STUDIES ON THE SEEDBORNE NATURE OF URDBEAN LEAF CRINKLE VIRUS (ULCV) IN URDBEAN (*Vigna mungo* L. Hepper)

Thesis

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By

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Dedicated

to

My Beloved Parents

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CERTIFICATE

This is to certify that the thesis entitled **"STUDIES ON THE SEEDBORNE NATURE OF URDBEAN LEAF CRINKLE VIRUS (ULCV) IN URDBEAN (*Vigna mungo* L. Hepper)"**, submitted in partial fulfilment of the requirements for the degree of **DOCTOR OF PHILOSOPHY** with major in **Plant Pathology** and minor in **Entomology** of the College of Post-Graduate Studies, G. B. Pant University of Agriculture and Technology, Pantnagar, is a record of **bona fide** research carried out by **Mr. HIMANSHU NEGI, ID. No. 26622,** under my supervision, and no part of the thesis has been submitted for any other degree or diploma.

The assistance and help received during the course of this investigation and source of literature have been duly acknowledged.

(K. Vishunavat)
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CERTIFICATE

We, the undersigned, members of the Advisory Committee of **Mr. HIMANSHU NEGI, ID. No. 26622,** a candidate for the degree of **DOCTOR OF PHILOSOPHY** with major in **Plant Pathology** and minor in **Entomology,** agree that thesis entitled **"STUDIES ON THE SEEDBORNE NATURE OF URDBEAN LEAF CRINKLE VIRUS (ULCV) IN URDBEAN (*Vigna mungo* L. Hepper)"**, may be submitted in partial fulfilment of the requirements for the degree.

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CONTENTS

Introduction

One INTRODUCTION

Grain legumes are the most important nutritional component of Indian diet for vegetarian and also for poor among the population. Black gram (*Vigna mungo* L. Hepper), commonly known as urdbean is one of the 13 different grain legumes grown in India. It belongs to the family "Leguminosae" and has been called by different names such as urd, mash, urid and urad etc. In India urdbean has been cultivated since ancient times. India is a primary centre of origin of urdbean and central Asia as a secondary centre. According to Zukovskij (1962) urdbean originated from its wild progenitor *Phaseolus sublobatus* in India.

Urdbean is consumed in various forms, as dal (whole or split, husked and unhusked or parched), the whole plant is used as fodder for cattle and is also a green manure crop. The crop plants possess deep root system which binds soil particles and thus, prevents soil erosion. Many delicious food items can be prepared from urdbean eg. dosa, idli, curry, papad, bari (spiced balls), pudding (halwa) and imrati (a delicious sweet).

Urdbean seeds are a good source of proteins, minerals and energy. The reported value of protein content is about 24 per cent, fat 1.4 per cent, moisture 9.7 per cent, minerals 3.2 per cent and carbohydrates 57.3 per cent, respectively (Aykroyd and Doughty, 1964).

In India, urdbean is cultivated as summer, kharif and winter crop. The major states of urdbean production are Madhya Pradesh, Uttar Pradesh, Uttaranchal, Punjab, Maharashtra, West Bengal, Andhra Pradesh, Tamil Nadu and Karnataka. It is cultivated over 3.26 million hectare area with total production of 1.40 million tonnes and productivity of 455 kg/ha in India (Anonymous, 2003).

The crop is highly prone to a number of pathogens viz. fungi, bacteria, viruses and nematodes etc. which are responsible for its low production. Important diseases of urdbean affecting its productivity are powdery mildew (*Erisiphe polygoni*), anthracnose (*Colletotrichum lindemuthianum*), leaf spot (*Cercospora canescens*), rust (*Uromyces appendiculatus*), dry root rot (*Rhizoctonia bataticola*), bacterial blight (*Xanthomonas campestris* pv. *phaseoli*), leaf crinkle (urdbean leaf crinkle virus), yellow mosaic (mungbean

yellow mosaic virus), black gram mottle virus and bean common mosaic virus. Of the viruses reported, urdbean leaf crinkle virus (ULCV) seems much prevalent and in recent years has become a potential threat to the cultivation of urdbean crop in many states of India, as most of the high yielding varieties are susceptible to this virus.

Urdbean leaf crinkle disease was first reported from Delhi and Uttar Pradesh in the year 1966 by Williams *et al.*, (1968). Later in 1967, the disease appeared in *tarai* region of UP (Kolte and Nene, 1970). These workers, for the first time named the disease as urdbean leaf crinkle disease, proved the infectious nature of the pathogen and designated it as urdbean leaf crinkle virus (ULCV). There is no information available on the occurrence of the disease in any other country of the world except India (Williams *et al.*, 1968), Sri Lanka (Shivanathan, 1980) and Pakistan (Bashir *et al.*, 1991).

The most conspicuous symptoms of the disease are wavy appearance on the third trifoliate leaf followed by typical crinkling, shortening of petiole of the central leaflet and thickening of leaf vein. The diseased trifoliates increase in size, become leathery and green in colour than normal ones and show downward curling of

leaf margins. The plants infected at an early stage remain sterile due to stunted growth of flower buds resulting in sterility of inflorescence.

Though, the disease is restricted in its occurrence, it is economically important. The yield losses estimated may go up to 100 % depending upon the stage at which plants become infected (Nene, 1973; Singh, 1980; Kadian, 1982; Brar and Rataul, 1989 and Bashir *et al.*, 1991).

Urdbean leaf crinkle virus is both seed as well as sap transmissible (Kolte and Nene, 1972; Nene, 1973; Beniwal and Chaubey, 1979; Bhaktavatsalam *et al.*, 1983b; Beniwal *et al.*, 1984; Dubey and Sharma, (1985) and Brar and Rataul, (1986). Of the various methods, transmission of virus through seed is of considerable ecological significance for virus perpetuation, perennation and dissemination, as well as economic consequence for the plant grower. Virus perpetuation by infected seeds is seemingly the perfect survival strategy since it serves as a protective link between crop growing seasons.

Keeping in view the immense importance of seedborne inoculum of urdbean leaf crinkle virus as a sole source of primary

inoculum in the field, the present investigations were undertaken to study

(i) The detection of seedborne inoculum of urdbean leaf crinkle virus in urdbean seeds.

(ii) The location of the seedborne inoculum of urdbean leaf crinkle virus in different flower and seed parts of urdbean.

(iii) Seed transmission of urdbean leaf crinkle virus under field and glass house conditions.

(iv) The influence of mother plant infection on flower organelles and seed quality.

(v) Yield losses in urdbean due to the seedborne inoculum of urdbean leaf crinkle virus.

(vi) Purification and molecular weight of coat protein of urdbean leaf crinkle virus.

Review of Literature

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REVIEW OF LITERATURE

The literature pertaining to the various aspects of urdbean leaf crinkle virus is being reviewed in the following paragraphs.

2.1 Occurrence

Urdbean leaf crinkle, a disease of urdbean caused by urdbean leaf crinkle virus (ULCV) was first reported by Williams *et al.*, (1968) from Delhi. Since then it has been reported from various urdbean growing states of India viz. Uttar Pradesh (Nene, 1968; Kolte and Nene, 1970; Beniwal and Chaubey, 1979), Punjab (Khatri *et al.*, 1971; Brar and Rataul, 1986), Tamil Nadu (Narayanasamy and Jaganathan, 1975), Haryana (Singh, 1980; Kadian, 1983), Himachal Pradesh (Gupta, 1974; Dubey and Sharma, 1985), Gujarat (Mishra *et al.*, 1994; Patel *et al.*, 1999), Maharashtra (Mahajan and Joi, 1999), and North Eastern Hill Region of India (Sahay *et al.*, 1999). Apart from India, this disease has also been reported from Sri Lanka (Shivanathan, 1980) and Pakistan (Bashir *et al.*, 1991).

