Nucleotide sequences of inclusion body protein gene of *Tulip breaking virus* (TBV)

***Tulip breaking virus* (AM048878)**

GCATTGAGCAACACGTTCGAGCAGTTGGGTTTGAATTACAATTTTGACTCACGCACTACGAAGAAAGAAGACT
TGTGGTTTATGTCACATAAAGGCTTAGAGCGTGGTGGAATATACATACCAAAATTAGAGCCGGAGCGTATTGT
GTCAATTCTGGAGTGGGATAGGTCCATTGAACCAGTCCACAGATTGGAAGCTATCTGTGCATCGATGATAGAA
GCGTGGGGTTATACAGAATTGTTGCATGAAATACGGAGATTTTATTATTGGGTGTTAAATCAAGCGCCATACA
CGGAACTGTCTAAAGAAGGAAAAGCGCCTTATCTATCTGAAGTGGCACTGACGGCTCTATATATGGGCAAGGA
ATCAGAAAGTATTGAAATCGAAAAATATATCCACCAAATTGACAATTGGTGTGATTATGATGACATTGAATCA
GTTCAGTTTCAA

***Tulip mosaic virus* (X63630)**

GCATTGAGCAACACGTTCGAGCAGTTGGGTTTGAATTACAATTTTGACTCACGAACTACGAAGAAAGAAGACT
TGTGGTTTATGTCACATAAAGGCTTAGAGCGTGATGGAATATACATACCAAAATTAGAGCCGGAGCGTATTGT
GTCAATTCTGGAGTGGGATAGGTCCATTGAACCAGTCCACAGATTGGAAGCTATCTGTGCATCGATGATAGAA
GCGTGGGGTTATACAGAATTGTTACATGAAATACGGAGATTTTATTATTGGGTGTTAAATCAAGCGCCATACA
CGGAACTGTCTAAAGAAGGAAAAGCGCCTTATCTATCTGAAGTGGCACTGACGGCTCTATATATGGGCAAGGA
ATCAGAAAGTATTGAAATCGAAAAATATATCCACCAAATTGACAATTGGTGTGATCATGATGACATTGAATCA
GTTCAGTTTCAA

B. Amino acid sequence of inclusion body protein of *Tulip braking virus* (TBV)

***Tulip breaking virus* (AM048878**

ALSNTFEQLG LNYNFDSRTT KKEDLWFMSH KGLERGGIYI PKLEPERIVS ILEWDRSIEP
VHRLEAICAS MIEAWGYTEL LHEIRRFYYW VLNQAPYTEL SKEGKAPYLS EVALTALYMG
KESESIEIEK YIHQIDNWCD YDDIESVQFQ

***Tulip mosaic virus* (X63630)**

ALSNTFEQLG LNYNFDSRTT KKEDLWFMSH KGLERDGIYI PKLEPERIVS ILEWDRSIEP
VHRLEAICAS MIEAWGYTEL LHEIRRFYYW VLNQAPYTEL SKEGKAPYLS EVALTALYMG
KESESIEIEK YIHQIDNWCD HDDIESVQFQ

A. Nucleotide sequences of partial CP gene of *Tulip breaking virus* (TBV)

***Tulip breaking virus* (AM048878**

GCAGACGAAACAATCAATGCTGGTAGAAGAGACGTAGCATCAACTAGCGGTAGCAAATCAGTTGCCAAACCAG
CTGCTGAATCTTCTCAAAAAGACAAAGATGTTGATGCAGGCACAACAGCTACATTCGAAATTCCGAGACTGAA
GGCCATATCGTCAAAGTTGGTGCTACCAAAATTTCGTGGAAAGAAAATAGTAAACTTGGAACATCTATTAAA

***Tulip mosaic virus* (X63630)**

GCAGACGAAACAATCAATGCTGGTAGAAGAGACGTAGCATCAACTAGCGGTAGCAAATCAGTTGCCACACCAG
CTGCTGAATCTTCTCAAAAAGACAAAGATGTTGATGCAGGCACAACAGCTACATTCGAAATTCCAAGACTGAA
GGCCATATCGTCAAAGTTGGTGCTACCAAAATTTCGTGGAAAGAAAATAGTAAACTTGGAACATCTATTAAA

B. Amino acid sequences of partial coat protein (CP) of TBV

Tulip breaking virus **(AM048878**

ADETINAGRR DVASTSGSKS VAKPAAESSQ KDKDVDAGTT ATFEIPRLKA ISSKLVLPKF
RGKKIVNLEH LL

Tulip mosaic virus **(X63630)**

ADETINAGRR DVASTSGSKS VATPAAESSQ KDKDVDAGTT ATFEIPRLKA ISSKLVLPKF
RGKKIVNLEH LL

Fig. 4.4. A. Nucleotide sequence alignment of inclusion body protein gene

```
AM048878        GCATTGAGCAACACGTTCGAGCAGTTGGGTTTGAATTACAATTTTGACTCACGCACTACG
X63630          GCATTGAGCAACACGTTCGAGCAGTTGGGTTTGAATTACAATTTTGACTCACGAACTACG
                ************************************************** ******

AM048878        AAGAAAGAAGACTTGTGGTTTATGTCACATAAAGGCTTAGAGCGTGGTGGAATATACATA
X63630          AAGAAAGAAGACTTGTGGTTTATGTCACATAAAGGCTTAGAGCGTGATGGAATATACATA
                ********************************************** *************

AM048878        CCAAAATTAGAGCCGGAGCGTATTGTGTCAATTCTGGAGTGGGATAGGTCCATTGAACCA
X63630          CCAAAATTAGAGCCGGAGCGTATTGTGTCAATTCTGGAGTGGGATAGGTCCATTGAACCA
                ************************************************************

AM048878        GTCCACAGATTGGAAGCTATCTGTGCATCGATGATAGAAGCGTGGGGTTATACAGAATTG
X63630          GTCCACAGATTGGAAGCTATCTGTGCATCGATGATAGAAGCGTGGGGTTATACAGAATTG
                ************************************************************

AM048878        TTGCATGAAATACGGAGATTTTATTATTGGGTGTTAAATCAAGCGCCATACACGGAACTG
X63630          TTACATGAAATACGGAGATTTTATTATTGGGTGTTAAATCAAGCGCCATACACGGAACTG
                ** ***********************************************************

AM048878        TCTAAAGAAGGAAAAGCGCCTTATCTATCTGAAGTGGCACTGACGGCTCTATATATGGGC
X63630          TCTAAAGAAGGAAAAGCGCCTTATCTATCTGAAGTGGCACTGACGGCTCTATATATGGGC
                ************************************************************

AM048878        AAGGAATCAGAAAGTATTGAAATCGAAAAATATATCCACCAAATTGACAATTGGTGTGAT
X63630          AAGGAATCAGAAAGTATTGAAATCGAAAAATATATCCACCAAATTGACAATTGGTGTGAT
                ************************************************************

AM048878        TATGATGACATTGAATCAGTTCAGTTTCAA
X63630          CATGATGACATTGAATCAGTTCAGTTTCAA
                 ****************************
```

B. Amino acid sequence alignment of inclusion body protein

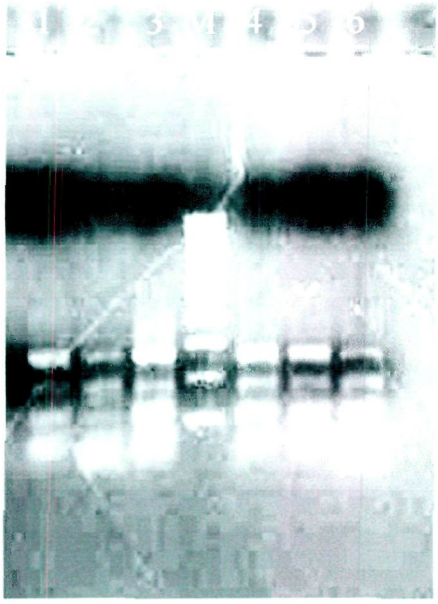
```
AM048878        ALSNTFEQLGLNYNFDSRTTKKEDLWFMSHKGLERGGIYIPKLEPERIVSILEWDRSIEP
X63630          ALSNTFEQLGLNYNFDSRTTKKEDLWFMSHKGLERDGIYIPKLEPERIVSILEWDRSIEP
                **********************************.************************

AM048878        VHRLEAICASMIEAWGYTELLHEIRRFYYWVLNQAPYTELSKEGKAPYLSEVALTALYMG
X63630          VHRLEAICASMIEAWGYTELLHEIRRFYYWVLNQAPYTELSKEGKAPYLSEVALTALYMG
                ************************************************************

AM048878        KESESIEIEKYIHQIDNWCDYDDIESVQFQ
X63630          KESESIEIEKYIHQIDNWCDHDDIESVQFQ
                ********************:*********
```

Fig. 4.5 A Nucleotide sequence alignment of partial CP gene of *Tulip breaking virus* (TBV)

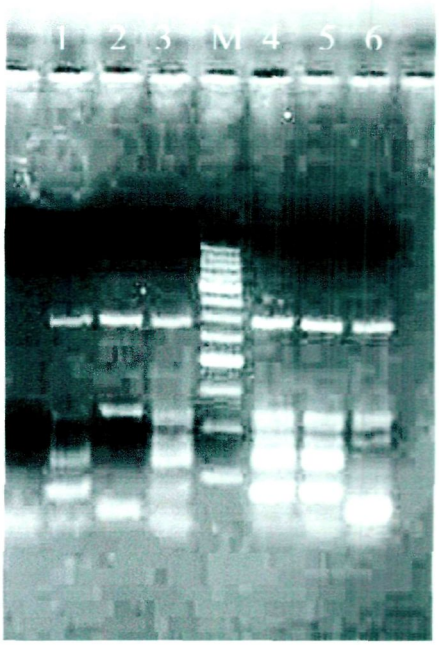
```
AM048878        GCAGACGAAACAATCAATGCTGGTAGAAGAGACGTAGCATCAACTAGCGGTAGCAAATCA
X63630          GCAGACGAAACAATCAATGCTGGTAGAAGAGACGTAGCATCAACTAGCGGTAGCAAATCA
                ****************************************************************

AM048878        GTTGCCACACCAGCTGCTGAATCTTCTCAAAAAGACAAAGATGTTGATGCAGGCACAACA
X63630          GTTGCCACACCAGCTGCTGAATCTTCTCAAAAAGACAAAGATGTTGATGCAGGCACAACA
                ****************************************************************

AM048878        GCTACATTCGAAATTCCAAGACTGAAGGCCATATCGTCAAAGTTGGTGCTACCAAAATTT
X63630          GCTACATTCGAAATTCCAAGACTGAAGGCCATATCGTCAAAGTTGGTGCTACCAAAATTT
                ****************************************************************

AM048878        CGTGGAAAGAAAATAGTAAACTTGGAACATCTATTAAA
X63630          CGTGGAAAGAAAATAGTAAACTTGGAACATCTATTAAA
                ***************************************
```

