# STUDIES ON INHERITANCE OF IMMUNITY IN DIFFERENT VARIETIES OF COWPEA, {*Vigna unguiculata* (L.) Walp.} AGAINST BLACKEYE COWPEA MOSAIC VIRUS.

BY

**MUHAMMAD ARSHAD**
**B.Sc. (HONS.) AGRI.**

**A THESIS SUBMITTED TO**

THE QUAID-I-AZAM UNIVERSITY IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
**MASTER OF PHILOSOPHY IN BIOLOGY**
(GENETICS)

**DEPARTMENT OF BIOLOGICAL SCIENCES**
**QUAID-I-AZAM UNIVERSITY**
**ISLAMABAD**
**1996**

O, ALLAH,

OPEN OUR EYES,

TO SEE WHAT IS BEAUTIFUL,

OUR MIND TO KNOW WHAT IS TRUE,

OUR HEARTS TO LOVE WHAT IS

GOOD

# *CERTIFICATE*

*This thesis submitted by* **Muhammad Arshad** *is accepted in its present form by the Department of Biological Sciences as satisfying the thesis requirement for the degree of Master of Philosophy in Biology (Genetics).*

***Internal Examiner:*** A.S. Qureshi

***External Examiner:*** Anwar Hussain

***Chairman :*** _____

***Dated :*** 2-4-1996

DEDICATED

TO

MY LOVING PARENTS

*The toil and sweat affectionate*

*parents as moral support enshirned*

*and grafted in the untiring to*

*get on to higher ideals of life*

MY BROTHER

*For his love, patience,*

*encouragement and understanding*

*what inspired me to accomplish*

*this humble effort*

MY LOVING

SONS

# CONTENTS

# PART 1

# ACKNOWLEDGMENTS

Up and above every thing "All Glory is to the Almighty Allah". Who is merciful and benevolent and whose bountiful blessings and exaltation flourished my thoughts and thrived my ambitions to have the cherish fruit of my modest efforts in the form of this write up from the blooming spring and blossoming knowledge and all respects to his Holy Prophet Hazrat Muhammad (peace be upon him) enlighting our conscience with the essence of faith in Allah, converging all the kindness and upon him.

I wish to express my grateful appreciation and indebtedness to Professor Dr. Afsari Sharif Qureshi, Department of Biological Sciences, Quaid-i-Azam University, Islamabad, for her immensely encouraging and constructively critical suggestions through the course of the investigation and meticulously going through the manuscript.

I have immense heartiest pleasure in expressing my gratitude to Dr. Bashir Ahmad Malik, Chief Scientific Officer/National Coordinator (Pulses Research Institute), National Agricultural Research Centre, Islamabad, for his fatherly love, valuable guidance, skillful suggestions, sympathetic attitude, keen interest and constructive criticism during my research work and also for providing ever possible facilities for research work. May Al-mighty Allah shower his kind and numerous blessings on Dr. Malik and enable him to steer the boat of pulses research to the height of pedestal.

I express my deep sense of gratitude to Dr. Muhammad Bashir, Senior Scientific Officer, (Pulses Research Institute), National Agricultural Research Centre, Islamabad under whose dynamic supervision, propitious guidance, illustrious advice, keen interest, philanthropic attitude and constructive criticism, the research work presented in this dissertation even in the midst of his multifarious duties. I cannot restrain myself from saying to him "May your light be long my guide".

I am taking this opportunity to express my heartfelt and respect to Dr. Abdul-Hafeez, Chairman, Department of Biological Sciences, Quaid-i-Azam University, Islamabad, for his dynamic, invaluable and immaculated appraisal of the manuscript.

I am extremely grateful to Dr. Ahmad Bakhsh Mahar, SSO. for his keen interest and encouragement during research work. I have no words to place on record for deepest sense of gratitude for Dr. A. M. Haqqani, M. Raiz Malik, , Dr. M. Tahir,

Dr. M. Ashraf ., Mr. M. Zubair, Mr. Abdul Ghafoor, Mr. and Mrs Khalique, Dr. Shaukat, and Mr. M. Anwar. I am also thankful to Mr. M. Zaman, M. Haq Nawaz and Mr. A. Hameed for their best cooperation during my study period.

With deep sense of honor and appreciation are also extend to Dr. Zahoor Ahmad, Dir./PSO. PGRI, and Mr. Zafar Riaz, and all other staff of PGRI. for their help and cooperation and brotherly behavior throughout my research work.

Although too numerous to mention individually, I extend my thanks to all well wishers, friends M. Aziz Ch., Wajid Ali, Rashid K., Qaisar M., my colleague Shazia and Ruqia S., and, M. Munir, M. Ashfaque, Ahmad Khan and M. Shafiq, who were alway helpful for me during my study.

Last but not least, I am gratefully indebted to my parents, brother & his family, cousins, my wife and sons for their love, immense orison, inspiration, well wishing, cooperation and keen interest, while hearten me to achieve success in every sphere of life.

**MUHAMMAD ARSHAD**

## ABBREVIATIONS

| | | |
|---|---|---|
| 1. | BCMV | bean common mosaic virus. |
| 2. | BlCMV | blackeye cowpea mosaic virus. |
| 3. | BC1 | back cross one. |
| 4. | CMV | cucumber mosaic virus. |
| 5. | CPYMV | cowpea yellow mosaic virus. |
| 6. | CABMV | cowpea aphid-borne mosaic virus. |
| 7. | DAC-ELISA | direct antigen coating; enzyme-linked immunosorbent assay. |
| 8. | DAS-ELISA | double antibody sandwish; enzyme-linked immunosorbent assay. |
| 9. | F1 | first filial generation. |
| 10. | F2 | second filial generation. |
| 11. | IITA | International Institute for Tropical Agriculture. |
| 12. | IRRI | International Rice Research Institute. |
| 13. | NS | Non-significant. |
| 14. | NARC | National Agricultural Research Centre. |
| 15. | OD | Optical density. |
| 16. | PGRI | Plant Genetic Resources Institute. |
| 17. | PBS | Phosphate buffer saline |
| 18. | PVP | Polyvinyl pyrrolidone |
| 19. | RNA | Ribosomal Nucleic Acid. |
| 20. | USA | United State of America. |
| 21. | US | United State. |
| 22. | $X^2$ | Chi-square. |

| S. No. | LIST OF FIGURES | PAGES |
|---|---|---|

S. No. LIST OF TABLES PAGES

S. No. LIST OF FIGURES PAGES

# PART 2

# CHAPTERS

# ABSTRACT

# ABSTRACT

Among the important viruses which infect cowpea {*Vigna unguiculata* (L.) Walp.} under field conditions blackeye cowpea mosaic virus (BlCMV) is more serious than others and can cause economic losses when occurs in epidemic proportions. Several approaches have been recommended to control BlCMV, but the use of resistant varieties is effective, economical and environmentally safe. Keeping in view the importance of resistant genes and their use in effective breeding programme for the development of resistant cowpea cultivars, this study was conducted with the objective to determine the genetic basis of immunity in the following six ccowpea lines; IT86F-2089-5, IT86D-880, IT90K-76, IT86D-1010, IT86F-2062-5 and BP1CP3.

All the experiments conducted in this study were carried out in insect-free greenhouse. Six immune and one susceptible cowpea lines were included as parents to make crosses. Direct, reciprocal and back crosses were attempted between the immune and susceptible parents to have F1 progeny. A pure culture of BlCMV was maintained to test the populations derived from various crosses. F1, F2 and back cross populations were tested against BlCMV by sap inoculation method. Immune plants were separated from the susceptible plants on the basis of disease symptoms and ELISA results.

All plants of F1 population of each cross were found susceptible to virus on mechanical inoculation. The number of immune and susceptible plants in F2 progenies of each cross segregated in a ratio of 1 immune : 3 susceptible. The observed ratios were compared with the expected monogenic recessive model for goodness of fit using chi-square test. The data from F1 population suggested dominant nature of susceptibility. Plants of the back crosses (F1BC) to susceptible parent were all susceptible. Based on the results obtained from F1, F2 and back crosses it is concluded that the "immunity" in the following six cowpea lines, IT86F-2089-5, IT86D-880, IT90K-76, IT86D-1010 IT86F-2062-5 and BP1CP3 is conditioned by a single homozygous recessive gene. The symbol for this gene is proposed as "bcm" (blackeye cowpea mosaic).

# INTRODUCTION

# CHAPTER 1

# INTRODUCTION

Cowpea {*Vigna unguiculata* (L.) Walp.} is an important tropical crop and belongs to the Leguminosae family. Cowpea is indigenous to Africa from which it was introduced into other tropical and subtropical countries (Cobley and Steele, 1975). It is suited to hot and humid climates as well as to semi-arid areas. The optimum temperature for growing cowpea is between 20 and 35° C (Kay, 1979). It can be grown in highly acidic to neutral soils but cannot tolerate alkalinity (Kippes, 1970).

It is now grown in many regions of Africa, India, Brazil, USA, the West Indies, Australia, Pakistan and parts of Europe and South America (Rachie and Roberts, 1974). Annual worldwide production of cowpea is estimated as 2.5 million tons of dry bean harvested from 9 million hectares. About 20 percent of the total grown cowpea is consumed as fresh vegetable. In Pakistan cowpea is planted as spring (March to June) or summer (July to October) crops. The total area under cowpea is estimated as about 16.9 thousands hectares, with annual production of 7.8 thousand metric tons. (Bashir, 1992).

The nutritional value of cowpea lies in its protein content of 20-25 %, which is double the protein value of most cereals (Stantion, 1966). Cowpea is a comparatively cheap source of quality protein, phosphorus, iron and vitamin and an excellent substitute for meat, eggs and other protein-rich foods when served as grains or vegetable (Carangal *et al.*, 1979). As a leguminous crop, cowpea cultivation plays an important role in maintaining the nitrogen balance in the soil (Shahjahah *et al.*, 1981).

Disease caused by viruses are among the major factors which contribute for low yields of cowpes. Virus diseases cause serious losses of yield and quality in cowpea in many cowpea growing countries. Worldwide, more than 20 viruses have been identified which infect cowpea under field or experimental conditions (Thottappillay and Rossel, 1985; Mali and Thottappillay, 1986). Numerous viruses are infectious to cowpea and are considered potential natural threats to cowpea production (Kuhn, 1990). In Pakistan previously only a whitefly-transmitted cowpea yellow mosaic virus (CPYMV) was known to occur on cowpea (Ahmad, 1978), but later five more seed transmitted viruses have been reported in cowpea (Bashir and Hamptom 1993). Among the seed transmitted viruses, blackeye cowpea mosaic (BlCMV) is more serious than others and can cause economic losses when occurs in epidemic proportions.

Several control strategies have been suggested for viral diseases which include, the use of virus-free seed or vegetative propagules, prevention of infection by breaks in cropping or control of weed hosts, prevention of transmission by vectors, and breeding for resistance. (Fraser, 1989). The last of these is undoubtedly the most important, economical and practical. However, studies of host resistance are also interesting, because this aspect of viral disease control address some fundamental questions e.g. how plants and viruses interact with each other, how the interaction evolve and what kinds of genes control the resistance against a particular viral pathogen.

Breeding for resistance has become an increasingly common practice on the

development of methods for the control of viral diseases in economically important crop diseases. Destruction of diseased plants, control of insect vectors, and planting in isolation have been principal means of suppressing viral epiphytotics in the past. These procedures will of course, continue to play an important role. However, such measures used to be repeated year after year. In most cases control measures through resistant varieties are effective, economical and environmentally safe. The only limitation to this method is that sometimes no sources of resistance in the related material are present, or because of difficulty in breeding certain plants. However, recent advances in molecular biology have made it easier. The use of resistant genes provide an effective and economical solution of such viral diseases. Genetic resistance is now available against a number of potyviruses (Bashir and Ahmad, 1995) including blackeye cowpea mosaic virus (BlCMV).

Historically, the majority of studies of the genetics of host-pathogen interactions have dealt with the fungal and bacterial pathogens of plants. The studies of resistance and virulence in plant-virus interactions are very rare when compared with fungal and bacterial pathogens. Many cases of resistance to plant viruses are under very simple genetic control, involving only a single locus. In other cases independent genes at two or more loci have been detected in host species (Bashir and Ahmad, 1995). The classical genetic analysis of resistance involves crossing of true breeding resistant and susceptible cultivars and then determining the disease reactions of F1, F2 and if necessary, back crosses generations to inoculation with virus. Observed segregation ratios for the reactions

are then compared with those predicted for various types of genetic control, using the chi-square test.