2.2 Symptomatology

According to Williams *et al.*, (1968) and Nene (1968) the urdbean leaf crinkle virus infected plants remained stunted,

developed rugosity, showed crinkling on affected leaves and produced few pods under field conditions. Khatri *et al.*, (1971) reported that the disease is characterized by leaf crinkling, reduction in leaf size and witches broom etc.

Kolte and Nene (1970, 1972) and Kolte (1971) described the symptoms of urdbean leaf crinkle virus in great detail both under natural and artificial conditions.

They stated that initial symptoms of the disease appeared three to four weeks after sowing on the third trifoliate leaf and were characterized by an increase in the size and a lighter green colour before typical crinkling became conspicuous a week later. The affected trifoliates showed enlargement of the leaflet followed by crinkled surfaces of the laminae. After the appearance of initial symptoms the affected trifoliates particularly 4^{th}, 5^{th} and 6^{th}, curved downward. In case of severely affected plants, two free stipules at the base of the affected trifoliate became thicker and broader than the normal ones. Stipules at the base of the terminal leaflets of the affected trifoliate were rudimentary or absent. The most conspicuous symptom was that the petiole behind the pulvinus of the terminal leaflet became so short that the basal portion of lamina touched the surfaces of the two side leaflets. In affected plants flowering was delayed by 8 to 10 days. The

peduncle of the inflorescence produced from the axil of the affected trifoliate bore large number of small sized flower buds. The sepals of the flower bud became thick, greener and covered half or all the buds giving a bushy appearance to the inflorescence.

ULCV adversely affected the pollen fertility. Kolte and Nene (1979) reported abnormal pollen grains to be present in the anthers of affected buds which contained 10 per cent sterile pollen. This resulted in reduction in pod formation to 41.77 per cent in diseased plants as against 84.96 per cent in healthy plants.

Beniwal and Chaubey (1979) found that infection by ULCV significantly affected number of pods per plant and pods from diseased plants produced shrivelled and light brown coloured seeds.

Brar and Rataul (1986) described the most characteristic symptoms of the disease as wavy appearance on the third trifoliate followed by typical crinkling, shortening of petiole of central leaflet and thickening of leaf veins. The leaf area of the diseased trifoliate was more than that of healthy ones. Presence of mixed infection of yellow mosaic virus and leaf crinkle virus together and partial infection of plants were the additional symptoms described by them.

Bashir *et al.*, (1991) reported that infection with ULCV reduced plant height by 8 per cent and decreased pod length, number of pods per plant, number of seeds per pod by 18.9 and 26 per cent, respectively.

2.3 Host Range

Urdbean is not the only host of leaf crinkle virus, but the virus has been found to infect *Phaseolus vulgaris, P. aureus, Nicotiana tabacum* (Gupta, 1974); *Vigna radiata, V. unguiculata, V. aconitifolia* (Kolte and Nene, 1975); *Cyamopsis tetragonoloba, Arachis hypogaea* (Narayanasamy and Jaganathan, 1974), *Cucumis sativus, Lagenaria cylindrica* (Beniwal *et al.*, 1983b), *Convolvulus arvensis, Datura* spp. (Kadian, 1983), *V. mungo, V. unguiculata, Vigna radiata, Cyamopsis tetragonoloba, Arachis hypogaea, Luffa aegyptica, Cucumis melon, Cucurbita maxima* and *Nicotiana tabacum* (Patel *et al.*, 1999).

Patel (1999) reported pumpkin and sponge gourd of Cucurbitaceae family as the new host for urdbean leaf crinkle virus in Gujarat.

2.4 Morphological Changes in ULCV Infected Plants

Kolte and Nene (1979) reported abnormal pollen grains in the anthers of ULCV affected plants.

Sharma and Dubey (1983) described the effect of disease on flower such as degeneration of androecium and gynoecium, incomplete development of pollen grains in pollen tetrads, non formation of ovules and abnormal ovarian cavity.

2.5 Anatomical Changes

Bhaktavatsalam *et al.*, (1983a) found virus like particles (VLP) in the nucleus, cytoplasm and chloroplasts of infected blackgram leaves. However, healthy cells were devoid of such particles. The VLP appeared to be spherical with a diameter of 25 to 30 nm. Hypertrophy of infected cells was observed. Some mitochondria were observed to be filiform as compared to normal spherical ones in healthy cells.

Sharma and Dubey (1985) reported several anatomical changes in the urdbean roots induced by the ULCV. The cells of epiblema, cortex, pith and endodermis were enlarged at the severely crinkled stage. This enlargement was observed to be less apparent in endodermis. They found that contrary to this, the virus caused reduction in cell size of phloem and xylem which was much conspicuous in xylem. Epiblema, cortex, endodermis, medullary rays and pith were found thickened. The pith region in diseased root appeared more pronounced with disintegrated

cortical and endodermal cells which were found to be deformed and irregularly arranged.

Patel (1999) observed the increase in leaf area and number of stomata in leaves of diseased plant.

2.6 Biochemical Changes

Sivaprakasam *et al.*, (1976) observed that leaf crinkle virus infection increased the accumulation of phosphorous and potassium content in the black gram leaves but there were reductions in the nitrogen, calcium and magnesium content in infected leaves.

Bhaktavatsalam *et al.*, (1982) reported a reduction in tryptophan and increase in the IAA (Indole Acetic Acid) in the leaves of *Vigna mungo* infected by urdbean leaf crinkle virus (ULCV). They postulated that reduction in tryptophan in ULCV infected leaves may be due to its utilization for the biosynthesis of IAA, as tryptophan is precursor for IAA. The increased IAA content in diseased leaves indicate that IAA might be involved in leaf expansion and distinct crinkling of the leaves.

Brar and Rataul (1990) observed that the total sugar content increased in plants infected with ULCV. The amino acid content of infected young leaves increased by 14.57 and 14.7 per cent at the

initial and late growth stages of diseased plants, while in old leaves it decreased by 22.7 and 16.2 per cent. Protein content decreased by 7.59 and 12.05 per cent at the initial stage infection of young and old leaves, respectively. However, it decreased more i.e. 16.1 and 17.09 per cent at the late stage of infection. The chlorophyll content reduced in infected young leaves but increased in older leaves.

Malik *et al.*, (2002) reported that urdbean variety T 9 susceptible to urdbean leaf crinkle virus showed enhanced total chlorophyll content and total sugar but reduced total phenol, total protein content and nitrate reductase (NR) activity.

2.7. Physical Properties of Urdbean Leaf Crinkle Virus

The physical properties of ULCV have been studied by several workers.

2.7.1 Thermal Death Point

Various workers reported different Thermal Death Point (TDP) of their isolates of ULCV such as 70-75°C (Gupta, 1974), 60°C (Narayanasamy and Jaganathan, 1974) and 60-70°C (Kolte and Nene, 1975). Dubey *et al.*, (1983) reported TDP as 60-75°C, whereas, Patel *et al.*, (1999) reported that the TDP of ULCV was 63-65°C.

2.7.2 Dilution End Point

The Dilution End Point (DEP) of ULCV was reported as 1:1000 by Gupta (1974); Kolte and Nene (1975), 1:10000 by Kadian (1980), between 1:1000 to 1:100000 by Dubey *et al.*, (1983). However, Narayanasamy and Jaganathan (1974) reported DEP as 1:50000 and Patel *et al.*, (1999) as 1:100000, respectively.