B. Amino acid sequence alignment of partial coat protein (CP)

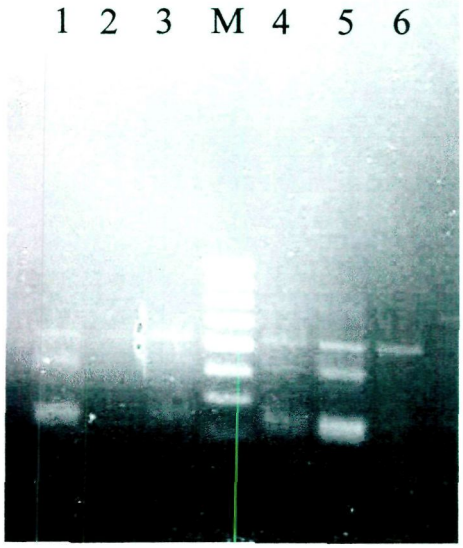
```
AM048878        ADETINAGRRDVASTSGSKSVAKPAAESSQKDKDVDAGTTATFEIPRLKAISSKLVLPKF
X63630          ADETINAGRRDVASTSGSKSVATPAAESSQKDKDVDAGTTATFEIPRLKAISSKLVLPKF
                ****************************.***********************************

AM048878        RGKKIVNLEHLL
X63630          RGKKIVNLEHLL
                ************
```



(A)



(B)



(C)

Fig. 4.6 Agarose gel electrophoresis (1.0%) showing cleaved amplified polymorphism of cloned TBV isolates using A) Hae-III, B)Taq-I, C)Alu-I (all 4 bp cutters). Lane 1: Chamba, Lane 2: Cultivar IHBT (Purple), Lane 3: Sundernagar, Lane 4: Solan, Lane 5: Cultivar IHBT (Red) and Lane 6: Cultivar IHBT (Yellow).

4.2 *Lily mottle virus* (LMoV)

4.2.1 Natural symptoms

Infected lilies in the experimental field of Floriculture Division (IHBT, Palampur) exhibited mosaic pattern on leaves, reduced plant size and distorted growth (Plate 4.3).

4.2.2 *Serological detection of potyviruses from infected lily*

Lily mottle virus (LMoV) is highly immunogenic and as a result serological methods can be applied reliably. In the present study, DAS-ELISA was used for LMoV detection in lily.

Double antibody sandwich-Enzyme Linked Immunosorbent assay (DAS-ELISA)

Infected lilies from different locations were screened for LMoV. Out of 30 lily cultivars, nine were found positive for LMoV, which includes four Asiatic hybrid lily cultivars such as London, Romano, Alaska, Nova Cento; two Oriental hybrid lily cultivars such as Galieli, White Mero Star; and three other lilies such as Spider lily, *L. longiflorum* and *L. tigrinum*. (Table 4.2). The absorbance value for tested samples near positive control or 3-4 times the negative control was considered positive for the virus. The absorbance values for positive and negative controls were found to be 1.042 and 0.127 respectively.

Plate 4.3 Mosaic pattern on leaves of Asiatic (a) and Oriental lily (b)

Table 4.2 *Lily mottle virus* detection by DAS-ELISA in Lily cultivars and other plants

S. No.	Cultivars	Result	S. No.	Cultivars	Result
Asiatic hybrids					
1	Pollyanna	-	9	Adelina	-
2	Ucaris	-	10	Cavi	-
3	Nerone	-	11	Alaska	+
4	London	+	12	Prato	-
5	Romano	+	13	Monobracia	-
6	America	-	14	Nova Cento	+
7	Brunello	-	15	Tascarna	-
8	Nova Cento	-			
Oriental hybrids					
16	Expression	-	19	White Mero Star	+
17	Galeili	+	20	Star Gizer Max	-
18	Woodriff's	-	21	Mediterranee	-
Other lilies					
22	Zentedeschia	-	24	Spider lily	+
23	*L. tigrinum*	+	25	*L. longiflorum*	+
Other plants					
26	Agapanthus	-	29	Hippeastrum	-
27	Hemerocallis	-	30	Tuberose	-
28	Liatris	-			

4.2.3 Detection of LMoV by Northern hybridization in lily samples

To detect LMoV in different lily cultivars, we used LMoV CP gene (~700bp) as radiolabelled probe in Northern hybridization detection method (Figure 4.7). Out of 15 isolates 3 were found positive for LMoV (Table 4.3).

Table 4.3 Results of Northern Hybridization with 15 isolates of lily

S. No.	Cultivars/Spp.	Reaction	S. No.	Cultivars	Reaction
1	Corida (A.H.)	+	9	London (A.H.)	-
2	*L. longiflorum*	+	10	Cavi (A.H.)	-
3	Brunello (A.H.)	+	11	Expression (O.H.)	-
4	White Mero Star (O.H.)	-	12	Nerone (A.H.)	-
5	Mediterranee (O.H.)	-	13	Adelina (Tissue-culture) (A.H.)	-
6	Nova Cento (A.H.)	-	14	Prato (A.H.)	-
7	Romano (A.H.)	-	15	Pollyanna (A.H.)	-
8	Star Gazer Max (O.H.)	-	16	*L. tigrinum* (positive sample)	+
A.H-Asiatic Hybrid			O.H-Oriental Hybrid		

4.2.4.1 Amplification of CP gene of LMoV by RT-PCR

Group specific primers of potyvirus coat protein gene were used for amplification of partial CP gene of LMoV from total RNA isolated from various lily cultivars (Table 4.4). Amplicon of ~ 700 bp was observed on agarose gel in virus infected plants (Fig.4.8).

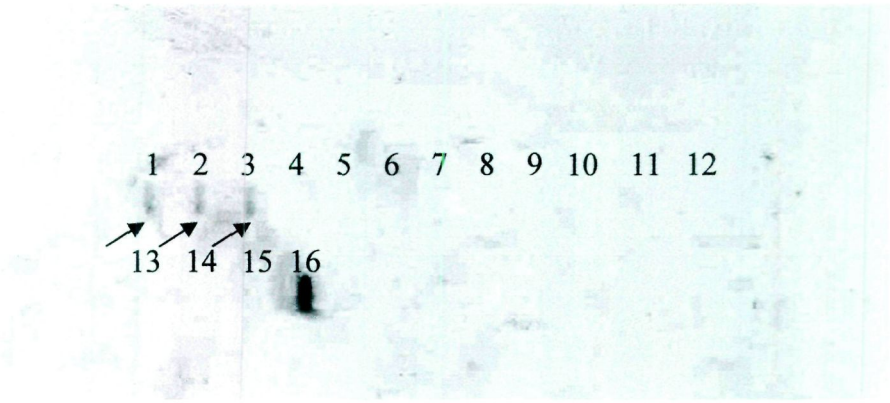


Figure 4.7 **Northern hybridization used for the detection of LMoV in lily samples using radiolabeled Tiger-lily CP gene probe. Out of 15 cultivars tested 3 cv. Corida (No.1), *L.longiflorum* (No.2), Brunello (No.3) found positive for LMoV. No. 16 is Tiger-lily RNA as positive sample**

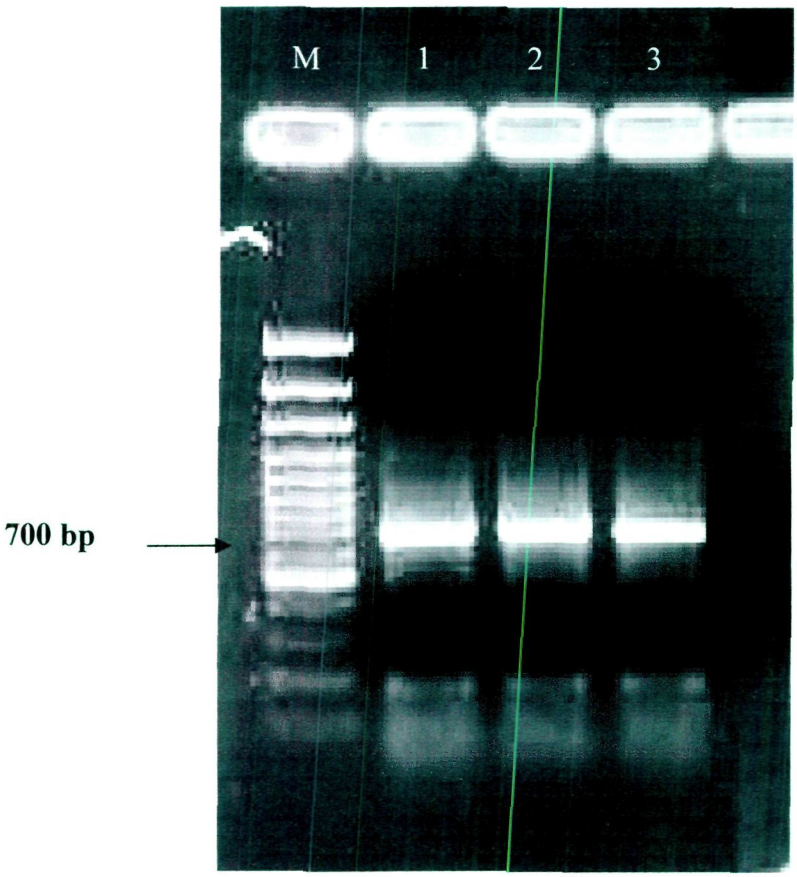


Figure 4.8 Agarose gel electrophoresis (1%) showing RT-PCR products of LMoV. Lane M: DNA marker (100 base pair ladder). Lane 1, 2 & 3: Amplified products from infected leaf samples. Primers used were potyvirus group specific. The approximately 700 bp band in lane 1, 2 & 3 was indicative of potyvirus infection

4.2.4.2 Cloning of RT-PCR amplified products

PCR product i.e. DNA of partial LMoV CP genes (~700bp) from tiger-lily was cloned in pGEM-T Easy vector (Promega, USA). Clone was selected on LB agar plates supplemented with ampicillin. The clone was checked by restriction digestion with *Eco* RI to confirm the presence of ligated CP genes in plasmids. (Fig. 4.9) shows photograph of the gel showing *Eco* RI digested recombinant plasmid DNA. It gave fragment of approximately 700 bp in case of LMoV CP gene in Lane 1, 3, 4 and 5.