Keeping in view the importance of resistance genes and their use in effective breeding programme for the development of resistant cowpea cultivars, this study was conducted with the objective to determine the genetic basis of immunity in six cowpea genotypes; IT86F-2089-5, IT86D-880, IT90K-76, IT86D-1010, IT86F-2062-5, and BP1CP3, which were found immune to blackeye cowpea mosaic virus (BlCMV) in a previous study (Bashir *et al.*, 1995). These information will help to develop a cowpea breeding programme to evolve BlCMV resistant cowpea cultivars with other desirable characters.

# REVIEW OF LITERATURE

# CHAPTER 2

# REVIEW OF LITERATURE

The potato virus Y or potyvirus group constitutes the largest group of plant viruses and collectively cause the greatest agricultural losses. There may be over 100 different members of this group (Dougherty and Carrington, 1988). Potyviruses are among the most prevalent and important viruses in leguminous crops because they are seed-borne and are readily transmitted non-persistently by many species of aphids. Economic losses attributed to potyvirus infection can be significant, however, potyviruses are frequently present as a part of the natural pathogen population causing chronic reduction in yield and quality (Hollings and Brunt, 1981). Among potyviruses naturally infecting cowpea crop under field conditions, blackeye cowpea mosaic (BlCMV) is a seed-borne virus (Kuhn, 1990; Purcifull and Gonsalves, 1985; Mali *et al.*, 1988), and is considered economically most important. Literature pertaining to BlCMV is reviewed in the following paragraphs.

## 3.1. History and geographical distribution:

Blackeye cowpea mosaic virus (BlCMV) was first reported on cowpea in the U.S. in 1955 (Anderson, 1955). Initially there was some confusion on the status of BlCMV. BlCMV was assumed to be a strain of bean yellow mosaic virus (Corbett, 1956), while Uyemoto *et al.*, (1973) reported that BlCMV and bean common mosaic virus (BCMV) were serologically identical. On the basis of serological, cytological and biological studies (Lima *et al.*, 1979; Taiwo *et al.*, 1982) BlCMV is now considered to be neither of the above, but a member of potyvirus group (Hollings and Brunt, 1981; Matthews, 1982).

BlCMV was thought earlier to have restricted geographical distribution, but is now occurring worldwide. It has been reported in USA (Anderson, 1955; Lima *et al.*, 1979; Murphy *et al.* 1987), Kenya and Nigeria (Taiwo *et al.*, 1982), Brazil (Lin *et al.*, 1981), India (Mali and Kulthe, 1980, Sekar and Sulochana, 1983, Mali. *et al.*, 1988), Japan, (Hino, 1960), Taiwan (Chang, 1983), Thailand and Malaysia (Tsuchizaki *et al.*, 1984), and Pakistan (Bashir and Hampton, 1993). This clearly indicates that BlCMV occurs wherever cowpea is grown.

## 3.2. Incidence and yield losses:

A 25-90 % incidence of naturally BlCMV-infected cowpea plants was reported by Kuhn (1990). A survey of cowpea diseases in South Carolina (USA), conducted in 1981 and 1982 indicated an incidence of BlCMV infection ranging from 0.5 to 56.5 % (Collins *et al.*, 1984). Mixed infection of BlCMV and cucumber mosaic virus (CMV) under greenhouse conditions caused synergistic reactions which had a devastating effect (42-85 %) on cowpea yield (Harrison and Gudauskas, 1968; Pio-Ribeiro *et al.*, 1978). Simultaneous infection with CMV is common in the eastern states of U.S.A, and results in cowpea plant stunting (Pio-Ribeiro *et al.*, 1978) and rugose mosaic of asparagus bean (Chang, 1983). In Pakistan information on the incidence and losses caused by BlCMV under field conditions are not yet available.

## 3.3. Virus particle morphology:

BlCMV is characterized by flexuous filamentous particles with a modal length of 743-765 nm (Mali *et al.*, 1988; Murphy, 1984). The coat protein

constitutes 95 % of the particle weight based on $A_{260}/A_{280}$ ratio. Nucleic acid is single stranded, positive sense RNA, with a molecular weight of 2.9 X $10^6$ (Murphy, 1984). RNA is infectious component of the virus (Taiwo *et al.*, 1982; Murphy, 1984). The thermal inactivation point in sap from the infected cowpea plant is 60-65° C, longevity in vitro is 1-2 days, and the dilution end point is between $10^{-3}$-$10^{-4}$ (Lima *et al.*, 1979).

### 3.4. Host range and virus-induced symptoms:

BlCMV has been reported to infect 40 species in 22 genera, including 34 species in 18 genera of Leguminosae family (Edwardson and Christie, 1986). The type of symptoms and susceptibility of BlCMV depends on the host species and cultivar and on viral strains being tested (Anderson, 1955; Kuhn, 1990). BlCMV produces both localized and systemic symptoms on cowpea. Localized symptoms include large reddish, often ring-like lesions which typically spread along the veins, forming a reddish-net like pattern. Systemic symptoms include mottle, green vein banding often with interveinal chlorosis, stunting, and leaf distortion (Anderson, 1955; Thottappillay and Rossel, 1985). The virus naturally infects cowpea (*Vigna unguiculata*), asparagus bean (*Vigna unguiculata* ssp. *sesquipedalis*), *Crotalaria spectabilis*, Alyce-clover (*Alysicarpus vaginalis*) and Desmodium, with cowpea being a major natural host. When BlCMV occurs in mixed infection with CMV it causes severe stunting of plants (Kuhn,1990).

### 3.5. Viral strains/variants:

Symptoms and host range variants of BlCMV have been reported (Bock, 1973; Bock and Conti, 1974, Taiwo *et al.*, 1982, Murphy *et al.*, 1987; Murphy,

1984). A major symptom variant is an isolate of BlCMV which causes red, necrotic ring spots and reddish veinal necrosis in cowpea cv Knuckle Purple Huall (Murphy *et al.*, 1987). Other differences in host range between this and another isolate of similar origin to that of Lima *et al.*, (1979), were also reported. Isolates from different parts of the world are closely related serologically (Chang, 1983; Murphy, 1984). Pathogenic variation among various isolates has also been reported (Bashir, 1992). Reports on the existence of pathotypes are lacking.

## 3.6. Transmission through seeds and by vectors:

BlCMV is seed-borne in cowpea. Seed transmission is dependent on both cowpea cultivars and viral isolates, and ranges from 3.5 to 30 % (Mali *et al.*, 1983, Mali *et al.*, 1988). The highest seed transmission rate (55 %) of BlCMV with Pusa isolate in cowpea cv Pusa Phalguni has been reported (Bashir *et al.*, 1995). Cowpea cultivars resistant to seed transmission are reported (Zettler and Evan, 1972; Ladipo, 1977; Bashir *et al.*, 1995). Bashir and Hampton (1994) reported seed-transmission of 0 to 55 % of various isolates of BlCMV in different cowpea cultivars.

BlCMV is readily transmitted mechanically in a non-persistent manner by the aphids; *Aphis craccivora*, *A. gossypii*, *Macrosiphum solinifolii*, and *Myzus persicae* (Anderson, 1955; Mali and Kulthe, 1980; Pio-Ribeiro *et al.*, 1978; Zhao *et al.*, 1991). Individuals of *M. persicae* were able to acquire and transmit both BlCMV and CMV from doubly-infected asparagus bean plants (Chang, 1983).

## 3.7. Sources of resistance:

Host resistance is the most economical and practical approach to control plant virus diseases. Several source of resistance to BlCMV in cowpea are reported (Kuhn *et al.*, 1965; Lima *et al.*, 1979; Mali *et al.*, 1988; Walker and Chambliss, 1981; Strniste, 1987; Kuhn *et al.*, 1966; Collin *et al.*, 1985); Lima, *et al* (1979) reported cowpea varieties Bola de Ouro, Crowder pea, Serodo, Snapper Long Pod, and Sete Sewars as resistance to BlCMV. Taiwo *et al.*, (1981) reported cowpea lines TVu-2480, TVu-2740, TVu-33237, TVu-2657, and TVu-2845 resistant to BlCMV. Recently Bashir *et al.*, (1995) reported the following ten cowpea genotypes immune to BlCMV. IT86F-2089-5, IT86D-880, IT90K-284-2, IT86D-1010, IT86F-2062-5, BP1CP3, IT90K-76, IT87D-611-3, TVU-7676 and PAK 45443.

## 3.8. Genetic basis of resistance:

Resistance to BlCMV in cowpea was found to be governed by a single gene in TVu-2480 (Taiwo *et al.*, 1981) and in "Worthmore" (Walker and Chambliss, 1981). Strniste (1987) found resistance in "Pinkeye Purple Hull-BVR" to be controlled by a single dominant gene. This gene was not allelic to the gene for resistance to BlCMV in "Worthmore". In a common bean (*Phasulus vulgaris* ) cultivar "Black Tourtle Soup" the resistance to BlCMV has been reported to be controlled by a single dominant gene. (Provvidenti *et al.*, 1983). Understanding the genetic basis of immunity to BlCMV in cowpea genotypes recently reported in Pakistan (Bashir *et al.*, 1995) will probably be helpful to cowpea breeders to develop BlCMV resistant cultivars in the country.

# MATERIALS AND METHODS

# CHAPTER 3

# MATERIALS AND METHODS

The research work presented in this dissertation was conducted in Seed Health Laboratory, Plant Genetic Resources Institute (PGRI), under the kind supervision of Dr. Muhammad Bashir, Senior Scientific Officer, Pulses Programme, at National Agricultural Research Centre, Islamabad, and Prof. Dr. Asfari S. Qureshi, Quaid-i-Azam University, Islamabad, Pakistan.

## 4.1. Selection of parents for crossing:

In a previous study (Bashir *et al.*, 1995) 10 cowpea genotypes were identified as immune (no disease symptoms and no virus detection by enzyme-linked immunosorbent assay from virus inoculated plants) to BlCMV. Out of 10 genotypes the following six; IT86F-2089-5, IT86D-880, IT90K-76, IT86D-1010, IT86F-2062-5, and BP1CP3 were selected to study the genetic basis of immunity against BlCMV. The seeds of the first five cowpea lines were obtained from International Institute for Tropical Agriculture (IITA), Ibadan, Nigeria. The seeds of BP1CP3 were obtained from International Rice Research Institute (IRRI), Philippine through the courtesy of Dr. Bashir Ahmad Malik, CSO/Coordinator (Pulses Programme), NARC, Islamabad. One cowpea genotype "Pusa Phalguni" an Indian improved cultivar but susceptible to BlCMV was also included in this

study. The seeds of these cultivars were obtained from Dr. Muhammad Bashir, S.S.O. (Pulses), NARC, Islamabad. The characteristics of these cowpea genotypes are given in Table-1.

**Table 1: Characteristics of cowpea genotypes included as parent to study the genetics of inheritance of immunity to blackeye cowpea mosaic virus.**

| S. No. | Genotypes | Source | Characteristics of the genotypes |
|---|---|---|---|
| 1 | IT86F-2089-5 | IITA-Ibadan, Nigeria. | Immune to BlCMV, early maturing, medium height, high yielder and brown seed colour. |
| 2 | IT86D-880 | IITA-Ibadan, Nigeria. | Immune to BlCMV, medium maturing, tall, spreading type, good yielder and light dark seed colour. |
| 3 | IT90K-76 | IITA-Ibadan, Nigeria. | Immune to BlCMV, early maturing, small, erect type, high yielder and brown seed colour. |
| 4 | IT86D-1010 | IITA-Ibadan, Nigeria. | Immune to BlCMV, late maturing, tall, spreading type, low yielder, small seeded and white seed colour. |
| 5 | IT86F-2062-5 | IITA-Ibadan, Nigeria. | Immune to BlCMV, late maturing, tall, spreading type, good yielder and dark brown seed colour. |
| 6 | BP1CP3 | IRRI, Philippines | Immune to BlCMV, early maturing, small, erect type, high yielder and black seed colour |
| 7 | Pusa Phalguni | India | Highly susceptible to BlCMV, small seeded, Extra early maturing, high yielder and white seed colour. |

## 4.2. Maintenance of virus culture:

An isolate of BlCMV originally obtained by Bashir, *et al.*, (1995) from infected cowpea seeds was used in this study. The isolate was maintained on susceptible cowpea plants in insect-free greenhouse. For further propagation of

virus isolate, carborundum (600 mesh) dusted, fully expanded primary leaves of susceptible seedlings of cowpea were inoculated with extract from virus-infected leaves homogenized in inoculation buffer (0.2 M phosphate buffer, pH 7.0). The leaves of the seedlings were rubbed with virus inoculum placed on ice with fore-finger of the right hand. The plants were washed with tap water after inoculation. Additionally, the virus isolate was also maintained at -30° C in desiccated infected cowpea tissue.