2.7.3 Longevity *in vitro*

The Longevity *in vitro* (LIV) of ULCV has been reported to be 2 days at room temperature (Gupta, 1974; Narayanasamy and Jaganathan, 1974), while Kolte and Nene (1975) and Kadian (1980) observed it to be 3 days at room temperature and 9 days at 5 ^{0}C. Dubey *et al.*, (1983) reported *in vitro* longevity of ULCV up to 5 days at room temperature, whereas ULCV was infective up to 108 hours at room temperature and 156 hrs at 5^{0}C temperature (Patel *et al.*, 1999).

2.7.4 Effect of Various Chemicals on Infectivity of ULCV

Bhaktavatsalam *et al.*, (1983b) reported the infectivity of ULCV to be pH dependent with a maximum at 7.8. They reported that calcium tribasic and dibasic phosphates and EDTA completely inhibited virus infectivity, whereas, magnesium sulphate had no effect. Actinomycin D and cyclohexamide

decreased infectivity but tetracycline hydrochloride did not affect it. Phenol treatment considerably reduced viral infectivity index suggesting that ULCV is not a viroid. ULCV is ether sensitive and does not possess lipids. They further observed that addition of 5% sucrose +1% mercaptoethanol to inoculum increased infectivity as well as longevity of ULCV.

Chowdhury and Chowdhury (1983) studied the infectivity of sap extracted with different buffers and also at different pH from *Vigna mungo* plants infected with urdbean leaf crinkle virus.

Malik and Rathi (2002) conducted the experiment to determine the effect of additives on the infectivity of ULCV and reported that sucrose (5%), mercaptoethanol (0.5%), sucrose + mercaptoethanol (5 + 0.5%), disodium ethylene dithiocarbamate (1.5%), thioglycolic acid (0.15%), DIECA +thioglycollic acid (1.5 + 0.15%), ethylene diamine tetraacetate (1.5%) and DIECA + EDTA (1.5 +1.5%) significantly increased the disease transmission, while DIECA +thioglycollic acid gave the highest transmission (73.3%).

2.8 Purification of Urdbean Leaf Crinkle Virus

Bhaktavatsalam (1976) developed a modified polyethylene glycol (PEG) method to purify ULCV. Two combinations of PEG and NaCl i.e. 6% PEG + 0.1 M NaCl and 8% PEG + 0.1 M NaCl

precipitated virus which resulted in 66.7 per cent infectivity. Concentrations of NaCl above 0.1 M were found to be lethal for ULCV infectivity. He separated host proteins and nucleic acid from ULCV by using cellulose column chromatography. He worked out that the ULCV contained 17 per cent nucleic acid.

2.9 Properties of Purified Virus

Bhaktavatsalam (1976) observed virus like particles (VLP) in the infected cells of urdbean leaves, scattered throughout the chloroplast and cytoplasm as dense particles. These particles were spherical in shape and 25 to 30 nm in diameter similar to turnip crinkle virus. He suggested that VLP might develop inside the nucleolus and pass into the cytoplasm through nuclear pores.

Kadian (1980) observed spherical viral particles with an average diameter of about 50 nm consisting of 15.24 per cent nucleic acid. This virus has similarities with pea enation mosaic virus.

Dubey *et al.* (1983) reported that electron microscopic observations of purified virus preparation and 'leaf dip method' revealed the particles to be isometric with an average diameter of 32 nm.

Patel *et al.*, (1999) observed a single light scattering zone (white opaque band of 2 mm) of purified virus after density

gradient centrifugation and purified virus suspension showed isodiametric virus particles in electron microscopy. The average size of virus particles was approximately 16-20 nm. Molecular weight of coat protein of urdbean leaf crinkle virus was found to be 28 k Da.

2.10 Serological Relationship

Dubey *et al.*, (1983) reported that virus did not show any serological relationship with the antisera of eleven isolates of viruses belonging to bromovirus (3 isolates of broad bean mottle virus), potyvirus, comovirus and black gram leaf mottle virus. Similarly, Beniwal and Bharathan (1980) also got negative serological relationship of ULCV with some members of comovirus, tymovirus, bromovirus and potyvirus groups.

Serologically ULCV could not be related to bean pod mottle, bean rugose mosaic, bean yellow stripe, black gram mottle, cowpea chlorotic mottle, southern bean mosaic, squash mosaic, turnip crinkle and turnip yellow mosaic virus (Beniwal and Nene, 1990).

Patel *et al.*, (1999) observed the mild reaction of ULCV with SqMV (Squash mosaic virus) in ELISA and in western blot.

2.11 Transmission of Urdbean Leaf Crinkle Virus

Like many other viruses, urdbean leaf crinkle virus spreads mainly through sap, seed and insect vectors.

2.11.1 Mechanical Transmission

Kolte and Nene (1972) were the first to report successful mechanical transmission of ULCV using 0.1 M potassium phosphate buffer, pH 7.6 and claimed that the leaf crinkle is caused by a virus. When primary leaves of 6 days old plants were inoculated, the transmission was successful to the extent of 100 per cent. Later, Bindra (1971); Khatri *et al.*, (1971); Gupta (1974); Narayanasamy and Jaganathan (1974); Beniwal *et al.*, (1983b); Kadian (1983); Patel (1995, 1999) also reported the sap transmissibility of the urdbean leaf crinkle virus.

2.11.2 Seed Transmission

Kolte (1971) and Kolte and Nene (1972) reported 18.39 per cent transmission of ULCV through seeds in urdbean cultivar T 9.

Gupta (1974) also observed seed transmission of ULCV in addition to sap and graft transmission.

Narayanasamy and Jaganathan (1975) observed that plant susceptibility and percentage of seed transmission of the ULCV

reduced as the age of plants increased. Higher percentage of infection in young infected plants induced a higher rate of seed transmission.

According to Beniwal *et al.*, (1980, 1983a) seed transmission of urdbean leaf crinkle virus ranged from 0-15 per cent in different germplasms and varieties of urdbean. Plant age at the time of infection affected seed transmission as higher percentage of transmission occurred in plants infected at early growth stage than those infected later in the season. Seed transmission of ULCV was not affected by morphological abnormalities in seed, different stages of seed maturation and presence or absence of seed coat.

Chowdhury and Nath (1983) reported that ULCV could be inoculated through germinated seeds by shaking the seeds with prepared viral suspension for different time interval. Plant emerged from these seeds developed symptoms being highest after 30 second shaking.

Beniwal and Chaubey (1984) could detect ULCV in all the floral and seed parts. The virus was found distributed in all the five parts of flower i.e. epicalyx, calyx, corolla, adroecium and gynoecium. Similarly, it was detected in all the three parts of the

seed i.e. seed coat, cotyledon and primary axis, hence indicating the internal seedborne nature of virus.

Beniwal *et al.*, (1984) proposed that dry seed examination of urdbean could serve as a good indication of presence of the virus if oversized seeds are found in the seed lot and infection could be further confirmed by growing on and indicator inoculation test.

Dubey and Sharma (1985) confirmed that ULCV was seed transmitted up to 17.6 per cent in naturally infected plants. The virus survived in cotyledons and embryos of infected seeds and did not affect germination. They also found that highest transmission of 68 per cent occurred in seeds from 10 days inoculated plants. No transmission was obtained through seeds collected from 50 days old inoculated plants.

Brar and Rataul (1986) found ULCV to be seed transmissible to the extent of 77.64 and 45 per cent in severely diseased and partially diseased plants, respectively.

Kadian (1994) reported that the virus was seed transmitted (21%) and transmission rate increased when infected seeds were continuously reused. The transmission rate was higher in seeds collected from mechanically sap inoculated one week old plants and decreased with the plant age. No transmission occurred from seeds of the plants inoculated 7 weeks after sowing.