Table 4.4 Detection of LMoV in lily by RT-PCR

	Cultivars	**LMoV**
Asiatic hybrid lily	Brunello	-
	Alaska	-
	London	+
	Nova Cento	-
	Romano	-
	Pollyanna (Hissar isolate)	+
Oriental hybrid lily	Galieli	-
	Star Gazer Max	-
	White Mero Star	+
	Expression	-
Lilium tigrinum		+
Spider lily		-
Zentedeschia		-
L. longiflorum		-

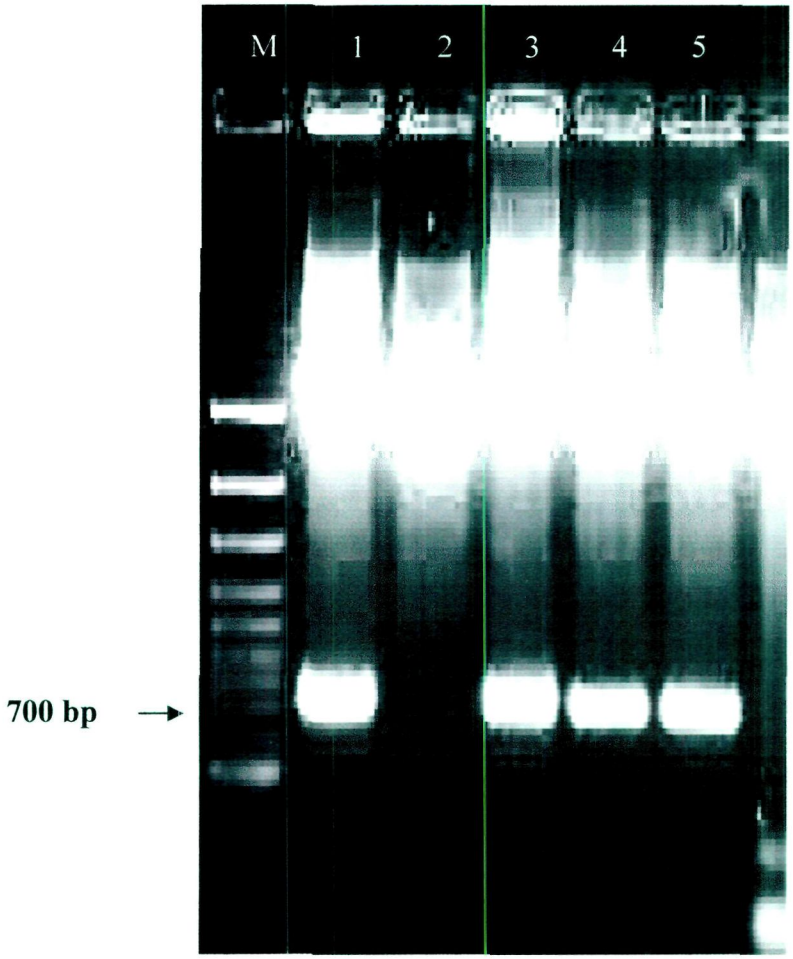


Figure 4.9 **Agarose gel electrophoresis (1.0%) of restriction digestion product of clones to check the identity of clones. Lane 1&3-4: Clones contains LMoV insert. Lane2: Clones without LMoV insert, Lane M: DNA marker (250 bp marker). The approximately 700 bp bands in lane 1&3-4 after restriction digestion reaction confirms the presence of LMoV insert in clones**

4.2.5 Sequencing

Partial LMoV CP gene obtained from tiger-lily was sequenced using T7 and SP6 primer in automated sequencer (ABI prism 310).

Sequence analysis

Multialign of the nucleotide and amino acid sequences was obtained with MULTALIN programme with other available sequences from database. Partial sequence of CP gene of LMoV was submitted in GenBank with accession number AJ879511 (Fig. 4.10). In BLAST search the sequence of CP gene of LMoV showed 90-97% homology with those of other available sequence from database. The amino acid sequence showed 68-73% homology with the other LMoV CP gene amino acid sequence (Fig. 4.11).

Fig. 4.10 Nucleotide and amino acid sequences of partial CP gene along with some 3' UTR region of *Lily mottle virus* (LMoV)

AJ879511	PALAMPUR
AB054886	JAPAN
AJ874696	CHINA
AJ874695	CHINA
AJ564636	UNITED KINGDOM
AJ748256	CHINA
AB053256	JAPAN

A. Nucleotide sequences of partial CP gene along with 3' UTR region of *Lily mottle virus* (LMoV

AJ879511

ATGGCGCGTGGCTCAAGATGGACGGAGATCAGCAAGTTGAATTTCCATTACGTCCCGAAATTGAACACGC
AAAACCGACGCTGCGCCAGATCATGGCGCATTTCTCACATCTTGCTGAAGCATATATTGAGAAACAAAAT
GCAGAGAAACCGTACATGCCTAGGTACGGCCTTCAGCGAAATCTCACCGATTTCACGCGAGCACGATTCG
CTTTCGTTTCTAAGAAGTTACTTCACGCACGCCCGCGCGAGCGAAGGAAGCACACTTCAGATGAAAACAG
CCGCCTTGCGCGGAAAACAATCGAAGCTATTTGGGTTAGATGGAAAGGTGACCACCCAAGAAGAGGACAC
GGAGAGGCATACAGCAGACGATGTCAAAAAAAACATGCACTCCTTACTTGGAATTTCTATTAAATCCTCG
AATAAACAGCACTAGTAACTTGCTAATATATCTACCTCGTTTATTCCGTAGTATCCTATCTTTTAGCCCA
ACTCTTTGTGCTATAGAGTGGTTTACCATGAACGCACATTGTAGGTGGGAACCGAGCTCCTTGTTAGTGT
CTAATAGCAGAGAGGTCGCCTCGCGTCTATCTAGCCACTAG

AB054886

ATGGCGCGTGGCTCATGATGGACGGAGATCAGCAAGTTGAATTTCCATTACGTCCCATAATTGAACACGC
AAAACCGACGCTGCGCCAGATCATGGCGCATTTCTCACATCTCGCTGAAGCATACATTGAGAAACAAAAT
GCAGAGAAACCGTACATGCCTAGGTACGGCCTTCAGCGAAATCTCACCGATTTCACGCTAGCACGATTCG
CTTTCGATTTCTATGAAGTTACTTCACGCACGCCCGCGCGAGCGAAGGAAGCACACTTCCAGATGAAAAC
AGCCGCCTTGCGCGGAAAACAATCGAAGCTATTTGGGTTAGATGGAAAGGTGACCACCCAAGATGAGGAC
ACGGAGAGGCATACAGCAGACGATGTCAATAAAAACATGCACTCCTTACTTGGAATTTCTATGTAAATCC
TCGAATAAACAGCACTAGTAACTTGCTAATATATCTATCTCGTTTATTCCGTAGTATCCTATCTTTTAGC
CCAACTCTTTGTGCTATAGAGTGGTTTACCATGAACGCACATTGTAGGTGGGAACCGAGCTCCTTGTTAG
TGTCTAATAGCAGAGAGGTCGCCTCGCGTCTATCTAGCCACTAG

AJ874696

ATGGCGCGTGGCTCATGATGGACGGAGATCAGCAAGTTGAATTTCCATTACGTCCCATAATTGAACACGC
AAAACCGACGCTGCGCCAGATCATGGCGCATTTCTCACATCTTGCTGAAGCATATATTGAGAAACAAAAT
GCAGAGAAACCGTACATGCCTAGGTACGGCCTTCAGCGAAATCTCACTGATTTCACGCTAGCACGATTCG
CTTTCGACTTCTATGAAGTTACTTCACGCACGCCTGCGCGAGCGAAGGAAGCACACTTCCAGATGAAAAC
AGCCGCTTTACGCGGAAAACAATCGAAGCTATTTGGGTTAGATGGAAAGGTGACCACCCAAGATGAGGAC
ACGGAGAGGCATACAGCAGACGATGTCAATAAAAACATGCACCCCTTACTTGGAATTTCTATGTAAATCC
TCGAATAAACAGCACTAGTAACTTGCTAATATATCTATCTCGTTTATTCCGTAGTATCCTATCTTTTAGC
CCAACTCTTTGTGCTATAGAGTGGTTTACCATGAACGCACATTGTAGGTGGGAACCGAGCTCCTTGTTAG
TGTCTAATAGCAGAGAGGTCGCCTCGCGTCTATCTAGCCACTAG

AJ874695

ATGGCGCGTGGCTCATGATGGACGGAGATCAGCAAGTTGAATTTCCATTACGTCCCATAATTGAACACGC
AAAACCGACGCTGCGCCAGATCATGGCGCATTTCTCACACCTCGCTGAAGCTTATATTGAGAAGCAAAAT
TTAGAGAAACCGTACATGCCTAGGTACGGCCTTCAGCGAAATCTCACCGACTTCAATCTAGCACGATTTG
CTTTTGATTTCTATGAAGTTACTTCACGCACGCCAGCACGCGCTAAAGAAGCACACTTCCAAATGAAAAC
AGCCGCTTTACGCGGAAAACAATCGAAGCTATTTGGGCTAGATGGAAAGGTGAACACCCAAGATGAGGAC
ACGGAGAGGCATACAGCAGATGATGTCAATAAAAACATGCACTCCTTACTTGGAATTTCTATGTAAATCC
TCGAATAAACAGCACTAGTAACCTGCTAATATATCTATCTCGTTTATTCCGTAGTATCCTATCTATTAGC
TCAACTCTTTGTGTTATAGTGTGGTTTACCATGAACGCACATTGTAGTTGAGAACCGAGCTCCCTGTTGG
TGTCTAGTTGCAGAGAGGTTTCCTCGCGTCTTCTTAGCCACTAGTCTTGGA

AJ564636

ATGGCGTGTGGCTCATGATGGACGGAGATCAGCAAGTTGAATTTCCTTTACGTCCTATACTTGAACACGC
AAAACCGACGCTGCGCCAAATCATGGCGCATTTCTCAAACCTCGCTGAAGCTTATATTGAGAAGCAAAAT
TTAGAGAAACCGTACATGCCTAGGTACGGCCTTCAGCGAAATCTCACCGACTTCAATCTAGCACGATTTG
CTTTTGATTTCTATGAAGTTACTTCACGCACGCCAGCACGCGCTAAAGAAGCACACTTCCAAATGAAAAC
AGCCGCTTTACGCGGAAAACAATCGAAGCTATTTGGGCTAGATGGAAAGGTGAACACCCAAGATGAGGAC
ACGGAGAGGCATACAGCAGATGATGTTAATAAAAACATGCACTCCTTACTTGGAATTTCTATGTAAATCC
TCGAATAAACAGCACTAGTAACTTGCTAATATATCTATCTCGTTTATTCCGTAGTATCCTATCTATTAGC
TCAACTCTTTGTGTTATAGTGTGGTTTACCATGAACGCACATTGTAGTTGAGAACCGAGCTCCTTGTTGG
TGTCTAGTTGCAGAGAGGTTTCCTCGCGTCTTCTTAGCCACTAG