## 4.3. Screening of parents against BlCMV virus:

Fifty seeds of each parent (10 seeds/pot, 5 pots/test genotype) were grown in earthen pots (40 cm diameter) filled with sterilized soil. When the seedlings were 7-8 days old and the primary leaves were fully expanded were inoculated with virus inoculum according to the procedure as described by Bashir *et al.*, (1995). Virus-infected leaves of (1:10 w/v) were tritiated in 0.2 M phosphate buffer (pH 7.0) in a motar and pestle. The inoculum was rubbed on the primary leaves of each plant dusted with carborundum powder (600 mesh). Ten non-inoculated plants of each parent served as control. Immediately after inoculation the plants were rinsed with tap water. After two weeks of first inoculation the symptomless plants were reinoculated to avoid any escape. Disease reaction were recorded at weekly interval following 0-4 point scale. Symptomless plants were also assayed by DAC-ELISA to separate immune plants from virus-infection symptomless plants if any.

### 4.3.1 Scoring scale for recording disease symptoms:

The scoring scale followed to record disease severity of individual inoculated plant was as follow:

0 : No visual disease symptoms at all, plants look healthy, virus is not recovered when inoculated and non-inoculated leaves of the same plant are tested by ELISA (immune).

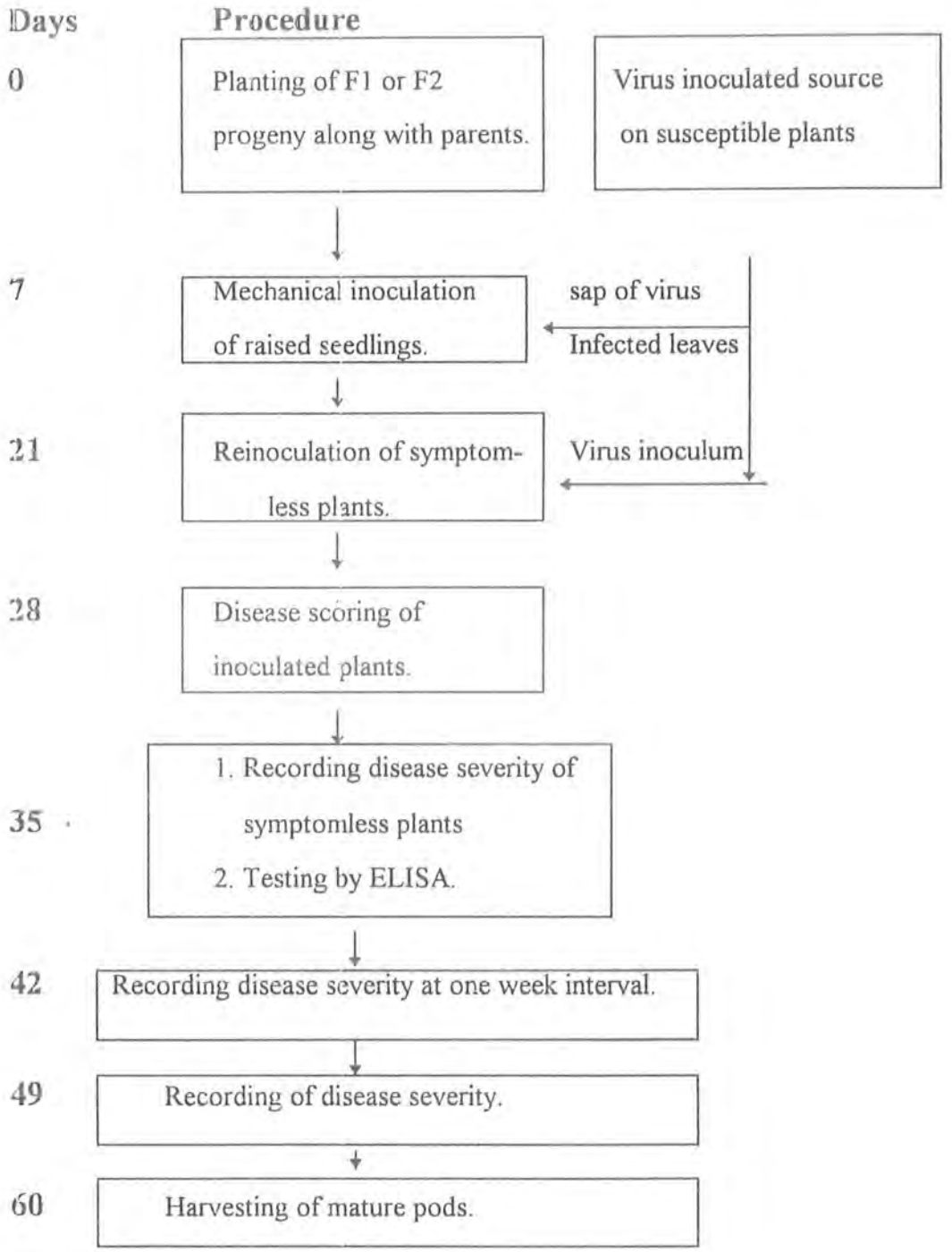
1 : Inoculated plants showing mild mosaic only, no vein chlorosis, and vein banding (systemic infection). When tested by ELISA virus was recovered (resistant).

2 : No visible disease symptoms, but virus is recovered in high titer when tested by ELISA (tolerant).

3 : Moderate mottling, mosaic, and interveinal chlorosis (systemic infection) (susceptible).

4 : Leaf distortion, curling, severe mosaic, vein banding and vein clearing (systemic infection) (highly susceptible).

Figure 1: Flow chart for screening procedure of F1 and F2 progenies of crosses between immune and susceptible parents and their reciprocal crosses.



## 4.4. Planting procedure for crossing:

All the experiments conducted during this study were carried out in an insect-free greenhouse, which was sprayed weekly with insecticides Karate 25 EC (Pyrethroid) (ICI) Pak. (miticide), Monitor 600-50EC (Organophosphate group) (insecticide) and Hostathion 40 EC (Triazophos) (miticide), Hoechst Pak. Ltd. Twenty five seeds of each parent (six immune and one susceptible cowpea genotypes) were planted in earthen pots (40 cms diameters), filled with sterilized soil mixture of silt, sand and farm yard manure in a ratio of 2:1:1 respectively. Five seeds per pot and five pots for each test genotype were used. The pots were kept in insect-free greenhouse and were maintained at proper temperature favorable for cowpea growth i.e., temperature, (28-30° C), lights (14 hr. photoperiod). The plants were regularly watered and properly fertilized.

## 4.5. Crossing procedure:

When the plants of each cowpea genotype were at flowering stage, they were crossed with a susceptible parent (Pusa Phalguni). Reciprocal crosses were also attempted, using susceptible parent as a female. The hybrid seeds were properly collected and maintained crosswise. The crosses were attempted as listed in Table 2.

Table 2: List of direct crosses.

| S. No. | Immune parents | Susceptible parent |
|---|---|---|
| 1 | IT86F-2089-5 | Pusa Phalguni |
| 2 | IT86D-880 | Pusa Phalguni |
| 3 | IT90K-76 | Pusa Phalguni |
| 4 | IT86D-1010 | Pusa Phalguni |
| 5 | IT86F-2062-5 | Pusa Phalguni |
| 6 | BP1CP3 | Pusa Phalguni |

Table 3: List of reciprocal crosses.

| S. No. | Susceptible parent | Immune parent |
|---|---|---|
| 1 | Pusa Phalguni | IT86F-2089-5 |
| 2 | Pusa Phalguni | IT86D-880 |
| 3 | Pusa Phalguni | IT90K-76 |
| 4 | Pusa Phalguni | IT86D-1010 |
| 5 | Pusa Phalguni | IT86F-2062-5 |
| 6 | Pusa Phalguni | BP1CP3 |

### 4.5.1. Emasculation and pollination:

The flowers on the resistant and the susceptible parents were emasculated between 1700 and 1900 hr. using forceps and were properly tagged. Next day early

in the morning the flowers were pollinated with pollen grains collected from their respective parents. All the process was conducted by hand according to the procedure as described by Quattara, (1991). During emasculation the forceps was sterilized with methylated spirt each time to avoid contamination and self-pollination. Each flower was properly tagged. To overcome the deficiency of desired flowers for emasculation and pollination the number of plants of the selected parents were increased so much that at a time they were used as male, as well as female parents.

### 4.5.2. Harvesting of hybrids seed:

The pod maturity took place after two to three weeks of crossing depending upon cowpea genotypes and environmental conditions. The hybrid seeds were collected from crossed pods, threshed, properly labeled and were stored at room temperature to raise next generation. After harvesting of hybrid seeds, they were prepared to raise F-1 generation. F2 progenies were obtained by allowing F1 plants to self pollinate under greenhouse conditions.

## 4.6. Planting of hybrid seeds to raise F-1 progeny:

The hybrid seeds harvested from the crossed flowers were sown without scarification in the clay pots filled with sterilized soil, to grow in insect-free greenhouse and were maintained at proper temperature and light as described before. The plants were regularly watered, properly fertilized, and weekly sprayed with insecticides to avoid insect infestation.

## 4.7. Screening of F1 progeny against virus:

The F1 progeny of each cross was raised from hybrid seeds while growing in earthen pots. When the seedlings of F1 progenies were 8-10 days old and the primary leaves were fully expanded, were mechanically inoculated with virus (BlCMV) inoculum prepared from virus-infected leaves in inoculation buffer. Virus-infected leaves (1:10 w/v) were tritiated in 0.2 M phosphate buffer (pH 7.0) in a motar and pestle. The inoculum was rubbed on the primary leaves of the seedling already dusted with 600 mesh carborundum powder. Ten plants of each parent (immune and susceptible) were also inoculated at the same time with the same inoculum. Non-inoculated plants served as negative control. Immediately after applying virus inoculum the plants were rinsed with tap water. One week after first inoculation all the plants were reinoculated to avoid any escape. The inoculated plants were kept under observations in an insect-free greenhouse at 28-30° C for 60 days.

## 4.8. Susceptibility/immunity criteria:

All the inoculated plants of F1 progeny of each cross and control plants were examined at 15 days interval for disease appearance, and individual plants was (~~parent~~) scored for the presence of virus symptoms. The plants showing mild or severe systemic symptoms of BlCMV were considered as "susceptible". The symptomless plants of each treatment were assayed for virus infection using direct antigen coating-enzyme-linked immunosorbent assay (DAC-ELISA) according to the procedure as described by Hobbs *et al.*, (1987). The symptomless plants with no virus detection by ELISA at all in replicated trials were considered as "immune" to BlCMV (Bashir and Hampton, 1992).

## 4.9. Direct antigen coating enzyme-linked immunosorbent assay (DAC-ELISA):

Currently ELISA is one of the most widely used serologically tests to test and identify plant viruses. There are two main categories of ELISA "direct ELISA" and "indirect ELISA". The direct ELISA (double antibody sandwich; DAS-ELISA) is highly strain specific, whereas the indirect ELISA (direct antigen coating; DAC-ELISA) is more sensitive than DAS-ELISA. DAC-ELISA is mainly used for virus detection in seedlings and leaf samples. Therefore, we used DAC-ELISA throughout this research work. Direct antigen coating enzyme-linked immunosorbent assay (DAC-ELISA) is the simplest of all indirect ELISA procedures. Antigen prepared in carbonate buffer, pH 9.6, is used for coating the wells of microtiter plates. This is followed by addition of crude antiserum at an optimum dilution prepared in antibody buffer pH 7.4. The majority of polyclonal antisera are produced in rabbits. Thus the rabbits r-globulins attached to antigen are probed with a suitable enzyme labeled anti-rabbit r-globulins produced in goat, called *goat-anti-rabbit r-globalins*-conjugate.