Mishra *et al.*, (1994) reported seed transmission of ULCV from Gujarat. The percentage varied from 2.0 to 31.25. Considerable reduction in pod size, number of seeds per pod and test weight in infected seeds of the *V. mungo* cv. T 9 was also observed.

Later on varying range of seed transmission viz. 1.16-16% (Mahajan and Joi, 1999), 10-30% (Patel *et al.*, 1999), 1-83% (Pushpalatha *et al.*, 1999) and up to 33% (Negi and Vishunavat, 2003) has also been reported by various workers.

2.11.3 Insect Transmission

Insects play an important role in spread of viral diseases by providing them attacking sites. There are contradictory reports in literature on the transmission of urdbean leaf crinkle virus by insects as various insect vectors of ULCV have been reported by different workers from different states of country.

Bindra (1971) reported an aphid *Aphis craccivora* and a leaf hopper *Circulifer tenellus* as the vector of the virus, whereas, Kolte (1971) failed to transmit the virus with black cowpea aphid *A. craccivora* and the cotton whitefly *Bemisia tabaci.*

Narayanasamy and Jaganathan (1973a) reported that the ULCV was transmitted by *B. tabaci* (25-67.5%) in Tamil Nadu.

According to Dhingra (1975) ULCV could be successfully transmitted by two aphid species viz. *A. craccivora* and *A. gossypii* with a short acquisition feeding period of 30 seconds to 2 minutes preceded by a pre-acquisition fasting which was necessary for successful viral transmission.

Beniwal and Bharathan (1980); Malik (1998) transmitted Pantnagar isolate of ULCV to urdbean plants through a beetle *Henosepilachna dodecagstigma.* They failed to transmit the virus by any of the insects previously reported as vectors such as aphids (*A. craccivora* and *A. gossypii*), leaf hopper (*Circulifer tenellus*) and white fly (*B. tabaci*).

Kadian (1980) reported from Haryana that the virus was readily transmitted through *A. craccivora* and *A. gossypii* in non-persistent manner.

Dhingra and Chenulu (1981) showed *Myzus persicae* to be an additional vector of urdbean leaf crinkle virus. They also confirmed the transmission of ULCV by *A. craccivora* in a non-persistent manner.

Dubey *et al.*, (1983) and Bhardwaj and Dubey (1984, 1986) reported the transmission of ULCV by *A. craccivora* and *Acyrthosiphon pisum* to the extent of 60 and 80 per cent, respectively. *A. pisum* was reported for the first time as an additional vector of the virus.

Nath *et al.*, (1986) reported the transmission of ULCV by *Lipaphis erysime* and *Hysteroneura setariae* in a non- persistent manner. They found that the highest percentage transmission occurred with an inoculation access time of 1 min.

Under field conditions, ULCV was found not having any relationship with the population of sucking insects (aphid and whitefly) observed on the crop (Brar and Rataul, 1987a).

Brar and Rataul, (1987b) tested four species of aphids viz. *A. craccivora, A. gossypii, Myzus persicae* and *Rhapalosiphum maidis*; a whitefly *B. tabaci*; a leaf hopper *Empoasca motti*; a beetle *Henosepilachna dodecagstigma* and a mite *Tetranychus telarius*, for their ability to transmit the virus in cage experiments. However, none of them could transmit the virus from diseased plants to the healthy ones.

Bhardwaj *et al.*, (1994) reported that plants of urdbean raised in clay or clay loam soil containing 7.67 per cent organic matter recorded highest transmission of ULCV by *A. craccivora.* Higher levels of phosphorous conferred immunity in urdbean plants to infection, whereas nitrogen created proneness.

According to Patel *et al.*, (1999) aphid (*A. gossypii*) transmitted the virus up to 40 per cent by acquiring virus from ULCV infected plants as well as by membrane feeding of purified virus preparation.

2.12 Epidemiology

Kolte (1971) reported that the ULCV affected plants appeared scattered in the field and also the spread of the disease was very slow.

Beniwal *et al.*, (1979) studied the nature and rate of spread of urdbean leaf crinkle disease under field conditions. According to them the low percentage (2.75%) of disease incidence provided an evidence for a very low rate of spread of the disease and most of the disease in field seems to develop by the seedborne virus or through rubbing of diseased leaves with healthy leaves might have served as a source of spread of the disease, particularly in the later part of the season.

Kolte and Nene (1979) reported that susceptibility of the urdbean plants to ULCV depended on the stage of plant growth. Inoculation at primary leaf stage produced 88.88 per cent infection and inoculation at first and second trifoliate leaf stage produced 71.11 and 66.66 per cent infected plants, respectively. Inoculation of the plants with the virus at sixth trifoliate and at post- bloom stages produced 23.14 and 10.74 per cent infected plants, respectively.

Kadian (1983) conducted a survey of urdbean (*V. mungo*) and mungbean (*V. radiata*) during 1978-1979 and revealed that

ULCV was less prevalent in summer crop than in kharif crop. It increased year by year throughout Haryana state and was more prevalent in northern region as compared to southern region.

Bansal *et al.*, (1984) during a survey of mung and mash observed that urdbean leaf crinkle virus and mungbean yellow mosaic viruses were fairly prevalent and ULCV was more common on urdbean than mungbean.

Brar and Rataul (1986) categorized the virus infected plants in to partially diseased and severely diseased plants. They concluded that the virus was not uniform in its distribution in the host plants; instead it moved erratically resulting in the crinkling of some branches and avoiding the others which looked apparently healthy.

2.13 Yield Losses

Kolte (1971); Nene (1973) and Kolte and Nene (1979) reported about 62 to 100 per cent yield losses in field grown urdbean plants due to urdbean leaf crinkle virus.

According to Beniwal and Chaubey (1979) ULCV infection significantly and adversely affected number of pods per plant and number of seeds per pod in urdbean cv. Pant Urd 30 and Pant Urd 26, whereas, 1000 seed weight remained unaffected.

Maximum yield reduction of 70.7 and 83.8 per cent was noticed when ULCV infection occurred at 10 days after planting in Pant U 30 and Pant U 26, respectively, which was however, progressively and significantly reduced with gradual delay in infection up to 60 days after planting. The infections at 60 DAP caused only 18.0 and 13.2 per cent losses in yield. The ULCV infection significantly affected the texture of urdbean seeds by increasing the percentage of shrivelled, oversized and brown coloured seeds.

Singh (1980) found that ULCV infection occasionally increased growth of the urdbean plants but caused a drastic decrease in yield.

Kadian (1982) reported that losses from leaf crinkle virus were 2.12 to 93.98 per cent in the *Vigna mungo* cv. Varsha and 2.82 to 95.17 per cent in *V. mungo* variety T 9. There was a significant decrease in yield in terms of pods per plant, seeds per pod and 1000 grain weight. He observed a direct correlation between the stage of plant growth at which infection occurred and loss in yield. The earlier the infection, the greater was the loss, which was mainly attributed to a reduction in the number of pods.

Beniwal *et al.*, (1984) observed that a temperature range of 20-37^{0}C with diffused light prevailing in the glass house during the month of July, August and September seemed to favour ULCV

infection and symptom development and expression only during these months.

Brar and Rataul (1989) recorded the yield losses due to ULCV in urdbean up to 84 per cent in severely infected and 55.2 per cent in partially infected urdbean plants.

Bashir *et al.*, (1991) reported that ULCV infection reduced plant height, number of pods per plant, pod length and number of seeds per pod by 8, 90, 18 and 26 per cent, respectively in urdbean. They found that on average, yield loss per plant was 81 per cent as compared with healthy plants.