AJ748256

ATGGCGTGTGGCTCATGATGGACGGAGATCAGCAAGTTGAATTTCCTTTACGTCCTATACTTGAACACGC
AAAACCGACGCTGCGCCAAATTATGGCGCATTTCTCAAACCTCGCTGAAGCTTATATTGAGAAGCAAAAT
TTAGAGAAACCGTACATGCCTAGGTACGGCCTTCAGCGAAATCTCACCGACTTCAATCTAGCACGATTTG
CTTTTGATTTCTATGAAGTTACTTCACGCACGCCGGCACGCGCTAAAGAAGCACACTTCCAAATGAAAAC
AGCCGCTTTACGCGGAAAACAATCGAAGCTATTTGGGCTAGATGGAAAGGTGAACACCCAAGATGAGGAC
ACGGAGAGGCATACAGCAGATGATGTCAATAAAAACATGCACTCCTTACTTGGAATTTCTATGTAAATCC
TCGAATAAACAGCACTAGTAACTTGCTAATATATCTATCTCGTTTATTCCGTAGTATCCTATCTATTAGC
TCAACTCTTTGTGTTATAGTGTGGTTTACCATGAACGCACATTGTAGTTGAGAACCGAGCTCCTTGTTGG
TGTCTAGTTGCAGAGAGGTTTCCTCGCGTCTTCTTAGCCACTAG

AB053256

ATGGCGTGTGGCTCATGATGGACGGAGATCAGCAAGTTGAATTTCCTTTACGTCCTATACTTGAACACGC
AAAACCGACGCTGCGCCAAATTATGGCGCACTTCTCAAACCTCGCTGAAGCTTATATTGAGAAGCAAAAT
TTAGAGAAACCGTACATGCCTAGGTACGGCCTTCAGCGAAATCTCACCGACTTCAATCTAGCACGATTTG
CTTTTGATTTCTATGAAGTTACTTCACGCACGCCAGCACGCGCTAAAGAAGCACACTTCCAAATGAAAAC
AGCCGCTTTACGCGGAAAACAATCGAAGCTATTTGGGCTAGATGGAAAGGTGAACACCCAAGATGAGGAC
ACGGAGAGGCATACAGCAGATGATGTCAATAAAAACATGCACTCCTTACTTGGAATTTCTATGTAAATCC
TCGAATAAACAGCACTAGTAACTTGCTAATATATCTATCTCGTTTATTCCGTAGTATCCTATCTATTAGC
TCAACTCTTTGTGTTATAGTGTGGTTTACCATGAACGCACATTGTAGTTGAGAACCGAGCTCCTTGTTGG
TGTCTAGTTGCAGAGAGGTTTCCTCGCGTCTTCTTAGCCACTAG

B. Amino acid sequences of partial coat protein (CP) of LMoV

AJ879511
GAWLKMDGDQ QVEFPLRPEI EHAKPTLRQI MAHFSHLAEA YIEKQNAEKP YMPRYGLQRN
LTDFTRARFA FVSKKLLHAR PRERRKHTSD ENSRLARKTI EAIWVRWKGD HPRRGHGEAY
SRRCQKKHAL LTWNFY

AB054886
GAWLMMDGDQ QVEFPLRPII EHAKPTLRQI MAHFSHLAEA YIEKQNAEKP YMPRYGLQRN
LTDFTLARFA FDFYEVTSRT PARAKEAHFQ MKTAALRGKQ SKLFGLDGKV TTQDEDTERH
TADDVNKNMH SLLGISM

AJ874696
GAWLMMDGDQ QVEFPLRPII EHAKPTLRQI MAHFSHLAEA YIEKQNAEKP YMPRYGLQRN
LTDFTLARFA FDFYEVTSRT PARAKEAHFQ MKTAALRGKQ SKLFGLDGKV TTQDEDTERH
TADDVNKNMH PLLGISM

AJ874695
GAWLMMDGDQ QVEFPLRPII EHAKPTLRQI MAHFSHLAEA YIEKQNLEKP YMPRYGLQRN
LTDFNLARFA FDFYEVTSRT PARAKEAHFQ MKTAALRGKQ SKLFGLDGKV NTQDEDTERH
TADDVNKNMH SLLGISM

AJ564636
GVWLMMDGDQ QVEFPLRPIL EHAKPTLRQI MAHFSNLAEA YIEKQNLEKP YMPRYGLQRN
LTDFNLARFA FDFYEVTSRT PARAKEAHFQ MKTAALRGKQ SKLFGLDGKV NTQDEDTERH
TADDVNKNMH SLLGISM

AJ748256
GVWLMMDGDQ QVEFPLRPIL EHAKPTLRQI MAHFSNLAEA YIEKQNLEKP YMPRYGLQRN
LTDFNLARFA FDFYEVTSRT PARAKEAHFQ MKTAALRGKQ SKLFGLDGKV NTQDEDTERH
TADDVNKNMH SLLGISM

AB053256
GVWLMMDGDQ QVEFPLRPIL EHAKPTLRQI MAHFSNLAEA YIEKQNLEKP YMPRYGLQRN
LTDFNLARFA FDFYEVTSRT PARAKEAHFQ MKTAALRGKQ SKLFGLDGKV NTQDEDTERH
TADDVNKNMH SLLGISM

Fig. 4.11 A. Nucleotide sequence alignment of partial CP gene along with 3'-UTR region of *Lily mottle virus* (LMoV)