**Various steps involved in DAC-ELISA were as follow:**

1. Two hundred mg leaf samples were collected from each test plant and were ground in 2 ml carbonate buffer (pH 9.6) with motar and pestle and poured in small test tubes. The sap was allowed to settle for half an hour at 4° C.
2. An aliquot of 200 µl of each sample was added in wells of microtiter plate in duplicate, along with positive (virus-infected sap) and negative (healthy sap) controls.

3. The plate loaded with samples was covered with plastic cover placed in a humidity chamber and incubated at 37° C for 2 hr or at 4° C overnight.
4. The plate was washed with washing buffer three times at 3 min interval.
5. For cross-absorbance healthy sap was prepared from healthy cowpea leaves (w/v. 500 X) while grinding in antibody buffer, pH 7.4, in a motar with pestle. The sap was sieved through a double layer of muslin cloth for cross-absorbance. Antibody solution was prepared at an appropriate dilution (1:1000) in healthy sap. Polyclonal antiserum to BlCMV was used for this purpose. Prior to adding microtiter plate the antibody-healthy sap solution was incubated at 37° C for 1 hr.
6. Added 200 µl of cross-absorbed polyclonal antiserum to BlCMV in a dilution of 1:1000 in each well of microtiter plate.
7. The plate was incubated at 37° C for 2 hr.
8. Repeated step 4 (washing of plate).
9. Added 200 µl per well of microtiter plate of *goat-anti-rabbit-IgG* (whole molecules) alkaline sphosphatase conjugate solution (Sigma Chemical Co. Product No. A-3687) diluted in antibody buffer (pH 7.4) at a dilution of 1:1000.
10. Repeated steps 3 and 4.
11. Added 200 µl per well of microtiter plate p-nitrophenyl phosphate substrate (Sigma Chemical Co. Product No. N-9389) solution prepared in substrate buffer (pH 9.6), (1 mg / ml 9.7 % diethanolamin + 0.2M sodium azide, pH 9.8) and kept at room temperature.

12. Optical density (OD) of each well was measured at 405 nm by an ELISA plate reader (Corona Electric MTP-32 Model, Made in Japan) at 30 min. and then at 1 hour after adding substrate solution.

13. Twice the valves of negative (healthy sap) control recorded at A 405 nm were considered as positive valves of the test samples.

Symptoms expression on the inoculated plants and enzyme-linked immunosorbent assay (ELISA) results were used to distinguish immune plants from susceptible plants in F1, F2 and back crosses. The inoculated plants with no disease symptoms and when tested by ELISA no virus was recovered were considered as "Immune" (Bashir, 1992), whereas the plants showing visible disease symptoms (mild to sever) and virus was recovered by ELISA were considered as "susceptible".

## 4.10 Buffers used for inoculation and in ELISA:

### 1. Inoculation buffer, pH 7.0

i. Potassium dihydrogen phosphate ($KH_2PO_4$) (Monobasic)= 2.72 gm

ii. Di-potassium hydrogen phosphate anhydrous

($K_2HPO_4$)(Dibasic) = 3.48 gm

In one liter flask containing 500 ml distilled water, both $KH_2PO_4$ and $K_2HPO_4$ were dissolved by stirring on electric stirrer with magnetic bar and volume was made up to 1000 ml by adding more distilled water, pH was adjusted as pH 7.0, and keep at 4° C.

## 2. Phosphate buffer saline (PBS), pH 7.4

| | |
|---|---|
| i. Sodium chloride (NaCl) | = 40 gm |
| ii. Potassium dihydrogen phosphate ($KH_2PO_4$) (Monobasic) | = 1 gm |
| iii. Sodium phosphate anhydrous ($Na_2HPO_4$) (Dibasic) | = 5.8 gm |
| iv. Potassium chloride (KCl) | = 1 gm |
| v. Sodium Azide ($NaN_3$) | = 1 gm |

In one liter flask containing 500 ml distilled water, the above mentioned chemicals were dissolved by stirring on electric stirrer with magnetic bar, and volume was made up to 1000 ml by adding more distilled water, pH was adjusted as 7.4 and keep at room temperature.

## 3. Carbonate buffer, pH 9.6

| | |
|---|---|
| i. Sodium carbonate ($Na_2CO_3$) | = 1.59 gm |
| ii. Sodium bicarbonate ($NaHCO_3$) | = 2.93 gm |
| iii. Sodium diethyldithiocarbamate (Na-DIECA) | = 1.71 gm |

Dissolved both $Na_2CO_3$ and $NaHCO_3$ in 800 ml of distilled water and then adjusted pH as 9.6 with 5N HCl and made volume upto 1000 ml and stored at 4° C. This buffer was used to prepare samples (antigens).

## 4. Serum buffer or antibody buffer, pH 7.4

| | |
|---|---|
| i. Phosphate buffer saline (PBS) | = 200 ml |
| ii. Distilled water | = 400 ml |
| iii. PVP (Polyvinyl pyrrolidone) (MW=25000) | = 20 gm |
| iv. Chicken egg lbumin | = 2 gm |
| v. Tween 20 | = 0.5 ml |

First dissolved polyvinylpyrrolidone (PVP) and chicken egg albumin in phosphate buffer saline (PBS), made the volume up to one liter, adjusted pH as 7.4 and stored at 4 °C. This buffer was used both for making antiserum and conjugate solutions.

## 5. Substrate buffer, pH 9.8

| | |
|---|---|
| i. Diethanolamin ($C_4H_{11}NO_2$) | = 47 ml |
| ii. Distilled water | = 400 ml |

Diethanolamine was added little by little into distilled water by stirring with magnetic bar on electric stirrer. Made volume upto 500 ml, by adding distilled water adjusted pH as 9.8 with 5N HCl and stored at 4 °C. Substrate solution was made each time fresh before used.

### 6. Washing buffer, pH 7.0

i. Phosphate buffer saline (PBS) = 200 ml

ii. Tween-20 = 1 ml

iii. Distilled water = 800 ml

Dissolved PBS in 500 ml distilled water and dissolved Tween-20 by stirring with magnetic bar on electric stirrer. Made volume upto 1000 ml, by adding distilled water adjusted pH as 7.0, stored at room temperature.

## 4.11. Back Crosses:

F-1 progeny of each cross was also back crossed with susceptible (Pusa Phalguni) parent as follow:

**Table 4: List of back crosses.**

| S. No. | Back cross | |
|---|---|---|
| | F1 progeny | Susceptible parent |
| 1 | F1 (IT86F-2089-5 X Pusa Phalguni) | Pusa Phalguni |
| 2 | F1(IT86D-880 X Pusa Phalguni) | Pusa Phalguni |
| 3 | F1(IT90K-76 X Pusa Phalguni) | Pusa Phalguni |
| 4 | F1(IT86D-1010 X Pusa Phalguni) | Pusa Phalguni |
| 5 | F1(IT86F-2062-5 X Pusa Phalguni) | Pusa Phalguni |
| 6 | F1(BP1CP3 X Pusa Phalguni) | Pusa Phalguni |

## 4.12. Evaluation of F2 progenies:

The seeds obtained from all F1 crosses (direct, reciprocal and back crosses) and parents were scarified as without scarification the germination percentage was very low). To get good germination, the scarified seeds were first germinated in moist roll of blotter paper by putting them in incubator having temperature ranging from 24-25° C. After 72 hr, individual seedling was transplanted in earthen pots filled with sterilized soil. The plants were allowed to grow in an insect-free greenhouse with 28-32° C temperature. After two weeks of transplanting, when the primary leaves of the established seedlings were fully expanded, were inoculated with virus culture. Inoculum was prepared and applied as has been described under F1 progeny testing. After two weeks of first inoculation, the symptomless plants of each cross were reinoculated to ensure virus infection and to avoid any escape. Individual plant of each cross was scored for disease severity following 0-4 point scale, at 15 days interval. After four weeks of first inoculation the symptomless plants were tested by enzyme-linked immunosorbent assay (ELISA) to detect virus presence and to separate immune plants from susceptible plants.

## 4.13. Application of chi-square test for goodness of fit:

The chi-square test is most commonly used to test hypothesis concerning the frequency distribution of one or more populations. In agricultural research this test is very useful for obtaining an objective approximation of goodness of fit of the attribute data (the attribute data is concerned with a finite number of discrete classes) obtained from a particular experiment.

There are three important application of the chi-square test in the analysis of attribute data.

(i). Test for a fixed-ratio hypothesis.

(ii). Test for independence in a contengency table.

(iii). Test for homogeneity of ratio.

In this study we used chi-square test for a fixed-ratio hypothesis. Chi-square test for a fixed-ratio hypothesis is a technique for deciding whether a set of attribute data conforms to a hypothesized frequency distribution that is specified on the basis of biological phenomenon.

The procedure followed to compute chi-square values of the data obtained from F2 segregation population of each cross was followed as described by Gomes and Gomes (1984).

To compute chi-square ($X^2$) values the following formula was used.

$$X^2 = \sum_{i=1}^{p} \frac{\{(|o-e|)-1/2\}^2}{e}$$

where

$\Sigma$ : Sum of squared deviations of all classes.

$X^2$ : Chi-square value.

p : is the number of classes.

o : is the observed number of units falling in each class.

e : in the number of units expected to fall in each class assuming that the hypothesized ratio holds.

As there were only two classes "susceptible" and "immune" in our data so the degree of freedom used to check from chi-square-table was 1. Our hypothesis in this study was ***"does the observed ratios as listed in table 8 and 9 against each cross (i.e., one immune : three susceptible) significantly deviate from the hypothesized ratio of 1 : 3.***

The following steps were followed to calculate $X^2$ values in each cross.

## Step 1:

Computed the $X^2$ value, depending on class number. In this study we have only two class numbers i. e., susceptible and immune

$$X^2 = \sum_{i=1}^{p} \frac{\{(|o-e|)-1/2\}^2}{e}$$

The $X^2$ values obtained using this formula in each cross of F2 segregating populations are given in Table 8 and 9.

## Step 2:

Compared the computed $X^2$ value of each cross given in Table 8 and 9 with the tabular $X^2$ values obtaining from Appendix - 1, with (p - 1) = 2 - 1 = 1

degree of freedom at the probability level of significance (5 % and 1 %) given in the last column of Table 8 and 9. If the computed $X^2$ values exceeds the corresponding tabular $X^2$ value at 5 % or 1 % level of significance, the hypothesis was rejected,

i.e., there was significant difference between the observed ratio and expected ratio and the data obtained does not hold good for fitness. On the other hand if the calculated $X^2$ value is less than the tabular value, the "hypothesis i. e. there is no difference between the observed ratio and expected ratio of 1 : 3" and is accepted and this means that the data hold good for fitness.

**Note :** The reduction of 1/2 from the observed-expected number in the formula is known as the "Yates Correction Factor" and adds to the accuracy of chi-square determinations when the number of the expected classes is small (Strickberger, 1969).

# RESULTS

# CHAPTER 4

# RESULTS

## 5.1. Response of parent plants to virus inoculation:

The results of screening of seven parents against BlCMV inoculation have been summarized in Table 1. Fifty plants of each parent were tested by sap inoculation method. It was observed that all the plants of six parents i. e., IT86F-2089-5, IT86D-880, IT90K-76, IT86D-1010 IT86F-2062-5 and BP1CP3 were symptomless except in case of genotypes IT86D-880 and IT86F-2062-5, where we found three and two plants susceptible to BlCMV respectively. In case of genotype "Pusa Phalguni" all the plants showed systemic infection on the trifoliolates. The susceptible plants exhibited disease symptoms of vein-clearing, vein-banding, malformation and blistering of the trifoliolate leaves. Various types of symptoms observed in susceptible plants have been shown in figures 1 and 2. In susceptible plants symptoms started appearing the first trifoliate leaves two weeks after first inoculations. All symptomless plants of each of the six parents when tested by DAC-ELISA, virus was not recovered in the inoculated as well as non-inoculated leaves, which confirmed the immunity of these cowpea lines to BlCMV before crossing.