Mishra *et al.*, (1994) reported a reduction of 0.6, 1.3 and 16.6 per cent in terms of pod size, number of seeds per pod and 1000 grain weight, respectively of ULCV infected urdbean plants.

Patel *et al.*, (1999) reported 28.9 per cent reduction in yield of urdbean plants as a result of ULCV.

2.14 Management

2.14.1 Thermotherapy

Beniwal *et al.* (1983a) reported that no seed transmission occurred in a seed lot receiving hot water treatment either at 60°C for 10, 20 and 30 min. or 70°C for 10 and 20 min. Dry heat

treatment at 70°C for 10, 20 and 30 min was also found equally effective. Sharma and Dubey (1984) found that the seedborne inoculum of ULCV was completely eliminated by treating urdbean seeds in a water bath for 30 minutes at 55°C without any adverse effect on seed germination.

2.14.2 Chemotherapy

Bhardwaj *et al.*, (1982) reported that drenching urdbean plants with benlate (benomyl) at 1% or more prior to inoculation mechanically or by *Aphis craccivora* and *Acyrthosiphon pisum* prevented symptom expression, however, less than 1 per cent concentration of benomyl was not so effective. Post inoculation drenching was reported to be less effective. The aphids failed to acquire the virus from infected plants already drenched with 2 per cent or more concentration of the fungicide.

Sharma and Dubey (1984) reported that pre inoculation drenching of urdbean plant with 10 per cent suspension of benlate or bavistin fully checked disease appearance, whereas, thiouracil and decoction of tea and coffee delayed the symptom appearance.

Malik (1998) indicated that topsin M and carbendazim significantly reduced the ULCV infection when used prior to

mechanical inoculation (pre inoculation drenching) as well as pre and post inoculation drenching.

2.14.3 Effect of Natural Oils and Plant Extracts

Chowdhary and Saha (1985) tested 15 crude plant extracts against ULCV and found that *in vitro*, ginger extract gave the highest percentage of inhibition after 1 hr incubation and turmeric after 2 hr. They also reported that the effect of 4 extracts on the virus *in vivo* was less marked than *in vitro* and onion had the greatest effect followed by turmeric, ginger and garlic.

Bhardwaj and Dubey (1986) also tested different plant oils for their effectiveness to prevent transmission of urdbean leaf crinkle virus by *Aphis craccivora.* They found that mustard, rapeseed, til (sesame) and groundnut oils at 1 per cent (foliar spray) reduced transmission of the virus significantly and 2.0 per cent (foliar spray) emulsions of rapeseed and sesame oils completely prevented the transmission of the virus.

Malik (1998) found neem oil to be the best inhibitor of seedborne inoculum of ULCV as it checked the symptom development.

According to Patel (1999) phyto extracts from *Clerodendrum inerme* gave higher inhibitory effect in all the three treatments (pre-inoculation, mixed-inoculation and post-

inoculation) i.e. 80, 90 and 35 per cent inhibition as compared to *Curcuma longa* which gave 60, 70 and 30 per cent inhibition, respectively of urdbean leaf crinkle virus.

Thirumalaisamy *et al.*, (2003) reported that extract from *Zingiber officinale*, *Piper longum* and *Prosopis juliflora* possessed the most potent anti ULCV properties.

2.14.4 Genetic Resistance

Germplasm screening work has been done by various workers by mechanical inoculation and under natural conditions.

Kolte (1971) tested 13 cultivars of urdbean against ULCV and found all of them to be susceptible.

Narayanasamy and Jaganathan (1973b) observed black gram varieties Karaikal, Mattikalai, Palladam, Parvathipuram, BR 16 and BR 68 to be highly resistant against ULCV with out showing any symptoms, while varieties Kahandur, PHM 8, P 33, P 49, P 58 and P 223 showed only less than 5 per cent infection of ULCV.

Kadian (1980) tested 338 varieties of urdbean for resistance against ULCV but only two varieties DLU-90 and DLU-487 were found resistant. Varieties HPU 19, 33, 55, 56, 72, 75, 91, 109, 167, 200, 232, 240, 246, 252, 264, 269 and 277 were found moderately resistant to ULCV infection.

Out of 280 varieties and germplasms screened by Sharma and Dubey (1984), urdbean cultivars HPU 27, 102, 164 and 315 were found to be highly resistant to ULCV.

Annapan *et al.*, (1988) observed that Co-5 was moderately resistant to ULCV besides powdery mildew (*Erysiphe polygoni*), tip blight and pod borer.

Iqbal *et al.*, (1991) screened 19 genotypes/ cultivars of mash selected from local races against urdbean leaf crinkle virus for two consecutive years (1988-1989) under natural infection conditions. Four genotypes S 210, MM 5-60, S 250 and Mash Sialkot were found resistant, while the others a moderate reaction to leaf crinkle virus disease.

Narendra Urd 1 was reported to be resistant against mungbean yellow mosaic virus (MYMV) and urdbean leaf crinkle virus (Singh and Singh, 1994).

According to Prasad *et al.*, (1998) out of 25 varieties of urdbean screened for ULCV, the maximum disease was observed in T 9-150 (39.5%) and no disease was recorded in case of NDU-94-6. Also the difference in the disease incidence between the varieties was found to be significant.

Malik (1998) observed minimum infection of ULCV (0.67%) in Pant Urd 35, while the infection was maximum (5.35%) in Pant Urd 30.

Joshi (1988) found 10 germplasm accessions viz. shU 9504, shU 9505, shU 9511, shU 9513, shU 9515, shU 9516, shU 9519, shU 9520, shU 9522 and shU 9528 to be resistant against ULCV infection both under field as well as glass house conditions.

According to Patel *et al.*, (1999) germplasm lines of urdbean i.e. GU-90-47, GU-90-60, GU-90-69, GU-90-741, GU-90-72 were found moderately resistant, while GU-90-54, GU-90-66, GU-90-61 as resistant and GU-90-44 highly resistant against ULCV infection.

Materials and Methods

Three MATERIALS AND METHODS

The details of materials used and procedures followed to carry out the present investigation on the "Seedborne nature of urdbean leaf crinkle virus (ULCV) in urdbean (*Vigna mungo* L. Hepper), are described below.

3.1 Experimental Site

Pantnagar falls in the *tarai* belt, adjoining the foot hills of Shivalik range of Himalayas with an altitude of 343.84 m above mean sea level. It is located at 29°N latitude and 79.3°E longitude. The climate of Pantnagar is humid subtropical.

The field trial was conducted in plot number B4 of the Crop Research Centre of the G. B. Pant University of Agriculture and Technology, Pantnagar during kharif of 2002 and 2003, while glass house experiments were carried out in glasshouses of Department of Plant Pathology. Experiments related to ELISA, virus purification and molecular weight study of viral coat protein were conducted at Plant Virus Laboratory, Department of Plant Pathology, B. A. College of Agriculture, G. A. U. Anand, Gujarat.

3.2 Experimental Materials

3.2.1 Soil

Fallow soil collected from Crop Research Centre (CRC) and homogenized with farm yard manure and sand in 4:1:1 ratio was used for raising the plants in pots under glass house conditions.

3.2.2 Pots

For growing on test, seedlings were raised in six inches plastic pots.

3.2.3 Seeds

The seeds of different urdbean varieties and germplasms were obtained from Seed Production Centre (SPC), Crop Research Centre (CRC) and from adjoining farmer's fields at Pantnagar. The desired seeds were also collected from glass house and field after harvest. Urdbean variety Pant Urd 19 was used as a test variety through out the studies.

3.2.4 Fungicidal Seed Treatment

Prior to planting, Thiram containing 75 % active ingredient was used @ 2.5 gm/kg seed as dry seed treatment.