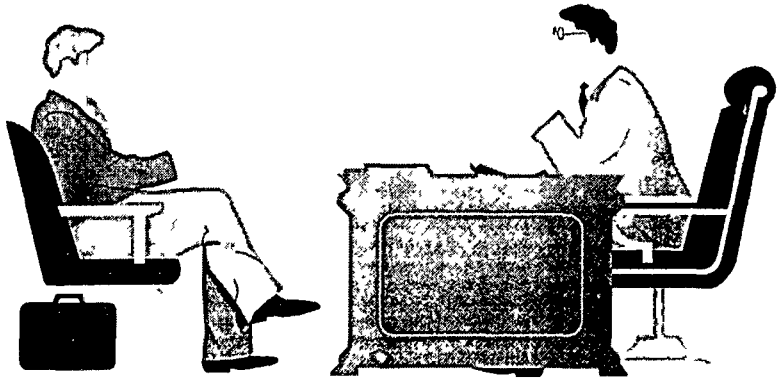
```
AJ748256        ATGGCGTGTGGCTCATGATGGACGGAGATCAGCAAGTTGAATTTCCTTTACGTCCTATAC
AB053256        ATGGCGTGTGGCTCATGATGGACGGAGATCAGCAAGTTGAATTTCCTTTACGTCCTATAC
AJ564636        ATGGCGTGTGGCTCATGATGGACGGAGATCAGCAAGTTGAATTTCCTTTACGTCCTATAC
AJ879511        ATGGCGCGTGGCTCAAGATGGACGGAGATCAGCAAGTTGAATTTCCATTACGTCCCGAAA
AJ874695        ATGGCGCGTGGCTCATGATGGACGGAGATCAGCAAGTTGAATTTCCATTACGTCCCATAA
AB054886        ATGGCGCGTGGCTCATGATGGACGGAGATCAGCAAGTTGAATTTCCATTACGTCCCATAA
AJ874696        ATGGCGCGTGGCTCATGATGGACGGAGATCAGCAAGTTGAATTTCCATTACGTCCCATAA
                ****** ********:*********************************** .:*.
```

```
AJ748256        TTGAACACGCAAAACCGACGCTGCGCCAAATTATGGCGCATTTCTCAAACCTCGCTGAAG
AB053256        TTGAACACGCAAAACCGACGCTGCGCCAAATTATGGCGCACTTCTCAAACCTCGCTGAAG
AJ564636        TTGAACACGCAAAACCGACGCTGCGCCAAATCATGGCGCATTTCTCAAACCTCGCTGAAG
AJ879511        TTGAACACGCAAAACCGACGCTGCGCCAGATCATGGCGCATTTCTCACATCTTGCTGAAG
AJ874695        TTGAACACGCAAAACCGACGCTGCGCCAGATCATGGCGCATTTCTCACACCTCGCTGAAG
AB054886        TTGAACACGCAAAACCGACGCTGCGCCAGATCATGGCGCATTTCTCACATCTCGCTGAAG
AJ874696        TTGAACACGCAAAACCGACGCTGCGCCAGATCATGGCGCATTTCTCACATCTTGCTGAAG
                ****************************.** ******** ******.* ** *******

AJ748256        CTTATATTGAGAAGCAAAATTTAGAGAAACCGTACATGCCTAGGTACGGCCTTCAGCGAA
AB053256        CTTATATTGAGAAGCAAAATTTAGAGAAACCGTACATGCCTAGGTACGGCCTTCAGCGAA
AJ564636        CTTATATTGAGAAGCAAAATTTAGAGAAACCGTACATGCCTAGGTACGGCCTTCAGCGAA
AJ879511        CATATATTGAGAAACAAAATGCAGAGAAACCGTACATGCCTAGGTACGGCCTTCAGCGAA
AJ874695        CTTATATTGAGAAGCAAAATTTAGAGAAACCGTACATGCCTAGGTACGGCCTTCAGCGAA
AB054886        CATACATTGAGAAACAAAATGCAGAGAAACCGTACATGCCTAGGTACGGCCTTCAGCGAA
AJ874696        CATATATTGAGAAACAAAATGCAGAGAAACCGTACATGCCTAGGTACGGCCTTCAGCGAA
                *:** ********.******  ************************************

AJ748256        ATCTCACCGACTTCAATCTAGCACGATTTGCTTTTGATTTCTATGAAGTTACTTCACGCA
AB053256        ATCTCACCGACTTCAATCTAGCACGATTTGCTTTTGATTTCTATGAAGTTACTTCACGCA
AJ564636        ATCTCACCGACTTCAATCTAGCACGATTTGCTTTTGATTTCTATGAAGTTACTTCACGCA
AJ879511        ATCTCACCGATTTCACGCGAGCACGATTCGCTTTCG-TTTCTAAGAAGTTACTTCACGCA
AJ874695        ATCTCACCGACTTCAATCTAGCACGATTTGCTTTTGATTTCTATGAAGTTACTTCACGCA
AB054886        ATCTCACCGATTTCACGCTAGCACGATTCGCTTTCGATTTCTATGAAGTTACTTCACGCA
AJ874696        ATCTCACTGATTTCACGCTAGCACGATTCGCTTTCGACTTCTATGAAGTTACTTCACGCA
                ******* ** ****. * ********* ***** *  *****:****************

AJ748256        CGCCGGCACGCGCTAAAGAAGCACACTTCCAAATGAAAACAGCCGCTTTACGCGGAAAAC
AB053256        CGCCAGCACGCGCTAAAGAAGCACACTTCCAAATGAAAACAGCCGCTTTACGCGGAAAAC
AJ564636        CGCCAGCACGCGCTAAAGAAGCACACTTCCAAATGAAAACAGCCGCTTTACGCGGAAAAC
AJ879511        CGCCCGCGCGAGCGAAGGAAGCACACTTCAGATG-AAAACAGCCGCCTTGCGCGGAAAAC
AJ874695        CGCCAGCACGCGCTAAAGAAGCACACTTCCAAATGAAAACAGCCGCTTTACGCGGAAAAC
AB054886        CGCCCGCGCGAGCGAAGGAAGCACACTTCCAGATGAAAACAGCCGCCTTGCGCGGAAAAC
AJ874696        CGCCTGCGCGAGCGAAGGAAGCACACTTCCAGATGAAAACAGCCGCTTTACGCGGAAAAC
                **** **.**.** **.************...:  *********** **.**********

AJ748256        AATCGAAGCTATTTGGGCTAGATGGAAAGGTGAACACCCAAGATGAGGACACGGAGAGGC
AB053256        AATCGAAGCTATTTGGGCTAGATGGAAAGGTGAACACCCAAGATGAGGACACGGAGAGGC
AJ564636        AATCGAAGCTATTTGGGCTAGATGGAAAGGTGAACACCCAAGATGAGGACACGGAGAGGC
AJ879511        AATCGAAGCTATTTGGGTTAGATGGAAAGGTGACCACCCAAGAAGAGGACACGGAGAGGC
AJ874695        AATCGAAGCTATTTGGGCTAGATGGAAAGGTGAACACCCAAGATGAGGACACGGAGAGGC
AB054886        AATCGAAGCTATTTGGGTTAGATGGAAAGGTGACCACCCAAGATGAGGACACGGAGAGGC
AJ874696        AATCGAAGCTATTTGGGTTAGATGGAAAGGTGACCACCCAAGATGAGGACACGGAGAGGC
                ***************** ***************.*********:****************

AJ748256        ATACAGCAGATGATGTCAATAAAAACATGCACTCCTTACTTGGAATTTCTATGTAAATCC
AB053256        ATACAGCAGATGATGTCAATAAAAACATGCACTCCTTACTTGGAATTTCTATGTAAATCC
AJ564636        ATACAGCAGATGATGTTAATAAAAACATGCACTCCTTACTTGGAATTTCTATGTAAATCC
AJ879511        ATACAGCAGACGATGTCAAAAAAAACATGCACTCCTTACTTGGAATTTCTAT-TAAATCC
AJ874695        ATACAGCAGATGATGTCAATAAAAACATGCACTCCTTACTTGGAATTTCTATGTAAATCC
AB054886        ATACAGCAGACGATGTCAATAAAAACATGCACTCCTTACTTGGAATTTCTATGTAAATCC
AJ874696        ATACAGCAGACGATGTCAATAAAAACATGCACCCCTTACTTGGAATTTCTATGTAAATCC
                ********** ***** **:************* ******************* *******

AJ748256        TCGAATAAACAGCACTAGTAACTTGCTAATATATCTATCTCGTTTATTCCGTAGTATCCT
AB053256        TCGAATAAACAGCACTAGTAACTTGCTAATATATCTATCTCGTTTATTCCGTAGTATCCT
AJ564636        TCGAATAAACAGCACTAGTAACTTGCTAATATATCTATCTCGTTTATTCCGTAGTATCCT
AJ879511        TCGAATAAACAGCACTAGTAACTTGCTAATATATCTACCTCGTTTATTCCGTAGTATCCT
AJ874695        TCGAATAAACAGCACTAGTAACCTGCTAATATATCTATCTCGTTTATTCCGTAGTATCCT
AB054886        TCGAATAAACAGCACTAGTAACTTGCTAATATATCTATCTCGTTTATTCCGTAGTATCCT
AJ874696        TCGAATAAACAGCACTAGTAACTTGCTAATATATCTATCTCGTTTATTCCGTAGTATCCT
                ********************** ************** **********************
```

```
AJ748256        ATCTATTAGCTCAACTCTTTGTGTTATAGTGTGGTTTACCATGAACGCACATTGTAGTTG
AB053256        ATCTATTAGCTCAACTCTTTGTGTTATAGTGTGGTTTACCATGAACGCACATTGTAGTTG
AJ564636        ATCTATTAGCTCAACTCTTTGTGTTATAGTGTGGTTTACCATGAACGCACATTGTAGTTG
AJ879511        ATCTTTTAGCCCAACTCTTTGTGCTATAGAGTGGTTTACCATGAACGCACATTGTAGGTG
AJ874695        ATCTATTAGCTCAACTCTTTGTGTTATAGTGTGGTTTACCATGAACGCACATTGTAGTTG
AB054886        ATCTTTTAGCCCAACTCTTTGTGCTATAGAGTGGTTTACCATGAACGCACATTGTAGGTG
AJ874696        ATCTTTTAGCCCAACTCTTTGTGCTATAGAGTGGTTTACCATGAACGCACATTGTAGGTG
                ****:***** ************ *****:*************************** **

AJ748256        AGAACCGAGCTCCTTGTTGGTGTCTAGTTGCAGAGAGGTTTCCTCGCGTCTTCTTAGCCA
AB053256        AGAACCGAGCTCCTTGTTGGTGTCTAGTTGCAGAGAGGTTTCCTCGCGTCTTCTTAGCCA
AJ564636        AGAACCGAGCTCCTTGTTGGTGTCTAGTTGCAGAGAGGTTTCCTCGCGTCTTCTTAGCCA
AJ879511        GGAACCGAGCTCCTTGTTAGTGTCTAATAGCAGAGAGGTCGCCTCGCGTCTATCTAGCCA
AJ874695        AGAACCGAGCTCCCTGTTGGTGTCTAGTTGCAGAGAGGTTTCCTCGCGTCTTCTTAGCCA
AB054886        GGAACCGAGCTCCTTGTTAGTGTCTAATAGCAGAGAGGTCGCCTCGCGTCTATCTAGCCA
AJ874696        GGAACCGAGCTCCTTGTTAGTGTCTAATAGCAGAGAGGTCGCCTCGCGTCTATCTAGCCA
                .************ ****.*******.*:********** ********** :  ******

AJ748256        CTAG-------
AB053256        CTAG-------
AJ564636        CTAG-------
AJ879511        CTAG-------
AJ874695        CTAGTCTTGGA
AB054886        CTAG-------
AJ874696        CTAG-------
                ****
```

B. Amino acid sequence alignment of partial coat protein (CP) of LMoV.

```
AJ748256        GVWLMMDGDQQVEFPLRPILEHAKPTLRQIMAHFSNLAEAYIEKQNLEKPYMPRYGLQRN
AB053256        GVWLMMDGDQQVEFPLRPILEHAKPTLRQIMAHFSNLAEAYIEKQNLEKPYMPRYGLQRN
AJ564636        GVWLMMDGDQQVEFPLRPILEHAKPTLRQIMAHFSNLAEAYIEKQNLEKPYMPRYGLQRN
AJ874695        GAWLMMDGDQQVEFPLRPIIEHAKPTLRQIMAHFSHLAEAYIEKQNLEKPYMPRYGLQRN
AB054886        GAWLMMDGDQQVEFPLRPIIEHAKPTLRQIMAHFSHLAEAYIEKQNAEKPYMPRYGLQRN
AJ874696        GAWLMMDGDQQVEFPLRPIIEHAKPTLRQIMAHFSHLAEAYIEKQNAEKPYMPRYGLQRN
AJ879511        GAWLKMDGDQQVEFPLRPEIEHAKPTLRQIMAHFSHLAEAYIEKQNAEKPYMPRYGLQRN
                *.** ************* :***************:********** **************

AJ748256        LTDFNLARFAFDFYEVTSRTPARAKEAHFQMKTAALRGKQSKLFGLDGKVNTQDEDTERH
AB053256        LTDFNLARFAFDFYEVTSRTPARAKEAHFQMKTAALRGKQSKLFGLDGKVNTQDEDTERH
AJ564636        LTDFNLARFAFDFYEVTSRTPARAKEAHFQMKTAALRGKQSKLFGLDGKVNTQDEDTERH
AJ874695        LTDFNLARFAFDFYEVTSRTPARAKEAHFQMKTAALRGKQSKLFGLDGKVNTQDEDTERH
AB054886        LTDFTLARFAFDFYEVTSRTPARAKEAHFQMKTAALRGKQSKLFGLDGKVTTQDEDTERH
AJ874696        LTDFTLARFAFDFYEVTSRTPARAKEAHFQMKTAALRGKQSKLFGLDGKVTTQDEDTERH
AJ879511        LTDFTRARFAFVSKKLLHARP-RERRKHTSDENSRLARKTIEAIWVRWKGDHPRRGHGEA
                ****. *****   ::    * * :. * . :.: *  *  : : :  *     ..  .

AJ748256        TADDVNKNMHSLLGISM
AB053256        TADDVNKNMHSLLGISM
AJ564636        TADDVNKNMHSLLGISM
AJ874695        TADDVNKNMHSLLGISM
AB054886        TADDVNKNMHSLLGISM
AJ874696        TADDVNKNMHPLLGISM
AJ879511        YSRRCQKKHALLTWNFY
                :   :*:   *
```



Discussion

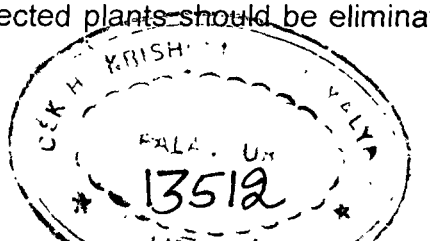
DISCUSSION

The importance of flower growing from environment and economic point of view was not understood properly till seventies. However from the last two decade it has progressed to a global industry with commercial production of flower. Tulip and lily ranked among top five potential cut flower in the world. Because of their attractive flower colours, both are grown in beds, borders and pots. Although tulip and lily have long vase life and are hardy in nature, a large variation in broken colour of flowers along with quality degradation have been observed due to various abiotic and biotic factors. The biotic factors include bacteria, fungi, nematodes and viruses. Of these entire factors, viruses are most economically important factors. Due to vegetative propagation of the plant, the various viruses once infect the plants perpetuate long way.