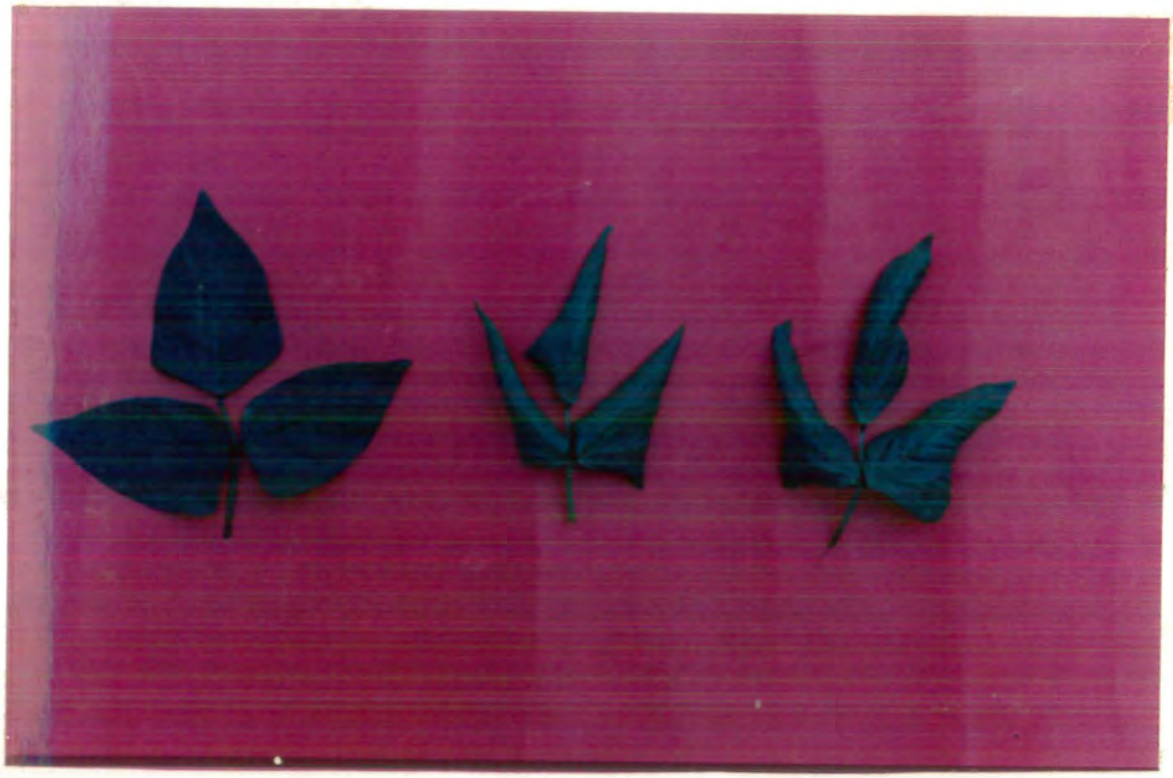


**Fig. 1: Symptoms induced by BlCMV isolate in susceptible parent, Pusa Phalguni (right), healthy control (left).**

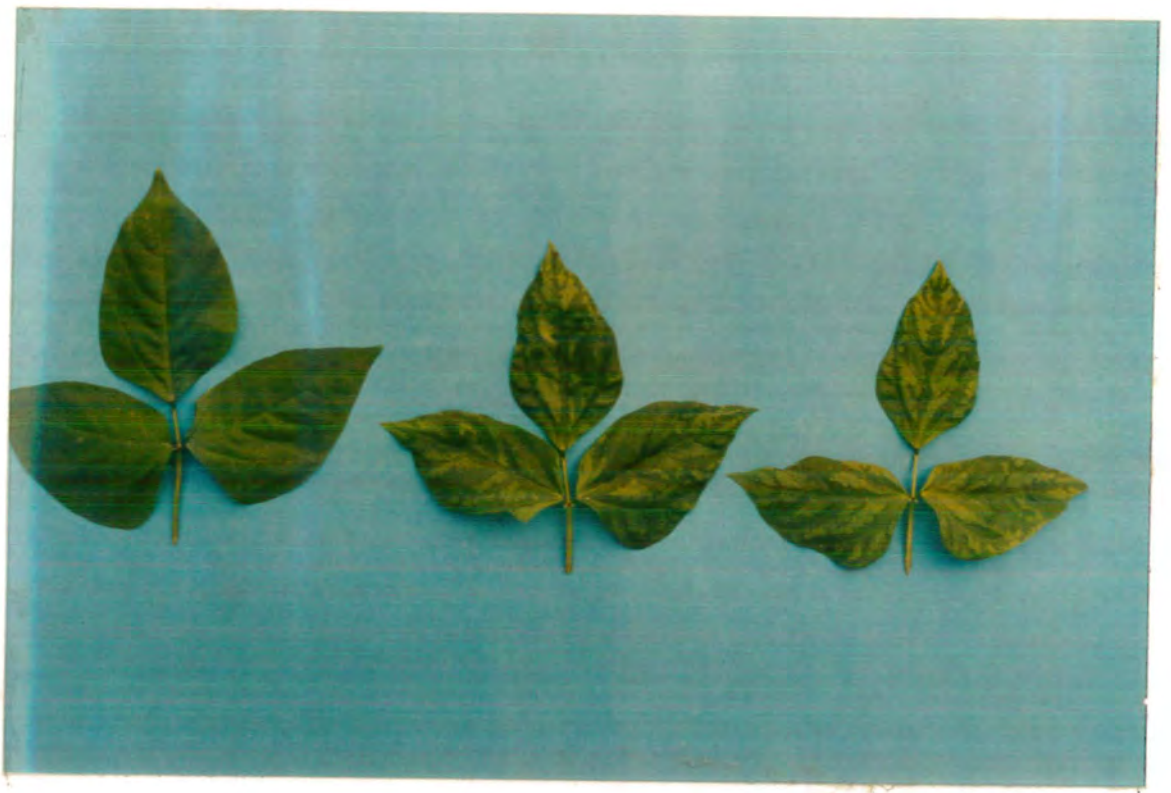


**Fig. 2: Symptoms of BlCMV in severe form induced in susceptible parent Pusa Phalguni (right, healthy control (left).**

**Table 1: Response of cowpea genotypes to blackeye cowpea mosaic virus (BlCMV) inoculation selected as parent for genetic study.**

| S. No. | Parents | No. of plants tested | Disease reaction | No. of plants found | | ELISA Results $A_{405nm}$ |
|---|---|---|---|---|---|---|
| | | | | Susceptible | Immune | |
| 1 | IT86F-2089-5 | 50 | - | 0 | 50 | 0.021 * |
| 2 | IT86D-880 | 50 | - | 3 | 47 | 0.032 * |
| 3 | IT90K-76 | 50 | - | 0 | 50 | 0.024 * |
| 4 | IT86D-1010 | 50 | - | 0 | 50 | 0.015 * |
| 5 | IT86F-2062-5 | 50 | - | 2 | 48 | 0.026 * |
| 6 | BP1CP3 | 50 | - | 0 | 50 | 0.032 * |
| 7 | Pusa Phalguni | 50 | +++ | 50 | 0 | 1.822 * |

\- : No disease symptoms at all, and virus was not recovered in symptomsless plants when tested by ELISA.

+++ : Sever systemic infection of BlCMV on leaves showing susceptibility, with characteristic BlCMV virus symptoms.

* : Absorbance value recorded at $A_{405\ nm}$ (Mean value of 50 plants).

## 5.2. Compatibility of parents in crosses:

Crossing procedure was the same in each cross. The number of hybrid seeds obtained from each cross was variable depending upon the parental compatibility and seed setting ability. Maximum seed-setting i.e., 109 hybrid seeds were obtained with the cross IT90K-76 X Pusa Phalguni, followed by BP1CP3 X Pusa Phalguni with 86 hybrid seeds. Minimum number of hybrid seeds were obtained with the cross IT86D-1010 X Pusa Phalguni (Table 2). It was found that all the parents synchronized with respect to flowering and no lack of flower problem was observed. The time to maturity was different in each cross combination. The maturity time in the crosses; IT86F-2089-5 X Pusa Phalguni; IT90K-76 X Pusa Phalguni and BP1CP3 X Pusa Phalguni was shorter (early) as compared to the rest of the crosses (late).

**Table 2: Results of seed setting (hybrid seeds) in direct crosses.**

| S. No. | Cross | | No. of hybrid seeds obtained from each cross | Maturity time |
|---|---|---|---|---|
| | Immune parent | Susceptible parent | | |
| 1 | IT86F-2089-5 | Pusa Phalguni | 22 | early |
| 2 | IT86D-880 | Pusa Phalguni | 48 | late |
| 3 | IT90K-76 | Pusa Phalguni | 109 | early |
| 4 | IT86D-1010 | Pusa Phalguni | 13 | late |
| 5 | IT86F-2062-5 | Pusa Phalguni | 42 | late |
| 6 | BP1CP3 | Pusa Phalguni | 86 | early |

### 5.2.1. Reciprocal crosses:

In reciprocal crosses maximum number of hybrid seeds were obtained with the cross Pusa Phalguni X IT86D-880 followed by Pusa Phalguni X BP1CP3. The range of seed setting in other crosses was from 23 to 45. The number of hybrid seeds harvested from each cross is given in Table 3. Flower drop was one of the reasons for low setting of hybrid seeds.

**Table 3: Results of seed setting in reciprocal crosses.**

| S. No. | Cross | | No. of hybrid seeds obtained |
|---|---|---|---|
| | Susceptible | Immune | |
| 1 | Pusa Phalguni | IT86F-2089-5 | 45 |
| 2 | Pusa Phalguni | IT86D-880 | 75 |
| 3 | Pusa Phalguni | IT90K-76 | 23 |
| 4 | Pusa Phalguni | IT86D-1010 | 30 |
| 5 | Pusa Phalguni | IT86F-2062-5 | 25 |
| 6 | Pusa Phalguni | BP1CP3 | 61 |

## 5.3. Testing of F1 progenies against virus inoculation:

The testing results against BlCMV inoculation obtained from the F1 populations from all the direct and reciprocal crosses are presented in Table 4 and 5 respectively. In F1 tests, all plants in the six crosses (direct and reciprocal) were found susceptible to BlCMV. F1 plants developed systemic symptoms (Fig. 3) that

**Fig. 3: Systemic symptoms developed in F1 plants three weeks after virus inoculation.**

closely resembled those exhibited by susceptible parent plants, indicating that immunity was inherited recessively. This condition was confirmed by the reaction of F2 progenies, which segregated in the ratio 1 immune : 3 susceptible. However, it was observed that the inoculated plants in reciprocal crosses exhibited more disease severity than the plants of indirect crosses. Variation in disease symptoms (mild to severe) was observed not only among the crosses but also within the same cross. The plants showing mild systemic infection in some of the crosses when tested by ELISA, virus was recovered in high titer. The number of plants tested in each cross was also variable depending upon the availability of hybrid seeds. Reciprocal F1 populations from all crosses (Table-5) were also susceptible to BlCMV, have no maternal (cytoplasmic) effect was observed. Expression of susceptibility to BlCMV in reciprocal F1 populations was identical to the F1 from direct crosses. Based on disease symptoms recorded on individual plant and ELISA results it was found that all the plants of F1 of each cross were susceptible to BlCMV, indicating that susceptibility was dominant over immunity suggesting the monogenic recessive model of inheritance.

We also observed virus infected seedlings emerging from hybrid seeds without virus inoculation at low percentage (i.e., 4-5 %). Typical BlCMV symptoms appeared on the first primary leaves (Fig. 4). Virus was also detected by ELISA in these seedlings. This was due to seed-transmission of the virus.