3.2.5 Insecticide Application

Carbofuron 3 G was used as soil application and metasystox (@ 0.1 %) as spray to keep the glass house free from insects which might enter accidentally.

3.2.6 Cages

Insect proof cages made up of nylon mosquito net were used for raising seedlings both under glass house and field conditions.

3.2.7 Virus

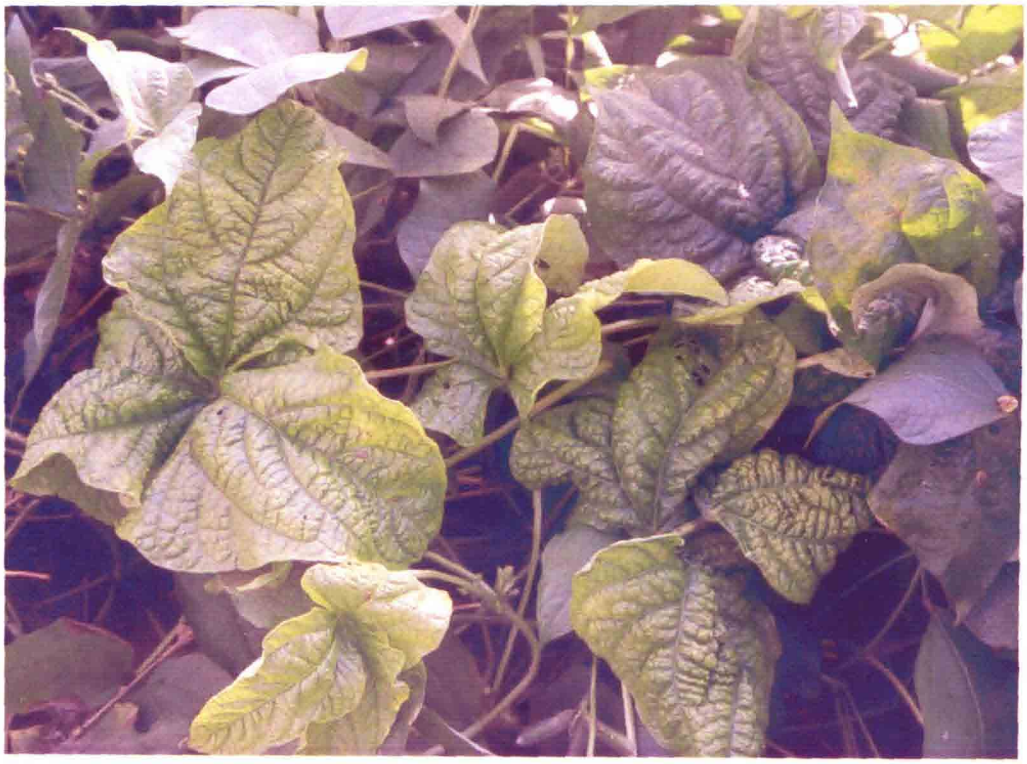
The virus used in the present study was Pantnagar isolate of urdbean leaf crinkle virus (ULCV) (Plate 1). It was maintained in glass house on urdbean plants of variety Pant Urd 19.

3.3 Detection of Seedborne Inoculum of Urdbean Leaf Crinkle Virus in Urdbean Seeds

3.3.1 Dry Seed Examination

Seed samples from urdbean leaf crinkle virus infected plants of urdbean variety PU 19 were carefully examined in the dry state for the presence of (i) shrivelled, (ii) off-coloured, (iii) oversized and, (iv) apparently healthy looking seeds. These seeds were further used for growing on test under glass house conditions to study the per cent seed transmission of ULCV in different categories of seed abnormality.

A- Field view of urdbean leaf crinkle virus infected plant



B- Characteristic symptoms of ULCV infection on urdbean leaves

Plate 1

3.3.2 Growing on Test

This test was conducted in glass house (with diffused light) with a temperature range of 25-30 ^{0}C. The plants were raised in plastic pots of 6 inch size. Counted seeds were sown in each pot containing soil: farmyard manure: sand mixture (4:1:1). On germination, seedlings were recorded for characteristic symptoms of leaf crinkle at the first, second, third and fourth trifoliate leaf stages.

3.4 Location of Seedborne Inoculum of Urdbean Leaf Crinkle Virus in Urdbean Seed

3.4.1 Association of ULCV with Different Flower and Seed Parts

The study was carried out using both the indicator inoculation test and serological method i.e. enzyme linked immunosorbent assay (ELISA) as described below.

3.4.1.1 Indicator Inoculation Test

The presence of urdbean leaf crinkle virus associated with different parts of flower and seed was studied by preparing the extracts of different parts for assays on urdbean plants under glass house conditions. Various steps involved in this method are described below.

3.4.1.1.1 Raising of Plants

Insect proof glass house was used for raising urdbean plants. The glass house was sprayed with systemic insecticides routinely at an interval of 10-15 days to keep it free from insects which might enter accidentally. All experimental test plants were raised in a soil: farmyard manure: sand mixture (4:1:1) in plastic pots of 6 inch. The seeds were treated with thiram (@ 0.25 %) before planting. After, sowing pots were transferred to insect proof nylon cages and confined there till the experiment was over. The plants were watered regularly to maintain moisture for growth.

3.4.1.1.2 Preparation of Virus Inoculum

3.4.1.1.2.1 Disinfection

Disinfection of glasswares, mortar, pestle etc. was done by rinsing them with trisodium phosphate solution (10%) and with tap water. After inoculation with infective sap, hands were washed with trisodium phosphate solution or soap. This was followed by washing with tap water. This measure was followed in all the experiments where sap was used as a medium of inoculation.

3.4.1.1.2.2 Preparation of Potassium Phosphate Buffer

Potassium phosphate buffer (0.1 M) solution of pH 7.6 was prepared by adding 1 part of 0.1 M K_2HPO_4. $3H_2O$ solution to 9

parts of 0.1 M KH_2PO_4 solution. Above two solutions were mixed together and shaken thoroughly.

3.4.1.1.2.3 Recording the pH

The pH of the phosphate buffer solution was adjusted to 7.6 by using pH meter with glass electrodes.

3.4.1.1.2.4 Preparation of Extract

(a) Flower Parts

Fifty unopened flower buds were collected from systemically urdbean leaf crinkle virus infected plants of variety PU 19. These flower buds were separated in to five parts i.e. epicalyx, calyx, corolla, androecium and gynoecium with the help of forceps, needle and blade. The forceps, needle and blade were thoroughly disinfected with 10 per cent solution of trisodium phosphate (Na_3PO_4) followed by rinsing in soap water and finally in tap water after each handling of flower parts. These parts were separately washed in running tap water for 20 min., then in sterilized water and were kept frozen for 15 min. to facilitate virus extraction. Later, each component was separately grounded in a mortar with a pestle in 0.1 M phosphate buffer (pH 7.6). After a thorough grinding, the extract was stained through cheese cloth and separately kept in a test tube. These extracts were used for inoculation.

(b) Seed Parts

Fifty seeds obtained from systemically ULCV infected plants of PU 19 were first soaked in tap water. These were then soaked in distilled water for 24 hours, after which each seed was separated in to three parts i.e. seed coat, cotyledon and embryo with the help of needle, forceps and blade. These parts were separately washed in distilled water to avoid contamination before extraction. The extracts of the three parts were prepared in the same way as the extracts of flower. The extracts from different seed parts were then used for inoculation.