5.1 *Tulip breaking virus* (TBV)

The various viruses found infecting tulips are CMV, LSV, LMoV, TuMV, TBV and SLRSV (Cohen *et al.*, 1985). Among these viruses, the most important ones are TBV, CMV and LSV (Dekker *et al.*, 1993). These viruses affect economically the quality and yield of cut flower (Lawson, 1981; Raju and Olsen, 1985). Virus infection causes irregular mottling and flecking of leaves, distorted and twisted growth, colour breaking in flower along with reduced plant size.

Tulip plants can be freed effectively from TBV by meristem tip culture combined with heat treatment. Virus tested tulips should be cultivated in insect proof greenhouses and infected plants should be eliminated as soon as possible.



Since TBV sometime occurs symptomless, visual examination is not reliable. Therefore, there is a need to develop some more molecular or serological detection techniques to identify virus infected plants.

5.1.1 Natural infection

Virus infected plants of tulip in the experimental fields of Institute of Himalayan Bioresource and Technology, Palampur were found to exhibit mottling of leaves along with reduced plant size. Although various symptoms such as mosaic of leaves and colour breaking in flower were observed in accordance to Phillips (1986) still most infected cultivars show neither leaf symptoms nor loss of vegetative vigour. Van Slogteren (1971) also observed that white and yellow varieties of tulip were incapable of breaking because they lack anthocyanin as their colour being determined by colourless or yellow plastids in the mesophyll layer.

5.1.2.1 Nucleic acid based detection

The most sensitive detection technique has found to be the PCR amplification, which could detect as little as 0.1 pg of RNA extracted from purified virus equivalent of 0.5 pg of infected leaf tissue (Antonia *et al.* 1997). We used the CP gene specific primers of TBV for the amplification of gene, which were already designed on the basis of other available CP gene sequences of TBV from GenBank. RT-PCR resulted in the amplification of ~700 bp gene which includes partial coat protein sequence plus some sequence of inclusion body protein gene. Samples of virus infected tulip collected from various districts of Himachal Pradesh were tested by RT-PCR, out of which Chamba, Solan, Sundernagar along with three other isolates from experimental fields of IHBT, Palampur found positive for TBV. Langeveld *et al.* (1991) also had used local areas of conserved amino acids sequences of the replicase and coat protein gene of potyvirus to select nucleotides

sequence for the construction of sets of degenerate oligonucleotides primers for amplification of DNA fragments.

5.1.2.2 Cloning

The PCR amplified products were ligated into pGEM-T Easy vector with T overhangs and *E.coli* cells were transformed. The recombinant clones were checked by restriction digestion method for TBV. The clone of Chamba isolate was sequenced in automated sequencer (IHBT) and analyzed.

5.1.2.3 Sequence analysis

In BLAST search, the sequence of amplified gene of TBV from tulip from Chamba revealed that the sequence contain partial inclusion body protein gene along with partial CP gene. Amplified inclusion body shows 99% homology while partial CP gene showed 100% homology and the deduced amino acid sequence of both partial inclusion body protein gene and partial CP genes showed 98% homology with the similar *Tulip mosaic virus* sequences (Acc. no X63630) from database. Change of amino acids from aspartate (polar, negatively charged) to glycine (non-polar, aliphatic) at 36^{th} position and histidine (polar, positively charged) to tyrosine (non-polar, aromatic) at position 14^{th} of inclusion body protein may play some vital role and it need to be further characterized. High homology of CP gene and amino acid sequence at 5'end (amplified portion) indicates the common origin of virus. Less variability at 5' end of CP was analysed with a change in single amino acid from threonine (polar, uncharged) to lysine (polar, positively charged) at 23^{rd} position.

cDNA synthesis, cloning and subsequent sequencing of 3'-terminus of TBV had also been reported by Ohira *et al.* (1994). He revealed that this sequence contains one open reading frame (ORF), followed by an untranslated region of 225

nucleotides and a poly (A) tail. The deduced amino acid sequences include C-terminus of the predicted RNA dependent RNA polymerase and the coat protein.

5.1.3 Cleaved amplified polymorphism

To check the scenario of TBV in tulip cultivars grown in HP, samples from Chamba, Solan, Sundernagar and Palampur were collected from which TBV CP gene was amplified and cloned. As we know sequencing is much more reliable and accurate but also a costly affair for differential studies of various isolates of TBV. To check the homology between different clones restriction digestion analysis of amplified product was performed (CAP) with three 4 bp cutters (such as Hae-III, Taq-I and Alu-I). One restriction enzyme analysis may not be productive enough so we used two other enzymes. Restriction pattern with these enzymes such as Taq-I and Alu-I was able to differentiate between various isolates of TBV depending on their sequence variability. This method is useful enough for differential studies of various isolates.

5.2 *Lily mottle virus* (LMoV)

5.2.1 Natural infection

Infected plants of lily in the experimental field of Floriculture Division (IHBT, Palampur) found to exhibit mosaic pattern on leaves. It was also observed that symptoms were more severe in mixed infection of LMoV and LSV in comparison when they get infected by LMoV alone. Although Lisa *et al.* (2002) has considered LMoV as an important virus of lily that also cause flower breaking in tulips, having natural host range restricted to family Liliaceae.

5.2.2 Serology based detection techniques

5.2.2.1 DAS-ELISA

Lily mottle virus (LMoV) is highly immunogenic so serological method can be a reliable method of detection. ELISA has been found to be best way for large scale routine testing of viruses due to its rapid, cost effective, simple and fast assay properties. Since the development of ELISA, it is one of the most popularly used techniques till today for detection of viruses and routine testing of bulbs (Zaidi *et al.,* 1993; Zaidi and Mukharjee, 1991). We used DAS-ELISA for LMoV detection in different lily cultivars. Out of 30 lily cultivars, nine were found positive for LMoV, which includes four Asiatic hybrid lily cultivars such as London, Romano, Alaska, Nova Cento; two Oriental hybrid lily cultivars such as Galieli, White Mero Star; and three other lilies such as Spider lily, *Lilium longiflorum* and *L. tigrinum.* Derks *et al.* (1994) reported that LMoV was distinguishable from TBV isolates with antisera prepared against potyvirus, inducing colour breaking in tulip. Niimi *et al.* (1999) reported the use of dot immunobinding assay (DIBA) and indirect ELISA to detect TBV-L in the scale segments of *Lilium**Enchantment. In our experiment even the plants not showing the visible symptoms were found positive by DAS-ELISA, which shows that serological detection has an advantage over morphological detection based on symptomology.

Sometimes it may have the limitation of low concentration of the virions in virus infected sample. Routine use of ELISA is the best technique available till date for viral pathogen detection, which is easy, economical in use and most widely adapted by growers worldwide. In the Netherlands, for large scale routine testing, a DAS-ELISA with polyclonal antiserum is generally used (Bowen and Zaayen, 1995). Asjes *et al.* (2000) also reported the inefficiency of detection of LMoV in bulbs of oriental hybrids, which was found to be practically true.

5.2.2.2 Northern Hybridization

The detection limits for ELISA procedure is ng of purified virus. Nucleic acid hybridization with either chemiluminescent or chromogenic substrates was found slightly more sensitive at detecting virus in tissue extracts (25 ng of infected tissue).Northern hybridization has found to detect virus concentration in pg compared to µg in ELISA. Northern hybridization with specific DNA probes is an alternative technique for identification and detection of viruses. It can help in further characterization of pathogens (Boonekamp *et al.* 1990). To screen different lily cultivars for LMoV, we used cloned LMoV CP gene (~700bp) as radioactive probe. Out of 15 lily samples, three (Corida, Brunello and *L. longiflorum*) were found positive for LMoV.

5.2.3 Sequence based detection technique

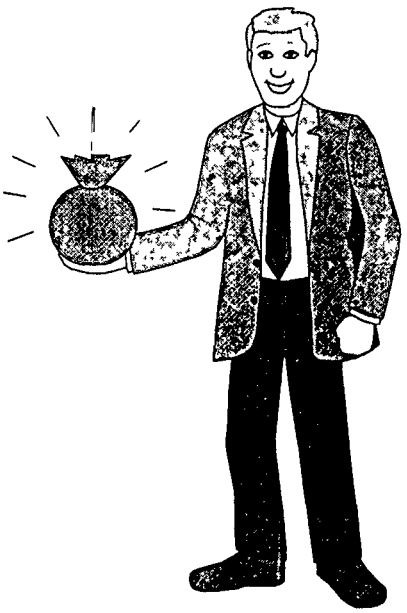
5.2.3.1 Amplification of LMoV CP gene

Limitation of ELISA to detect the virus may be overcome by the use of RT-PCR technique, which is more sensitive than ELISA and can detect very low concentration of virions. Niimi *et al.* (2003) also reported that RT-PCR is the more sensitive methods than ELISA to determine the presence of viruses in various *Lilium* spp. In our experiments, some plants which were not positive for DAS-ELISA, gave positive signal in RT-PCR showing that RT-PCR is more reliable than ELISA based detection. So group specific primers of potyvirus coat protein gene were used for amplification of partial CP gene of LMoV from total RNA isolated from lily. Amplicon of ~ 700 bp was observed on agarose gel in virus infected lilies (London, White Mero Star and *L. tigrinum).* Kim *et al.* (2001) had reported that virus detection on lily by one step RT-PCR was more rapid and reliable than by the conventional RT-PCR methods.

5.2.3.2 Cloning and sequencing

Amplified product (~700bp) from tiger-lily was cloned in pGEM-T Easy vector (Promega, USA). Selection was done on LB agar plates supplemented with ampicillin. Restriction digestion (with *Eco* RI) method was used to confirm the presence of ligated CP genes in plasmids. Cloned LMoV CP gene obtained from tiger-lily was sequenced using T7 and SP6 primer in automated sequencer (ABI prism 310).

The nucleotide and translated sequences (by use of Expasy tools obtained were multaligned with other available sequences from database. Partia sequence of CP gene of LMoV was submitted in GenBank with accession numbe AJ879511. There is a report that analysis of coat protein sequences of LMo\ isolates revealed two subgroups, corresponding to the earlier 'TBV-lily strain' anc 'TBBV' isolates (Zheng *et al.*, 2003). In our experiments on BLAST search, thi partial CP gene sequence (Accession no AJ879511) showed 90-97% homology while amino acid sequence showed 68-73% homology with available sequence from database with overall change of polarity of CP from non-polar to polar. Thi change need to be further characterized for detailed analysis of CP gene.