Table 4: Screening results of F1 progeny in direct crosses against BICMV by sap inoculation.

| S. No. | Cross | | No. of plants tested | No. of plants found | |
|---|---|---|---|---|---|
| | Immune | Susceptible | | Susceptible | Immune |
| 1 | IT86F-2089-5 | Pusa Phalguni | 17 | 17 | 0 |
| 2 | IT86D-880 | Pusa Phalguni | 39 | 39 | 0 |
| 3 | IT90K-76 | Pusa Phalguni | 76 | 76 | 0 |
| 4 | IT86D-1010 | Pusa Phalguni | 12 | 12 | 0 |
| 5 | IT86F-2062-5 | Pusa Phalguni | 38 | 38 | 0 |
| 6 | BP1CP3 | Pusa Phalguni | 67 | 67 | 0 |

Table 5: Screening results of F1 progeny in reciprocal crosses against BICMV by sap inoculation.

| S. No. | Cross | | No. of plants tested | No. of plants found | |
|---|---|---|---|---|---|
| | Susceptible | Immune | | Susceptible | Immune |
| 1 | Pusa Phalguni | IT86F-2089-5 | 39 | 39 | 0 |
| 2 | Pusa Phalguni | IT86D-880 | 73 | 73 | 0 |
| 3 | Pusa Phalguni | IT90K-76 | 18 | 18 | 0 |
| 4 | Pusa Phalguni | IT86D-1010 | 24 | 24 | 0 |
| 5 | Pusa Phalguni | IT86F-2062-5 | 18 | 18 | 0 |
| 6 | Pusa Phalguni | BP1CP3 | 45 | 45 | 0 |

## 5.4. Back crosses evaluation:

The F1 obtained from each cross was also back crossed with susceptible male parent "Pusa Phalguni". The number of seeds obtained in each back cross are given in Table 6. It was observed that seed setting in back crosses was poor. as compared to other crosses attempted. However, a reasonable number of seeds were obtained to have meaningful results for final conclusion.

**Table 6: Result of seed setting in each back cross.**

| S. No. | Back cross | | No. of seeds obtained |
|---|---|---|---|
| | F 1 | Susceptible parent | |
| 1 | F1 (IT86F-2089-5 X Pusa Phalguni) | Pusa Phalguni | 10 |
| 2 | F1(IT86D-880 X Pusa Phalguni) | Pusa Phalguni | 38 |
| 3 | F1(IT90K-76 X Pusa Phalguni) | Pusa Phalguni | 20 |
| 4 | F1(IT86D-1010 X Pusa Phalguni) | Pusa Phalguni | 15 |
| 5 | F1(IT86F-2062-5 X Pusa Phalguni) | Pusa Phalguni | 28 |
| 6 | F1(BP1CP3 X Pusa Phalguni) | Pusa Phalguni | 22 |

The results obtained after screening back cross populations against virus inoculation are presented in Table 7. The number of plants tested in each back cross was variable (10 - 38) depending on the availability of seeds. It was observed

that all the plants tested under each back cross were susceptible and characteristic virus symptoms appeared systemically (Fig. 5) to virus and no immune plants were identified from back cross populations. ELISA results also correlated with disease symptoms. The results of back crosses also supported a single recessive gene inheritance model.

Fig. 5: Typical symptoms of BICMV in susceptible plants three weeks after inoculation in form of vein clearing, vein chlorosis and blistering of leaves (Back cross population).

Table 7: Screening results of back cross progenies of each cross against BlCMV inoculum.

| S. No. | Back cross | | | No. of plants tested | No. of plants found | |
|---|---|---|---|---|---|---|
| | F1 progenies | Susceptible parent | Disease reaction | | Susceptible | Immune |
| 1 | F1 (IT86F-2089-5 X Pusa Phalguni) | Pusa Phalguni | +++ | 10 | 10 | 0 |
| 2 | F1(IT86D-880 X Pusa Phalguni) | Pusa Phalguni | +++ | 38 | 38 | 0 |
| 3 | F1(IT90K-76 X Pusa Phalguni) | Pusa Phalguni | +++ | 20 | 20 | 0 |
| 4 | F1(IT86D-1010 X Pusa Phalguni) | Pusa Phalguni | +++ | 15 | 15 | 0 |
| 5 | F1(IT86F-2062-5 X Pusa Phalguni) | Pusa Phalguni | +++ | 28 | 28 | 0 |
| 6 | F1(BP1CP3 X Pusa Phalguni) | Pusa Phalguni | +++ | 22 | 22 | 0 |

+++ : Highly susceptible, with characteristic BlCMV symptoms.

## 5.5. Evaluation of F2 population:

### 5.5.1. Direct crosses

The segregation patterns to virus infection observed from six direct crosses are presented in Table 8. The number of plants obtained from F1 seeds were variable and ranged from 41 to 610. The F2 population from each cross segregated in the ratio 1 immune : 3 susceptible. All the symptomsless plants of each cross when tested by DAC-ELISA, virus was not recovered from inoculated as well as non-inoculated leaves of the same plant. This group of plants in each cross was considered "immune" to BlCMV. The second group of plants which were regarded as "susceptible" showed variability in disease symptoms (Figs. 6 and 7). Throughout the trials we observed variability in disease symptoms ranging from mild to severe infection not only among the crosses, but also within the same cross. Virus titer was also different in plants of the same cross. Even virus titer was not uniform within the same plants when determined by ELISA. We also observed infecting seedlings (Fig. 8) from seeds indicating seed transmission of BlCMV, but at low rate (2-3 %). On the average ELISA results were highly correlated with the visual symptom scoring. ELISA reading for known positive (1.823 to 2.748) and negative (0.021 to 0.034) samples indicated adequate separation of BlCMV-infected (susceptible) and symptomless non-infected (immune) plants. Irrespective of the disease symptoms (mild to severe) in Fig. 9.

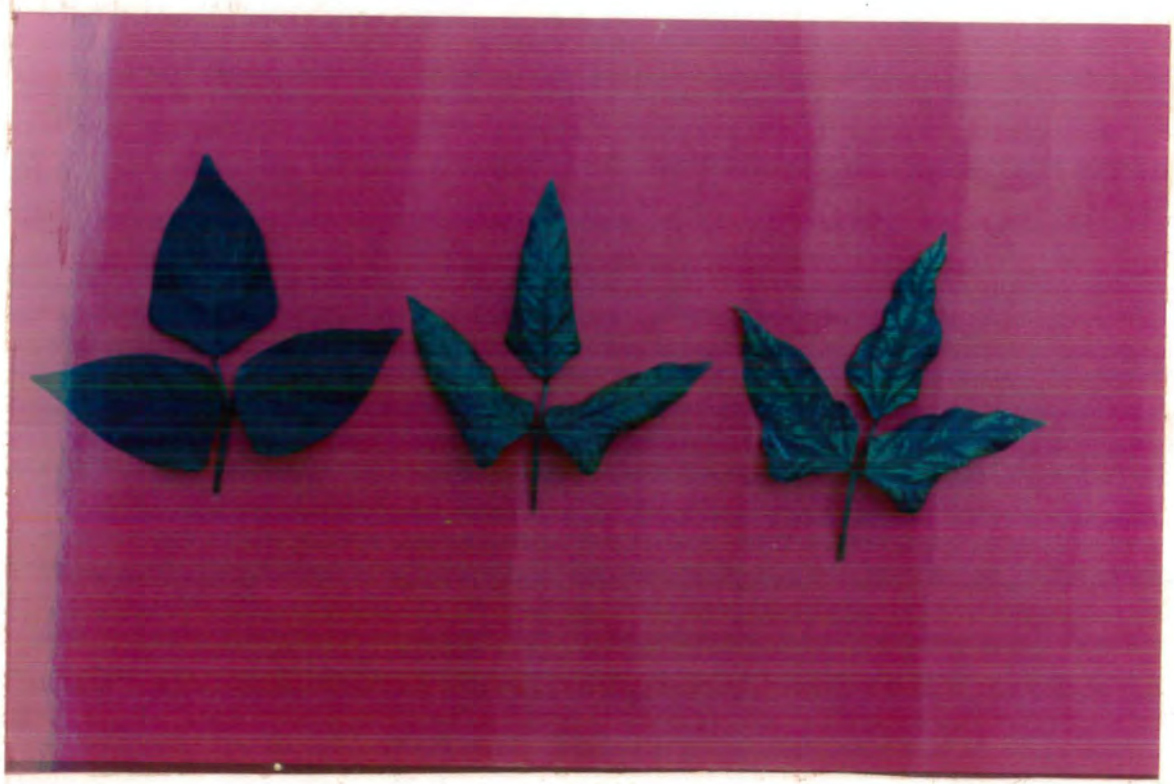


**Fig. 6: Severe symptoms induced by BlCMV in F2 segregants (right), healthy control (left).**

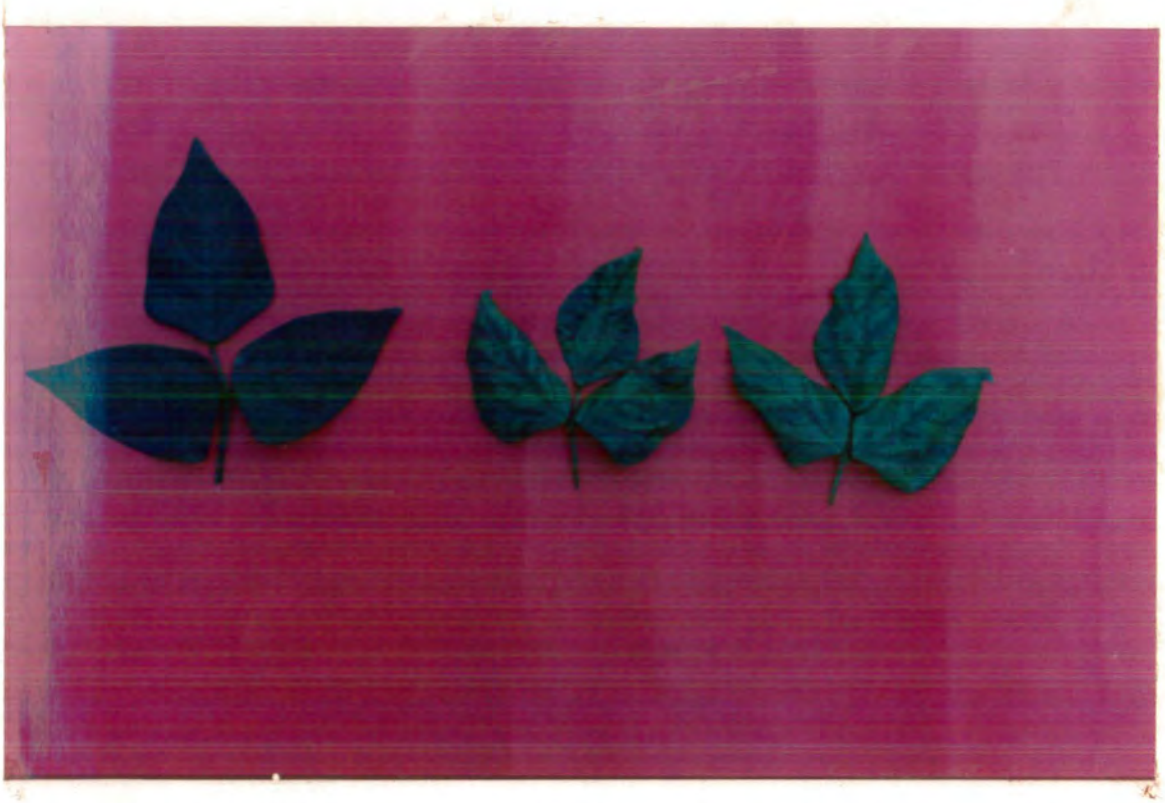


**Fig. 7: Mild symptoms induced by BlCMV in F2 susceptible plants.**

**Fig. 8: Virus symptoms on primary leaves of seedlings emerged from infected seeds (F2 plants).**

**Fig. 9: Segregation towards susceptibility/ immunity in F2 populations after three weeks of virus inoculation.**

and virus titer variability in plants of the same cross, this group of plants was considered as "susceptible". All plants of susceptible parent showed severe virus symptoms and positive ELISA values.

The number of immune and susceptible plants obtained in each cross of direct and reciprocal crosses were compared with those expected in monogenic recessive model for major gene and goodness of fit using chi-square test (Gomes and Gomes, 1987). Chi-square ($X^2$) values calculated on the basis of observed ratio (1 : 3) of each cross are given in Table 8. As the calculated Chi-square ($X^2$) value of each cross is less than the tabulated values at 5 % or 1 % level of probability, which indicated that the results are non-significant. These segregations (all observed ratios 1 immune : 3 susceptible) fit the hypothesis that immunity in these six cowpea genotypes. is dependent upon the homozygous condition of a single recessive gene pair.