3.4.1.1.2.5 Inoculation Process

Inoculation was carried out by gently rubbing 600 mesh carborundum dusted leaves with the help of fore fingers dipped in the inoculum. Primary leaves (cotyledonary leaves) before the emergence of true leaves were inoculated. The inoculated leaves were immediately rinsed with gentle stream of tap water with the help of plastic wash bottle. The leaves on the control plants were inoculated with only potassium phosphate buffer.

3.4.1.1.2.6 Recording Observations

The inoculated plants were observed daily and those showing typical leaf crinkling were recorded.

3.4.1.2 Serological Test Using ELISA Technique

The presence of seedborne inoculum of urdbean leaf crinkle virus in different categories of abnormal seeds such as shrivelled, oversized and off-coloured seeds, in various flower parts viz. epicalyx, calyx, corolla, androecium, gynoecium and seed parts as seed coat, cotyledons and embryo was also confirmed by DAC-ELISA method described below.

3.4.1.2.1 Enzyme Linked Immunosorbent Assay (ELISA)

1. A crude sap of various abnormal seeds and different flower and seed parts was prepared in ELISA coating buffer. The sap was filtered and filled in wells of ELISA plate (100 µl) in each well.
2. The plates were incubated in freeze for overnight or 18 hours
3. The ELISA plates were washed thrice with PBS tween at 5 minutes interval.
4. Each well was loaded with 100 µl of 0.1 % blotto (skimmed milk powder in PBS) and kept for 30 minutes at room temperature. Then the plates were washed thrice with PBS tween at 5 minutes interval.
5. Antibody raised against urdbean leaf crinkle virus and diluted in blotto was filled (100 µl) in each well of ELISA

plate (the concentration depending on the titre of the antiserum). It was kept for 1 hour at room temperature.

6. The plates were then washed thrice with PBS tween at 5 minutes interval.
7. Horse-radish peroxidase Goat Anti Rabbit (IgG) conjugate was diluted in blotto (1:1000 µl) and 100 µl was loaded in each well.
8. It was kept for 1 hour at room temperature.
9. Then washing of wells with PBS tween was done at 5 minutes interval.
10. Then substrate solution was prepared using (ABTS) 0.5 µg/ml in substrate buffer and H_2O_2 (2 µl/ml) was added just before the use.
11. 50 µl of the substrate solution was added to each well. The O.D. (490nm) was measured in ELISA plate reader (Biorads, USA) after about 10-15 min.

3.4.1.2.2 Composition of Buffers Used for ELISA

(1) Coating buffer (pH 9.6)

Na_2CO_3	1.59 gm
$NaHCO_3$	2.93 gm
Water	1000 ml

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

KCl	300 ml
NaCl	600 ml
KH_2PO_4	3000 ml
$Na_2HPO_4.12H_2O$	2.9 gm
$Na_2HPO_4.2H_2O$	1.44 gm

Made up to one liter

(3) PBS T (PBS- Tween 20), pH 7.4

PBS	1000 ml
Tween 20	0.8 ml

Made up to one liter

(4) HRP substrate buffer, pH 4.0 (Citrate/phosphate buffer, 0.1 M)

61.45 ml citric acid solution (0.1 M) + 38.55 ml sodium phosphate solution (0.2 M) OR 1.9 gm citric acid + 1.37 ml sodium phosphate /100 ml distilled water

3.4.2 Internal or External Nature of Seedborne Inoculum of ULCV

The internal or external nature of seedborne inoculum of ULCV was determined in the following three ways.

1. One hundred seeds from systemically ULCV infected PU 19 urdbean plants were first soaked in 10 per cent trisodium phosphate (Na_3PO_4) solution for five minutes and then

washed in distilled water for five minutes. The control seeds were soaked only in distilled water for five minutes. The percent transmission was determined by grow out test as described earlier. The seeds were planted in plastic pots containing soil: sand: farmyard manure mixture (4:1:1 ratio) and the percentage seed transmission was calculated by recording the number of plants showing symptoms of ULCV.

2. One hundred seeds obtained from systemically ULCV infected PU 19 urdbean plants were thoroughly shaken for five minutes in a 250 ml flask containing potassium phosphate buffer (0.1 M, pH 7.6) in 1:1 ratio. The surface washings were assayed for the ULCV by the indicator-inoculation test.

3. Per cent transmission in urdbean seeds with or with out seed coat was determined by growing on test. One hundred seeds obtained from systemically ULCV infected PU 19 urdbean plants were soaked overnight in distilled water. Next morning the seed coats of 50 seeds were removed with the help of forceps and needle. Such seeds were thoroughly washed in sterilized water. Fifty seeds with intact seed coats served as control. The percentage transmission was determined by growing on test.

3.5 Transmission of Seedborne Inoculum of Urdbean Leaf Crinkle Virus in Urdbean Seeds

3.5.1 In Seeds with Morphological Abnormalities

Urdbean seeds collected from urdbean plants showing systemic ULCV infection under field conditions were further sorted out in four categories viz. oversized, shriveled, off-coloured and mixed seeds, respectively. A total of 50 seeds from each category were sown separately in 6 inch plastic pots under glass house conditions. Data were recorded on percentage germination and seed transmission of ULCV.

3.5.2 In Different Germplasms and Varieties

A total of nineteen germplasms and varieties of urdbean were tested for per cent seed transmission of urdbean leaf crinkle virus through seed under field conditions. For this a fixed number of seeds were sown and data were recorded on per cent transmission of ULCV by observing number of plants showing typical crinkling symptoms.

3.5.3 In seeds Obtained from Plants Infected at Different Stages of Plant Growth

Seeds for experiment on the effect of plant age at the time of infection on subsequent seed transmission of ULCV were obtained in the following ways.

Urdbean plants of variety PU 19 were raised in 6 inch plastic pots containing soil: farmyard manure: sand mixture (4:1:1 ratio) under glass house conditions (20-37 ^{0}C with diffuse light). These plants were then sap inoculated by rubbing 600 mesh carborundum powder dusted primary leaves, by fore fingers dipped in sap prepared from urdbean leaf crinkle virus infected leaves in 0.1 M potassium phosphate buffer (pH 7.6) at 7, 14, 21, 28, 35, 42 and 49 days after sowing (DAS). The inoculated leaves were immediately rinsed with tap water with the help of plastic wash bottles. Check plants were inoculated with only phosphate buffer. Minimum four replications were taken in each treatment.

Observations on the number of plants infected, range of incubation period, expression of leaf crinkling symptoms at different stages of plant growth, number of pods per plant, pod length, number of seeds per pod, 1000 seed weight and yield per plant were recorded in each treatment. Seed texture, colour and seed size were also recorded in each category.

At maturity seeds were collected separately from each plant of different category of ULCV infection and grown separately in glass house. Observations on seed germination and number of plants exhibiting typical crinkling were made.

3.5.4 In Seeds Collected from Variously Infected Plants

In this experiment seeds were collected from three categories (T_1- T_3) of field grown PU 19 urdbean plants viz. plants showing systemic ULCV symptoms, plants showing ULCV symptoms at later stages and finally apparently healthy looking plants, respectively. Seeds, collected from such plants were separately sown in an insect proof screen house under field conditions in experiment number A. Data were recorded on per cent seed germination, mortality and seed transmission of ULCV in each treatment. Plants were observed up to 40 days after sowing for the symptom appearance and allowed to mature for the collection of seeds. At maturity seeds were collected from all the three treatments and were sown in next cropping season in experiment number B. Observations were made again on seed germination, mortality and seed transmission of ULCV in each treatment. Per cent seed transmission was calculated by dividing the number of plants showing disease symptoms by total germinated/sown seeds.