Summary

SUMMARY

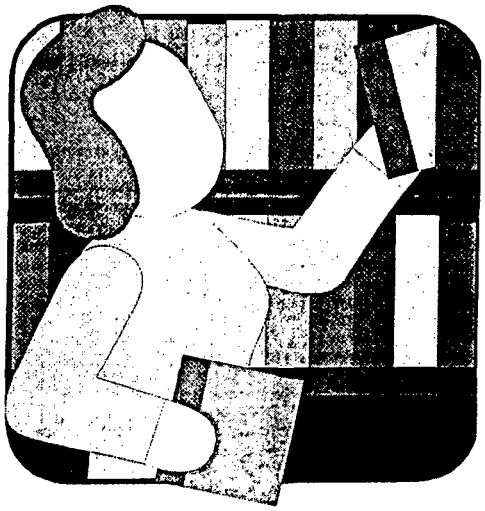
Lily (*Lilium* spp.) and tulip (*Tulipa* spp.) are popular ornamental bulbous plants regarding their trade in International floriculture market. Because of their attractive flower colour, they are grown in beds, borders and pots. Tulip and lily are infected by number of diseases caused by viruses which causes significant losses in their cut flower production. One of the major group of viruses infecting lily and tulip is the potyvirus group.

Potyviruses are single stranded, monopartite and positive RNA genome with particle length of 750-770 nm. Important potyviruses of tulip and lily are TBV, TBBV, TTBV, RTBV and LMoV. Out of these TBV and LMoV are aphid-borne viruses which economically affect the quality and yield of cut flower.

Lily samples procured from Palampur were screened with DAS-ELISA for LMoV. Out of 30 lily cultivars, nine were found positive for LMoV, which includes four Asiatic hybrid lily cultivars such as London, Romano, Alaska, Nova Cento; two Oriental hybrid lily cultivars such as Galieli, White Mero Star; and some other lilies such as Spider lily, *Lilium longiflorum* and *L. tigrinum*. Northern hybridization was also used for screening 15 lily isolates for LMoV, out of which 3 (Asiatic lily cvs. Corida, Brunello and *L. longiflorm*) found positive for LMoV using cloned LMoV cp gene was used as radioactively labeled probe. In Tiger lily (Palampur) partial CP gene of LMoV along with some 3'UTR region on RT-PCR with group specific primers, gave an amplicon of ~700 bp. On BLAST search, this partial CP gene sequence (Accession no AJ879511) showed 90-97% homology, while amino acid sequence showed 68-73% homology with available sequences from database.

In tulip isolates, partial TBV CP gene along with some inclusion body protein gene was amplified (~700 bp) with gene specific primers. This amplified product was cloned in pGEM-T Easy vector and sequenced (Accession no AM048878). In BLASTN search the sequences of inclusion body protein of TBV (Chamba) showed 99% homology, while CP gene of this isolate showed 100% homology with those of other available sequences from database. The deduced amino acid sequence of both inclusion body protein and coat protein showed 98% homology with amino acid of available sequences from database.

Scenario of potyvirus (es) in tulip samples from different parts of HP (Chamba, Solan, Sundernagar and Palampur) were studied by Cleaved amplified polymorphism. Amplified DNA was digested with available 4-basepair cutters and restriction pattern produced, was analyzed. Four bp cutters used were Hae-III, Taq-I and Alu-I. It was observed that restriction pattern with Taq-I and Alu-I restriction enzymes can differentiate various isolates of TBV. Restriction patterns observed in different tulip cultivars during Restriction digestion identify virus as TBV and also show high variability in all TBV isolates of Himachal Pradesh.



Literature Cited

LITERATURE CITED

Aarnes, H. 1994. *Tulip breaking virus*. Blyttia 52: 29-31.

Allen, T.C. 1975. Viruses of lilies and their control. Acta Horticulturae 47: 69-75.

Antonia, R., Figueira, D., Fitopatologia, U.F.L.A., Lavras, M.B., Leslie, L.. and Domier, U.S.D.A. 1997. Comparison of Techniques for Detection of *Barley Yellow Dwarf Virus*. Plant Disease 81:1236-1240.

Asjes, C.J. 1978. Viruses and viral diseases in tulips. Bloembollencultuur 88: 942-946.

Asjes, C.J., Barnhoorn, G.J.B. and Van Schadewijk, A.R. 2002. Effect of seasonal detection of LSV and LMoV on aphid-borne virus spread in *Lilium* in The Netherlands. Acta Horticulturae 568: 201-207.

Asjes, C.J., Barnhoorn, G.J., Piron, P.G.M., Harrewijn, P., Oosten, A.M., Van Oosten, A.M., Loebenstein, G., Hammond, J., Gera, A., Derks, A.F.L.M. and Zaayen, A. 1996. Control review of air-borne *Tulip breaking virus* and *Lily symptomless virus* in *Lilium* in The Netherlands. Acta Horticulturae 432: 290-297.

Asjes, C.J., Vos, M.P. and Van Slogteren, D.H.M. 1973. Brown ring formation and streak mottle, two distinct syndromes in lilies associated with complex infections of *Lily symptomless virus* and *Tulip breaking virus*. Netherlands Journal of Plant Pathology 79: 23-35.

*Beattie, D.J. and White, J.W. 1993. *Lilium* hybrids and species. *In:* The physiology of flower bulbs (eds. Hertogh, A.D. and Nard, M.L.). Elesvier Amsterdam 423-454.

Bellardi, M.G. and Bertaccini, A. 2001. First report of *Turnip mosaic virus* in lily. Journal of Plant Pathology 83: 69.

Bellardi, M.G., Nanni, G., Bertaccini, A. and Hammond, J. 2002. Old and new Viruses of lily in Italy. Acta Horticulturae 568: 215-220.

Bertaccini, A. and Mariani, F. 1982. Electron microscopy of two viruses and mycoplasma like organisms in lilies with deformed flowers. Phytopathologia Mediterranea 21: 8-14.

Boonekamp, P.M., Asjes, C.J., Derks, A.F.L.M., Van Doorn, J., Franssen, J.M., Linde, P.C.G.V., Vlugt, C.I.M.V., Bol, J.F., Van Gemen, B., Linthorst, H.J.M., Memelink, J. and Van Schadewijk, A.R. 1990. New technologies for the detection and identification of pathogens in bulbous crops with immunological and molecular hybridization techniques. Acta Horticulturae 266: 483-490.

Bouwen, I. and Van der Vlugt, R.A.A . 2000. Natural infection of *Alstroemeria brasiliensis* with *Lily mottle virus*. Plant Disease 84: 103.

Brandes, J. and Bercks, R. 1965. Gross morphology and serology as a basis for the classification of elongated plant viruses. Advances in Virus Research 11: 1-22.

Brierley, P. and Smith, F.F. 1944. Studies on lily viral diseases: mottle group. Phytopathology 34l: 718-746.

Brown, T. A. 1995. Cloning vectors based on *E. coli* plasmids. *In:* Gene cloning. (eds. Brown, T.A). Oxford 104-110.

Cayley, D.M. 1932. *Tulip breaking virus. In*: Brunt, A.A., Crabtree, K., Dallwitz, M.J., Gibbs, A. and Watson, L. Viruses of Plants. CAB International UK 1331-1333.

Chang, C.A., Chen, C.C., Deng, T.C., Chen, Y.W., Huang, S.C., Shen, C.S., Tsai, S.H. and Yih, M.S. 1998. Current status of lily virus research and the development of its detection techniques in Taiwan. Taichung District Agricultural Improvement Station 40: 151-158.

Chen. J., Chen, J. P. and Adams, M. J. 2002. Characterization of some Carla and Potyviruses from bulb crops in China. Archive Virology 147: 419-428.

Christie, R.G. and Edwardson, J.R. 1985. Light microscopy of plants virus induced inclusions. Acta Horticulturae 164: 119-130.

*Clark, M.F. and Adams, A.N. 1977. Characteristics of the microplate method of Enzyme Linked Immunosorbent Assay (ELISA) for the detection of plant viruses. Journal of General Virology 34: 478-483.

Cohen, D., Milne, K.S. and Hylano, M.I. 1985. *In vitro* manipulation of virus concentration in hybrid lilies. Acta Horticulturae 164: 319-324.

Corpet, F. 1988. Multiple sequence alignment with hierarchial clustering. Nucleic Acids Research 16: 10881-10890.

Dash, M. 1999. TulipoMania. Tulip breaking: Past, present and future (eds. Lesnaw, J.A. and Ghabrial, S.A.). Plant Disease 84: 1052-1060.

Dekker, E.L., Derks, A.F.L.M., Asjes, C.J., Lemmers, M.E.C., Bol, J.F. and Langeveld, S.A. 1993. Characterization of potyviruses from tulip and lily which causes flower breaking. Journal of General Virology 74: 881-887.

Derks, A.F.L.M. 1992. Some unusual serological reactions among potyviruses. Archives of Virology. Supplementum 5: 77-79.

Derks, A.F.L.M., Lemmer, M.E.C., Gemen, B.A., VanGemen, B.A., Mokra, V. and Brunt, A. 1994. *Lily mottle virus* in lily: Characterization and its differentiation from TBV in tulips. Acta Horticulturae 377: 281-288.

Derks, A.F.L.M., Lemmers, M.E.C. and Hollinger, T.C. 1997. Detectability of viruses in lily bulbs depends on virus, host and storage conditions. Acta Horticulturae 430: 633-640.

Derks, A.F.L.M., Vink Van den Abeele, J.L. and Van Schadewijk, A.R. 1982. Purification of *Tulip breaking virus* and production of antisera for use in ELISA (Enzyme-linked immunosorbent assay). Netherlands Journal of Plant Pathology 88: 87-98.

Eikelboom, W., Van Eijk, J.P., Peters, D. and Van Tuyl, J.M. 1992. Resistance to *Tulip breaking virus* in tulip. Acta Horticulturae 325: 631-636.

Franssen, J.M. and Van der Hulst, C.T. 1986. The possible application of monoclonal antibodies in ELISA for detection of TBV. Acta Horticulturae 177:111-114.

Hammond, J. and Chastagner, G.A. 1988. Natural infection of tulips with *Turnip mosaic virus* and another potyvirus isolate distinct from *Tulip breaking virus* in the USA. Acta Horticulturae 234: 235-242.

Hammond, J. and Chastagner, G.A. 1989. Field transmission of *Tulip breaking virus* and serologically related potyviruses in tulip. Plant Disease 73: 331-336.

*Hari, V., Siegel, A., Rozek, C. and Timberlake, W.E. 1974. The RNA of *Tobacco etch virus* contains poly (A). Virology 92: 568-571.

*Hill, J.H. and Benner, H.I. 1976. Properties of potyvirus RNAs: *Turnip mosaic virus, Tobacco etches virus* and *Maize dwarf mosaic virus*. Virology 75: 419-432.