Table 8: Segregation of blackeye cowpea mosaic virus (BlCMV) reaction in F2 populations of direct crosses.

| Cross | No. of plants tested | Observed values | | Calculated/ Expected values | Expected ratio | $\frac{(O-C)^2}{C}$ | Chi-square ($X^2$) values | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Classes | | | | | Calculated | Tab. (5%) | Tab. (1%) |
| **IT86F-2089-5 X Pusa phalguni** | 192 | Susceptible | 142 | 144 | 1 : 3 | 0.015 | 0.061 NS | 3.841 | 6.635 |
| | | Immune | 50 | 48 | | 0.046 | | | |
| **IT86D-880 X Pusa phalguni** | 269 | Susceptible | 196 | 201.75 | 1 : 3 | 0.136 | 0.214 NS | 3.841 | 6.635 |
| | | Immune | 73 | 67.25 | | 0.078 | | | |
| **IT90K-76 X Pusa phalguni** | 959 | Susceptible | 737 | 719.25 | 1 : 3 | 0.413 | 1.647 NS | 3.841 | 6.635 |
| | | Immune | 222 | 239.7 | | 1.234 | | | |
| **IT86D-1010 X Pusa phalguni** | 41 | Susceptible | 31 | 30.75 | 1 : 3 | 0.002 | 0.008 NS | 3.841 | 6.635 |
| | | Immune | 10 | 10.25 | | 0.006 | | | |
| **IT86F-2062-5 X Pusa phalguni** | 327 | Susceptible | 252 | 245.25 | 1 : 3 | 0.159 | 0.636 NS | 3.841 | 6.635 |
| | | Immune | 75 | 81.75 | | 0.477 | | | |
| **BP1CP3 Pusa phalguni** | 610 | Susceptible | 453 | 457.5 | 1 : 3 | 0.035 | 0.14 NS | 3.841 | 6.635 |
| | | Immune | 157 | 152.5 | | 0.105 | | | |

NS : Non-significant.

### 5.5.2. Reciprocal crosses:

Six reciprocal crosses were also attempt using susceptible parent as a female and immune parent as a male. Cytoplasmic effect towards immunity was not observed. The segregation pattern to virus infection of F2 populations of each reciprocal cross is presented in Table 9. When we used susceptible parent as a female, the percentage seed setting was high as compared to direct crosses. Therefore, more number of seeds were harvested from F1 plants. The number of plants tested in F2 population of each reciprocal cross also varied from 161 to 651.

F2 population of each cross in reciprocal crosses also segregated in a ratio of one resistant : 3 susceptible on the same pattern as we obtained in direct crosses. There was not parental effect, towards immunity.

Chi-square (X2) values were calculated similarly as in case of direct crosses and are given in Table 9. In each cross the results were non-significant and supported the hypothesis that the immunity in each cowpea genotype to BlCMV is conditioned by a single homozygous recessive gene.

Based on the results obtained from F1, F2 segregation pattern, and back crosses, it is concluded that immunity in the following six cowpea genotypes;

IT86F-2089-5, IT86D-880, IT90K-76, IT86D-1010, IT86F-2062-5 and BP1CP3 is conditioned by a single recessive gene for which we proposed the same symbol "bcm" (blackeye cowpea mosaic) which has already been suggested by Walker and Chambliss (1981).

Table 9: Segregation of blackeye cowpea mosaic virus (BlCMV) reaction in F2 populations of reciprocal crosses.

| Cross | No. of plants tested | Observed values | | Calculated/ Expected values | Expected ratio | $\frac{(O-C)^2}{C}$ | Chi-square ($X^2$) values | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Classes | | | | | Calculated | Tab. (5%) | Tab. (1%) |
| **Pusa phalguni X IT86F-2089-5** | 406 | Susceptible | 303 | 304.5 | 1 : 3 | 0.003 | 0.012 NS | 3.841 | 6.635 |
| | | immune | 103 | 101.5 | | 0.009 | | | |
| **Pusa phalguni X IT86D-880** | 651 | Susceptible | 485 | 488.25 | 1 : 3 | 0.015 | 0.061 NS | 3.841 | 6.635 |
| | | Immune | 166 | 162.75 | | 0.046 | | | |
| **Pusa phalguni X IT90K-76** | 263 | Susceptible | 198 | 197.25 | 1 : 3 | 0.001 | 0.0019 NS | 3.841 | 6.635 |
| | | Immune | 65 | 65.75 | | 0.0009 | | | |
| **Pusa phalguni X IT86D-1010** | 303 | Susceptible | 227 | 227.25 | 1 : 3 | 0.0002 | 0.001 NS | 3.841 | 6.635 |
| | | Immune | 76 | 75.75 | | 0.0008 | | | |
| **Pusa phalguni X IT86F-2062-5** | 161 | Susceptible | 118 | 120.75 | 1 : 3 | 0.042 | 0.167 NS | 3.841 | 6.635 |
| | | Immune | 43 | 40.25 | | 0.125 | | | |
| **Pusa phalguni X BP1CP3** | 482 | Susceptible | 348 | 361.5 | 1 : 3 | 0.467 | 1.869 NS | 3.841 | 6.635 |
| | | Immune | 134 | 120.5 | | 1.402 | | | |

NS : Non-significant

Fig. 10 : Pattern of inheritance of immunity in six cowpea genotypes to BICMV.

Parent rr X RR

(Immune) ↓ (Susceptible)

F1 Rr (All susceptible to BICMV virus)

Selfing (Rr X Rr)

↓

| | R | r |
|---|---|---|
| R | RR | Rr |
| r | Rr | rr |

F2 RR : Rr : rr

1 : 2 : 1

susceptible immune

Segregation 3 : 1

(susceptible) (immune)

Back cross:

Parents rr X RR

(Immune) ↓ (Susceptible)

F1 Rr

For BC1 (F1) Rr X RR

↓

Progenies from back crosses: RR, Rr

(All susceptible)

| | R | r |
|---|---|---|
| R | RR | Rr |

{RR and Rr} Susceptible

**Results: Immunity in the six cowpea genotypes is conditioned by a major single recessive gene.**

# DISCUSSION

# CHAPTER 5

# DISCUSSION

Breeding for resistance is one of the most economical and effective means of controlling virus diseases of plants. Genetic resistance has been found against a number of potyviruses including BlCMV. Resistance resources to BlCMV have been identified by several workers (Bashir *et al.*, 1995; Mali *et al.*, 1988; Taiwo *et al.*, 1982). Recently ten cowpea genotypes were identified as immune to a local strain of BlCMV in Pakistan (Bashir *et al.*, 1995) and six of them were included in this study to determine the genetic basis of immunity in these cowpea lines. Perhaps understanding the mode of inheritance of immunity to BlCMV in these cowpea lines will help to develop an effective future breeding programme to evolve resistant cowpea cultivars with desirable characters.

Before starting to make crosses we also confirmed the immunity /susceptibility of the seven parents which were included in this study by inoculation test while testing against BlCMV isolate. All the parents tested behaved uniformly towards immunity/susceptibility except that we found 3 and 2 susceptible plants (out of 50 plants each in IT86D-880 and IT86F-2062-5) respectively (Table 1). Whereas Bashir *et al.*, (1995) did not find any susceptible plant out of 30 - 32 plants tested against the same virus isolate. The appearance of two or three

susceptible plants in these cowpea lines may be either due to mixture of any other susceptible variety during harvesting, threshing or at the time of handing of these lines at any stage or these genotypes may not be homogenous and their immunity may be strain specific. Such variation to virus susceptibility in cowpea lines while testing against two isolates of cowpea aphid-borne mosaic potyvirus (CABMV) was also observed by Taiwo *et al.*, (1981), Ladipo and Allen (1979) and Taiwo *et al.*, (1982).

In this presentation we have used two terms "*susceptibility*" and "*immunity*" and we have also classified our data into two discrete classes based on reaction of parents lines, F1, F2 and back cross progenies to virus inoculation. A host plant in which a virus multiplies, and may cause visible disease symptoms is "*susceptible*" to that particular virus. On the other hand when a host plant does not support detectable virus multiplication when challenged to the virus inoculum is called "*immune*". The immunity may be two types i.e., non-host immunity and host immunity. The third term used is "*resistance*', which refers to various levels of virus multiplication (low to high) within the host with or without disease symptoms. Fraser (1990) has suggested that the term "*immunity*" should not be applied to plant of a normally susceptible species which show no detectable symptoms after virus inoculation because of the presence of a resistance gene. The

other school of thought did not agree with his proposal and have used and is being used the term "immunity" to plants which do not support virus multiplication and show no detectable virus and symptoms within the host species. The term "immunity" has been applied by several workers to plant species showing complete resistance to plant viruses (Kuhn *et al.*, 1968; Taiwo 1978; Ladipo and Allen 1979 and Arif, 1995).

During screening of F1, F2 and back cross progenies to virus infection symptom differences were apparent not only among the progenies from different crosses but also within the progeny of the same cross. Therefore, we recorded disease symptoms on individual plant using 0 - 4 scale. For convenience, the susceptible and highly susceptible segregants in the F2 population were pooled within each cross against moderately resistant segregants to work out the fitness to 1 immune : 3 susceptible. However, we did not find any variability in immunity among the plants of F1 and F2 progenies when tested by ELISA. Similar observations of differential symptoms on peas have been recorded as responses to inoculation with pea mosaic virus and other related viruses, and have been attributed to plant age, environmental conditions and variety, but not due to genetic factors (Yen and Fry 1956).

Plants of the parent lines of F1, F2 and back crosses populations were classified as immune or as susceptible according to visible disease symptoms and testing by ELISA. All F1 plants and all back crosses to susceptible parent were susceptible, indicating that immunity is recessive to susceptibility. Considering the over all F2 segregation pattern (1 immune : 3 susceptible) for reaction to BlCMV seems to follow the hypothesis of single recessive gene model.

Based on the results obtained from F1, F2 and back crosses of this investigation, we concluded that a single recessive gene controls immunity to BlCMV in the cowpea lines; IT86F-2089-5, IT86D-880, IT90K-76, IT86D-1010 IT86F-2062-5 and BP1CP3. The same mode of inheritance was implicated in the resistance to BlCMV in cowpea cultivar "Worthmore" (Walker and Chambliss 1981). Taiwo *et al.*, (1981) also reported a single recessive gene responsible for high level of resistance in cowpea lines; TVu-2740, TVu-3273, TVu-2657 and TVu-2845. In contrast to these results a single dominant gene for resistance to BlCMV in cowpea cultivar "White Acre-BVR" (Quattara and Chambliss, 1991), bean cultivar (*Phaseolus vulgaris*) "Black Turtle Soup" (Provvidenti *et al.*, 1983); and cowpea cultivar "Pinkeye Purple Hull BVR" (Strniste, 1987). has been reported. Although we do not find any report on polygeneic pattern of inheritance against BlCMV in cowpea in the literature, but there are a few reports on

quantitative inheritance of resistance against plants viruses in other crop species (Bashir and Ahmad 1995).

Although there are more chances for the breakdown of resistance/immunity controlled by major genes (vertical resistance) by evolution of new virulent virus strains with the passage of time than the resistance controlled by polygenes (horizontal resistance), but it is more convenient to transfer vertical resistance/immunity than horizontal resistance to develop improved cultivars.

Thus high level of resistance (immunity) to BlCMV in the six cowpea lines can be exploited easily in breeding programmes designed to transfer BlCMV-resistance to desirable commercial cowpea cultivars and to aid in the control of cowpea stunt disease which is caused by mixed infection of BlCMV and CMV under field conditions (Kuhn, 1990). It is interesting to mention that all the immune TVu lines which we tested in this study are also resistant to a local isolate of cowpea aphid-borne mosaic virus (CABMV) (Personal communication by Dr. Bashir : data not yet published).

# LITERATURE CITED

# LITERATURE CITED

Ahmad, M. 1978. Whitefly (*Bemisia tabaci*) transmission of a yellow mosaic disease of cowpea (*Vigna unguiculata*). Plant Dis. Rep. 62:224-226.

Anderson, C. W. 1955. *Vigna* and *Crotalaria* viruses in Florida II. Notations concerning cowpea mosaic virus (*Marmor vignae*). Plant. Dis. Rep. 39:349-353.

Arif, M. 1995. Studies on fungus transmission and molecular pathology of potato mop top furovirus. Ph. D. Thesis. The University of Edinburgh. UK.