*Holmes, D.S. and Quingley, M. 1981. A rapid boiling method for the preparation of bacterial plasmids. Annals of Biochemistry 114: 193-197.

*Hoog, M.H. 1973. On the origon of Tulip, Lilies and other Liliaceae. Royal Horticulture Society, London 47-64.

Hsu, H.T., Franseen, J.M., Van der, Hulst, C.T.C., Derks, A.F.L.M. and Lawson, R.H. 1988. Factors affecting selection of epitope specificity of monoclonal antibodies to *Tulip breaking potyvirus*. Phytophthology 78: 1337-1340.

*Hughes, M. 1931. *Tulip breaking virus*. Review of Applied Mycology (Abs) 36: 305.

Jeong, K.S., Hahn, Y., Shin, K., RyuYeol, R.S. and Lim Y.D. 2000. Occurrences of viruses in lilies (*Lilium* spp.) in highland area and their detection by one-step RT-PCR. Research in Plant Disease 7: 80-85.

Kim, S.J., Hahm, Y., Shin, K.Y., Ryu, S.Y., Jung, H.W., Choi, J.K. and Lim, Y.D. 2001. Occurrence of viruses in lily in highland areas and their detection by one step RT-PCR. Research in Plant Diseases 7: 80-85.

Kim, J.Y., Han, Y.H., Lee, H.S., Kim, S.J. and Ra, Y.J. 1998. Occurrence of lily viruses and damage of viral diseases in oriental lilies. RDA Journal of Crop Protection 40: 68-65.

Langeveld, S.A. 1991. Identification of potyvirus using the polymerase chain reaction with degenerate primers. Journal of General Virology 72: 1531-1541.

Lawson, R.H. 1981. Controlling virus diseases in major national flower and bulbs crop. Plant Disease 65: 780-786.

Lee, J.S., Nou H.S., Hong, D.K., Lee, K.K., Lee, Y.S. and Choi, J.K. 1998. Detection of viral diseases in *Lilium orientalis* plants using RT-PCR techniques. Journal of Crop Protection 40: 50-56.

Lesnaw, J.A. and Ghabrial, S.A. 2000. Tulip breaking: Past, present and future. Plant Disease 84: 1052-1060.

Lisa, V., Vetten, H.J., Lesemann, D.E. and Must, P. 2002. Occurrence of *Lily mosaic virus* in Escarole. Plant Disease 86: 329.

McWhorter, F.D. and Brierley, P. 1963. The principal virus disease of lilies. The lily yearbook of the North America lily Society 16: 89-106.

Mowat, W.P. 1985. *Tulip chlorotic blotch virus*, a second potyvirus causing tulip flower break. Annals of Applied Biology 106: 65-73.

*Mullis, K., Faloona, F., Scharf, S., Saiki, R., Horn, G. and Erlich, H. 1986. Specific enzymatic amplification of DNA *in vitro*. The Polymerase Chain Reaction. Quantitative Biology 51: 263.

Muramatsu, Y. 1971.Studies on the tulips inoculated with *Tulip mosaic virus:* Relation between tulip varieties and some breaking types. Seiken Ziho 22: 7-13.

Niimi, Y., Gondaira, T., Kutsuwada, Y. and Suji, H. 1999. Detection by ELISA and DIBA test of *Lily symptomless virus, Tulip breaking virus*-lily (TBV-L) and *Cucumber*

mosaic virus (CMV) in *Lilium* spp. grown in the field. Journal of the Japanese Society for Horticulture Science 68: 176-183.

Niimi, Y., Han, D.S., Mori, S., Kobayashi, H. and Han, D.S. 2003. Detection of *Lily symptomless virus* and *Lily mosaic virus* in *Lilium* spp. by RT-PCR technique. Scientia Horticulturae 97: 57-63.

Ohira, K., Namba, S., Miyagawa, M., Kusumi, T. and Tsuchizaki, T. 1994. Nucleotide sequences of the coat protein coding region of *Tulip breaking virus*. Virus Genes 8: 165-167.

Park, K., Choi, J.D., Park, I.S., Eum, S.J. and Kim, K.W. 2003. Virus infected status in imported bulbs of *Lilium* oriental hybrid. Korean Journal of Horticultural Science and Technology 21: 57-61.

Phillips, S. 1986. *Tulip breaking potyvirus. In:* Viruses of plants-description and list from the VIDE database (eds: Brunt, A.J. and Watson, L.). 1331-1333.

Raju, B.C. and Olsen, C.J. 1985. Indexing systems for producing clean stocks for disease control in commercial floriculture. Plants disease 69: 189-192.

Romanow, L.R., Van Eijk, J.P. and Eikelboom, W. 1986. Investigating resistance in tulip to *Tulip breaking virus* and its transmission. Acta Horticulturae 177: 235-239.

Romanow, L.R., Van Eijk, J.P., Eikelboom, W., Schadewijk, A.R., Peters, D., Van Eijk, J.P. and Van Schadewijk, A.R. 1991. Determing levels of resistance to *Tulip breaking virus* (TBV) in tulip (*Tulipa* L.) cultivars. Euphytica 51: 273-280.

Se, T. and Kanematsu, S. 2002. First report of *Tulip band breaking virus* in mosaic diseased tulip in Japan. Plant Disease 86: 1405

Sharma, A. 2002. Identification, isolation and characterization of Lily viruses. Ph.D. thesis. Guru Nanak Dev University, Amritsar.

*Singh, R.K. 1999. Clonal propagation of virus free *Lilium* spp. Ph.D. thesis. C.S.J.M. University, Kanpur.

Straathof, T.H.P., Eikelboom, W., Van Tuyl, J.M. and Peters, D. 1997. Screening for TBV resistance in seedling populations of *Tulipa* L. Acta Horticulturae. 430: 487-494.

Van Schadewijk, A.R. 1986. Detection of TBV and LSV in lily bulbs by means of ELISA. Acta Horticulturae 177:121-128.

Van Schadewijk, A.R. and Eggink, J. 1980. Detection of *Tulip breaking virus* in tulips by the enzyme-linked immunosorbent assay (ELISA). Acta Horticulturae 110:89-90

Van Slogteren, D.H.M. 1971. *Tulip breaking virus*. CMI/AAB description of plant viruses 71.

Van Slogteren, D.H.M. and Asjes, C.J. 1970. Virus diseases in tulip. Daffodil and tulip Yearbook 35: 85-97.

*Van Slogteren, D.H.M. and de Vos, N.P. 1966. In:Viruses of Plants (eds: Beemster, A.B.R. and Dijkstra, J.). 320.

*Villemson, S.V. 2001.Virus infection of lilies. Zashchita Karantin Rastenii 4: 36-37.

Ward, C.W. and Shukla, D.D. 1991. Taxonomy of potyviruses; Current problems and some solutions. Intervirology 32: 269-296.

Yamaguchi, A.1963. Current season symptoms in tulip flowers by *Tulip breaking virus*. Phytopathology 249: 355-361.

Yamaji, Y., Lu, X.Y., Kagiwada, S., Oshima, K., Namba, S. and Lu, X.Y. 2001.Molecular evidence that a lily-infecting strain of *Tulip breaking virus* from Japan is a strain of *Lily mottle virus*. European Journal of Plant Pathology 107: 833-837.

Zaidi, A.A. and Mukherjee, D. 1991. Immune-detection of plant viruses and their utility for production of virus-free ornamental plants. *In*: Biotechnology in crop protection (eds: Sen, C. and Dutta, S.). Kalayani Publication 93-101.

Zaidi, A.A., Ram, R., Zaidi, S.N.H. and Mukherjee, D. 1993. Diagnosis of viruses in some ornamental plants with special reference to serological methods: New developments. Indian Review of Life sciences 13: 157-174.

*Zheng, H.U., Chen, J., Zhao, M.F., Lin, L., Chen, J.P., Antoniw, J.F. and Adams, M.J. 2003. Occurence and sequences of *Lily mottle virus* and *Lily symptomless virus* in plants grown from imported bulbs in Zhejiang province. China. Archive Virology 148: 2419-2428.

* Originals are not seen.

APPENDIX

APPENDIX

Reagents used in DAS-ELISA:

1. Sample extraction buffer

Sodium sulphite (anhydrous)	13%
Polyvinyl pyrolidone (PVP)	2%
Sodium azide	0.02%
Powdered egg (chicken) albumin	0.2%
Tween 20	2%

pH was set to 7.4 before making the final volume to 100 ml in 1X PBST.

2. Coating buffer

Sodium carbonate (anhydrous)	0.159%
Sodium bicarbonate	0 293%
Sodium azide	0.02%

pH was adjusted to 9.6 before making the final volume to 100 ml with water.

3. PBST (phosphate buffer saline and Tween-20)

Sodium chloride	0.8%
Sodium phosphate dibasic (Na_2HPO_4 anhydrous)	0.115%
Potassium chloride	0.02%
Tween 20	0.05%
Potassium phosphate monobasic (KH_2PO_4 anhydrous)	0.02%

pH was adjusted to 7.4 before making up the volume to 100 ml with water.

4. ECI buffer

Bovine serum albumin (BSA)	0.2%
Polyvinyl pyrolidone (PVP)	0.2%
Sodium azide	0.02%

pH was adjusted to 7.4 before making the final volume 100 ml with 1x PBST and and stored at 4°C.

5. Substrate (p-nitro phenyl phosphate) buffer, pH 9.8

Magnesium chloride	0.01%
Sodium azide	0.02%
Diethanolamine	9.7%

Volume was adjusted to 100 ml with water.

Reagents used in agarose gel electrophoresis:

Sample loading dye (6X)

Ficoll 400	15%
Bromophenol blue	0.25%
Xylene cyanol	0.25%

TAE buffer (50X, per lit):

Tris base	242 g
Glacial acetic acid	57.1 ml
EDTA (0.5 M, pH 8)	100 ml

Reagent used in plasmid isolation:

STET buffer: 8% sucrose, 0.5% Triton X-100, 50 mM Tris-HCl (pH 8.0), and 50 mM EDTA (pH 8.0). Add Triton X-100 after autoclaving rest of the components in solution.

Reagent used in Northern hybridization:

Denaturation Solution: 1M NaCl and 0.5M NaOH.
Neutralizing Solution: 1.5 Tris HCl (pH 8) and 3M NaCl.
SSC (20x): 3M NaCl and 0.3M Trisodium citrate.
dNTP MIX (for -p dCTP as radioactive molecule, from 100 mM stock of d ATP, dGTP and dGTP):1µl+1µl+1µl+27µl water. 4.5µl of this mix was used for one reaction.
Buffer A: 500 Mm Tris HCl (pH 7.5), 500mM NaCl, 5mM EDTA and 0.5%SDS.