Bashir, M. and R. O. Hampton. 1992. Biological characterization of pathotypes of blackeye cowpea mosaic and cowpea aphid-borne mosaic potyviruses. Abstracts of Presentation. Abstract No. A-392. APS/MSA Joint Annual Meeting held in Portland, Oregon, U.S.A. August 8-12, 1992.

Bashir, M. 1992. Serological and biological characterization of seed-borne isolates of blackeye cowpea mosaic and cowpea aphid-borne mosaic potyviruses in *Vigna unguiculata* (L.) Walp. Ph.D. Thesis OSU/USA.

Bashir, M. and R. O. Hampton. 1993. Natural occurrence of five seed-borne cowpea viruses in Pakistan. Plant Diseases. 77:948-951.

Bashir, M., Zahoor, A., Zafar, A. and B. A. Malik. 1995. Sources of immunity in cowpea (*Vigna unguiculata*) against blackeye cowpea mosaic potyvirus. Phytopath. 7(2):94-97.

Bashir, M. and R. O. Hamptom. 1994. Seed and aphid transmission of isolates of blackeye cowpea mosaic and cowpea aphid-borne mosaic potyviruses. Pak. J. Phytopath. 6(2):140-146.

Bashir, M. and W. Ahmad. 1995. Resistant genes in some selected crops to potyviruses - A review. Science, Technology and Development. Pakistan Council for Science & technology. vol. 14(4):37-43.

Bock, K. R. 1973. East African strains of cowpea aphid-borne mosaic virus. Ann. Appl. Biol. 74: 75-83.

Bock, K. R. and M. Conti. 1974. Cowpea aphid-borne mosaic virus. No. 134. In: Description of Plant Viruses, Commonwealth Mycol. Inst., Kew, Surrey, England. 4 pp.

Carangal, V. R., A. C. S. Morales and E. C. Dranddiano. 1979. Cowpea (*Vigna unguiculata*) research in the Asian Cropping Network and the Philippines. Proc. Conf. IITA, Ibadan, Nigeria.

Chang, C. A. 1983. Rugose mosaic of asparagus bean caused by dual infection with cucumber mosaic virus and blackeye cowpea mosaic virus. Plant Protection Bulletin (Taiwan). 25:117-190.

Cobley, L. S. and W. M. Steele. 1975. An Introduction to the Botany of Tropicals. Longman, London. 371 pgs.

Collins, M. H., J. F. Murphy., W. Witcher and O. W. Barnett. 1984. Survey of cowpeas in South Carolina for six viruses. Plant Disease. 68:561-563.

Collins, M. H., W. Witcher., O. W. Barnett and W. L. Ogle. 1985. Reactions of 16 cowpea cultivars to six viruses. Plant Disease. 69: 18-20.

Corbett, M. K. 1956. Serological and morphological relationships of plant viruses. Fla. Univ. Agri. Expt. Stn. Annual Report. pp. 117-118.

Dougherty, W. G. and T. C. Carrington. 1988. Expression and function of potyviral gene products. Ann. Rev. Phytopath. 26:123-143.

Edwardson, J. R. and R. G. Christie. 1986. Viruses infecting forage legumes. Vol. II. Monograph No. 14. Agri. Expt. Stn. Univ. of Fla., Florida. Gainesville. U.S.A.

Fraser, R. S. S. 1989. Control of plant viruses. Plants Today. 2:100-105.

Fraser, R. S. S. 1990. The genetics of resistance to plant viruses. Annu. Rev. Phytopath.. 28:179-200.

Gomes, K. A. and A. A. Gomes 1984. Chi-square test. (Chapter -11). Statistical Procedure for Agricultural Research, (2nd Edition). In International Rice Research Institute Book. John Wily and Sons. pp: 458-478.

Harrison, A. N. and R. T. Gudauskas. 1968. Effects of some viruses on growth and seed production of two cowpea cultivars. Plant Dis. Rep. 52:509-511.

Hino, T. 1960. Studies on asparagus bean mosaic virus. Ann. Phytopath. Soc. Japan. 25: 178-186.

Hobbs, H. A., D. V. R. Reddy, R. Rajeshwari, and A. S. Reddy. 1987. Use of direct antigen and protein A coating ELISA procedure for detection of three peanut viruses. Plant Disease. 71(8):747-749.

Hollings, M. and A. A. Brunt. 1981a Potyviruses, pp 732-777. In "Handbook of Plant Viruses Infections and Comparative Diagnosis ed. E. Kurstak,. New York; Elsevier/North Holland. Biomedical.. 943, pp.

Hollings, M. and A. A. Brunt. 1981b Potyvirus Group. CMI/AAB. Description of Plant Viruses. No. 245.

Kay, D. E. 1979. Food Legumes. Crop and Product Digest, No. 3. Tropical products Institute, London.

Kippes, N. S. 1970. Production of Field Crops. A Text Book of Agronomy .Tata-McGraw Hill, Bombay, India.

Kuhn, C. W., B. B. Brantley. and G. Sowell Jr. 1965. Immunity to bean yellow mosaic virus in cowpea. Plant Dis. Rep. 49:879-881.

Kuhn. C. W., B. B. Brantley. and G. Sowell Jr. 1966. Southern pea viruses: Identification, symptomology and sources of resistance. Georgia Agric. Expt. Stn. Bull. Athens, Georgia, U.S.A. 157. 22 pp.

Kuhn, C. W., D. Sowell Jr., J. H. Chalkey and H. F. Stubbs. 1968. Screening for immunity to peanut mottle virus. Plant Disease. 52:467-469.

Kuhn, C. W. 1990. Cowpea virus diseases in the United States: A status report. pp :7-23. In: "Cowpea Research" a U.S. perspective. Proceedings of the Second Southern Pea (cowpea) Workshop held at Nashville, TN. Feb. 6, 1989. Miller, J. Jr., Miller, T. P. and Fery, R. L. (Eds.). 80 pgs.

Ladipo, J. L. 1977. Seed transmission of cowpea aphid-borne mosaic virus in some cowpea cultivars. Nigerian Journal of Plant Protection. 3:3-10.

Ladipo, J. L. and D. J. Allen. 1979. Identification of resistance to cowpea aphid-borne mosaic virus. Trop. Agric. (Trinidad) 56:353-358.

Lima, J. A. A., D. E. Purcifull. and E. Hiebert. 1979. Purification, partial characterization, and serology of blackeye cowpea mosaic virus. Phytopathology. 69:1252-1258.

Lima, J. A. A. and D. E. Purcifull. 1979. Tecnicas sorologicas de simples difusao em agar e de microscopia electronica para identificacao de virus do caupi. Fitopatologia Brasileira. 4:299-308.

Lin, M. T., A. A. Santos. and E. W. Kitajima. 1981. Host reactions and transmission of two seed-borne cowpea virus strains in Central Brazil. Fitopathologia Brasileira. 6:193-203.

Mali, V. R. and K. S. Kulthe. 1980. A seed-borne potyvirus causing mosaic of cowpea in India. Plant Disease. 64:925-928.

Mali, V. R., P. V. Khalikar. and D. H. Gaushal. 1983. Seed transmission of poty and cucumo viruses in cowpea in India. Indian Phytopathology. 36:343.

Mali, V. R. and G. Thottappillay. 1986. Virus diseases of cowpea in the tropics. Tropical Plant Pathololgy. 3:361-403.

Mali, V. R., G. E. Mundhe., N. S. Patil. and K. S. Kulthe. 1988. Detection and identification of blackeye cowpea mosaic and cowpea aphid-borne mosaic viruses in India. Int. J. Tropical Plant Diseases. 6:159-173.

Matthews, E. E. F. 1982. Classification and Nomenclature of Viruses. 4th Report of the International Committee for the Taxonomy of Viruses. Karger Basel.

Murphy, J. F. 1984. Viruses infecting cowpea in South Carolina and characterization of a blackeye cowpea mosaic strain. MS. Thesis. Clemson University. U.S.A. 84 pgs.

Murphy, J. F., Barnett, O. W. and W. Witcher, 1987. Characterization of a blackeye cowpea mosaic virus strain from South Carolina. Plant Disease. 71:243-248.

Pio-Ribeiro, G., S. D. Wyatt. and C. W. Kuhn. 1978. Cowpea stunt: a disease caused by a synergistic interaction of two viruses. Phytopathology. 68:1260-1265.

Provvidenti, R., D. Gousalves and M. A. Taiwo. 1983. Inheritance of resistance to blackeye cowpea mosaic virus and cowpea aphid-borne mosaic virus in *Phaseolus vulgaris*. The Journal of Heridity. 74:60-61.

Purcifull, D. and D. Gonsalves. 1985. Blackeye cowpea mosaic virus. CMI/AAB. Description of Plant Viruses. No. 305.

Quattara, S. and O. L. Chambliss. 1991. Inheritance of resistance to blackeye cowpea mosaic virus in " White Acre-BVR" cowpea. Hort. Science. 26:194-196.

Rachie, K. G. and L. M. Roberts. 1974. Grain legumes of the low land tropics. Adv. Agron. 26:1-32.

Stantion, W. R. 1966. Grain Legumes in Africa. FAO, Rome.

Shahjahan, M., D. Begum and M. C. Islam 1981. Biological nitrogen fixation by grain legumes. Proc. National Workshop on Pulses. BARI/IDRC, Dhaka.

Sekar, R. and C. B. Sulochana. 1983. Blackeye cowpea mosaic virus: Occurrence in India. Indian J. Plant Pathology. 1:38-44.

Strickberger, M. W. 1969. Probability and Statistical testing. (Chapter-8) p:126-152. In Genetics. The Macmillan Company. Collier-Macmillan Canada, Ltd. Ontario, USA.

Strniste, P. B. 1987. The inheritance and assessment of a second quantitative gene for blackeye cowpea mosaic virus in southern pea, *Vigna unguiculata* (L.) Walp. M.Sc. Thesis. Auburn Univ. Auburn, Alabama, U.S.A.

Taiwo, M. A. 1978. Sources of resistance in cowpea (*Vigna unguiculata* (L.) Walp. to cowpea aphid-borne mosaic and cowpea mosaic viruses. M.Sc. Thesis. Cornell University, USA.

Taiwo, M. R., R. Provvidenti and D. Gonsalves. 1981. Inheritance of resistance to blackeye cowpea mosaic virus in *Vigna unguiculata.* J. of Heridity. 72:433-434.

Taiwo, M. A., D. Gonsalves., R. Provvidenti and H. D. Thurston. 1982. Partial characterization and grouping of isolates of blackeye cowpea mosaic and cowpea aphid-borne mosaic viruses. Phytopathology. 72:590-596.

Taiwo, M. A. and D. Gonsalves. 1982. Serological grouping of isolates of blackeye cowpea mosaic and cowpea aphid-borne mosaic viruses. Phytopathology. 72:583-589.

Thottappillay, G. and H. W. Rossel. 1985. Worldwide occurrence and distribution of virus diseases. pp: 155-171. In: "Cowpea Research, Production and Utilization". Singh, S. R. and Rachie, R. O. (Eds.). John Wiley and Sons, Ltd. Chichester, England.

Tsuchizaki, T., T. Senboku., M. Iwaki., S. Pholauporn., W. Srithongchi., N. Deema. and C. A. Ong. 1984. Blackeye cowpea mosaic virus from asparagus bean (*Vigna sesquipedalis*) in Thailand and Malaysia, and their relationship to a Japanese isolate. Phytoptopathol. Soc. Japan. 50:461-468.

Uyemoto, J. K., R. Provvidenti and D. E. Purcifull. 1973. Host range and serological properties of a seed-borne cowpea virus. Phytopathology. 63:208-209. (Abstract).

Walker, C. A. Jr. and O. L. Chambliss. 1981. Inheritance of resistance to blackeye cowpea mosaic virus in cowpea (*Vigna unguiculata* (L.) Walp.). J. Amer. Soc. Hort. Sci. 106:410-412.

Yen, D. E. and P. R. Fry 1956. The inheritance of immunity to pea mosaic virus. Aust. J. Agri. Res. 7:272-281.

Zhao, G. S., D. D. Baltensperger., D. E. Purcifull, R. G., Christie., E. Hiebert. and J. R. Edwardson. 1991. Host range, cytology, and transmission of an alyce-clover isolate of blackeye cowpea mosaic virus. Plant Disease. 75:251-253.