3.5.5 In Different Stages of Seed Maturation

For experiment on transmission of urdbean leaf crinkle virus at different stages of seed maturation, 100 seeds were collected

from pods of systemically ULCV infected urdbean plants of PU 19 at 1, 2, 3 and four weeks after pod set and at harvest stage. The seeds collected at 4 week after pod set were physiologically mature, whereas, those collected earlier had been immature. The growing on test as described earlier was followed for the estimation of per cent transmission of seedborne inoculum of ULCV in different stages of seed maturation.

3.6 Influence of Mother Plant Infection with ULCV on Flower Organelles of Urdbean

3.6.1 Flower Morphology

Ten systemically ULCV infected PU 19 urdbean plants were used for study. Observations were taken for any type of abnormality that was visible in flower parts.

3.6.2 Pollen Viability

The viability of the pollen grains was determined with the help of acetocarmine stain. Fifty flower buds each from healthy and systemically ULCV infected PU 19 urdbean plants were stained separately with acetocarmine stain. After staining, pollen grains were examined under compound microscope. The pollen grains which did not take the stain were considered as sterile and those getting stained were recorded as viable, respectively.

3.7 Yield Losses Due to Seedborne Inoculum of ULCV

3.7.1 Effect of Plant Age at ULCV Infection on Yield Contributing Factors and Yield of Urdbean

To study the effect of plant age at the time of ULCV infection and its effect on yield contributing factors and yield of urdbean, the experiment was conducted as mentioned in section number 3.5.3. of Materials and Methods. Data on number of pods per plant, pod length, number of seeds per pod, 1000 seed weight, yield per plant and per cent yield reduction were recorded in each treatment. The per cent yield reduction was calculated by following formula (Nene, 1972).

$$Q = \frac{A - B}{A} \times 100$$

Q = per cent yield reduction

A = average yield of healthy plants

B = average yield of diseased plant

3.7.2 Effect of Different Categories of ULCV Infection on Yield of Urdbean under Field Conditions

Experiment to study the effect of different categories of plant infection viz., (i) plants showing systemic ULCV symptoms (T_1), (ii) plants showing ULCV symptoms at later stages (T_2) and (iii) apparently healthy looking urdbean plants (T_3) on yield under field

conditions, was laid down as mentioned under section 3.3.2 of Materials and Methods. Data were recorded on 1000 seed weight and per cent yield reduction, respectively in both the field experiments (A & B).

3.8 Purification of Urdbean Leaf Crinkle Virus

3.8.1 Selection of Host Plant

The choice of host plant in which a virus is multiplied for purification is often critical and it should be easily and quickly grown from seed. If possible, a host is selected which is free from inhibitors, gums, latex and phenolic compounds which might inactivate or interfere with the virus during purification and the virus should be easily separable from the host constituents and should multiply to high concentrations in the selected host. Urdbean cv. PU 19, susceptible to ULCV was selected for the purpose.

3.8.2 Propagation of Virus for Purification

Before isolation of virus, it was multiplied in pure form (without any mixed infection of other viruses) efficiently in selected host for purification by constant mechanical inoculation method.

3.8.3 Partial Purification of ULCV

In partial purification process the virus particles were extracted from the host cells and separated from the heavier host constituents. All the subsequent stages of purification process were carried out at temperature of 3 to 5°C.

(1) Virus Extraction and Clarification

(a) After 20 to 25 days of mechanical inoculation the first two leaves of infected urdbean plants were harvested (250 gm material). The whole material was pre-cooled for 1/2 hr. in a freeze before extracting the sap.

(b) The plant material (infected leaves) about 250 gm were homogenized in warring blender with phosphate buffer (60 mM $NaPO_4$, 10 mM Na DIECA, pH 9.0) + Mercaptoethanol (0.2 %). In the homogenized mixture 60 per cent chloroform ($CHCl_3$) was added by volume and then mixture was again homogenized and spun at 16400 g for 10 min.

(c) Supernatant was decanted out using water vacuum, filtered and volume was measured.

(d) Again chloroform (60 % w/v) was added, mixed thoroughly in warring blender and spun at 16400 g for 10 minutes.

(e) Supernatant was decanted out, filtered and volume was measured. In the supernatant, Tritan X-100 was added to 5 per cent of the volume and stirred for 1/2 hour in cold room.

(f) Spinning was done at 16400 g for 10 minutes, again supernatant was decanted out and filtered, then PEG (4 % of the volume) and 0.3 M NaCl were added to it. Whole mixture was stirred for 1/2 an hour and left as such for 1/2 an hour in cold room and again spun at 24600 g for 20 minutes.

(g) Supernatant was discarded and pellet was resuspended in 25 ml phosphate buffer, pH 7.8 (10 mM EDTA, 50 mM $NaPO_4$). Whole material was left overnight.

(h) Spun at 200000 g for 10 minutes and supernatant was collected.

3.8.4 Final Purification of ULCV

1. Finally density gradient was prepared (44.15 gm Sucrose 60%, 5.32 gm of 60 % $CsSO_4$ and 3.73 gm of resuspension buffer). All the chemicals were mixed properly and 6.65 gm of mixture was loaded in to Beckman SW-41 tubes. Rest of the tube was filled with partially purified virus in resuspension buffer. The tubes were balanced to approximately 16 gm each and spun at 162500 g for 20 hours.
2. Tubes were taken out and the band was collected drop by drop with the help of syringe and again spun at 190000 g for 100 minutes after addition of resuspension buffer.

3. Supernatant was discarded. Pellets washed once with water and resuspended in 300 µl of resuspension buffer and left overnight for softening.
4. The pellet was washed and homogenized thoroughly. Spun at 6150 g for 5 min.
5. Further the supernatant was loaded on to density gradient for removing the remaining impurities of host protein by repeating the above steps I to IV.
6. Finally the supernatant was taken out and stored. The purity of the virus was checked by SDS PAGE.

3.9 Molecular Weight of Coat Protein of ULCV

3.9.1 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS PAGE)

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was done for determining molecular weight of virus coat protein of urdbean leaf crinkle virus.

3.9.1.1 Sample Preparation:

The purified virus preparation or infected sap is used for SDS-PAGE. An aliquot of 50µl of virus preparation is taken in an eppendorf tube to which 50µl of Laemmli buffer is added. After vortexing for few minutes, it is heated at 100°C for 3 minutes. After cooling the sample is loaded on to the gel.

Mini- Protein Dual Slab Cell (Biorad)

1- Cleaning of all the parts is done with absolute alcohol.

2- Casting the gel (casting discontinuous polyacrylamide gels)

Discontinuous polyacrylamide gel consists of a resolving or a separating (lower gel) and a stacking (upper gel). The stacking gel acts to concentrate large sample volume resulting in better band resolution than is possible using the same volume on a gel without a stack. Molecules are then completely separated in the resolving gels.

(a) The separating gel monomer solution is prepared by combining all reagents and immediately poured in the glass plate of the gel sandwich. Then immediately thc monomer solution is overlaid with water for leveling.

(b) The gel is allowed to polymerize for 45 min - 1 hr. The overlaid water is rinsed off completely.

(c) The stacking gel monomer solution is prepared by combining all reagents and poured immediately after placing a comb plate.

The gel is allowed to polymerize for 30-45 min. The comb is removed by pulling it up straight slowly and gently.

2- The upper buffer chamber is removed by releasing clamp assemblies/gel sandwich from the casting stands. Then the clamp assembly is attached on the inner cooling core.

3.9.1.2 Loading the Sample

(a) A 300 ml of electrode buffer was prepared by combining 60ml of 5X electrode buffer with 240 ml of distilled water; approximately 115 ml of buffer is poured into the upper buffer chamber until the buffer reaches into the upper buffer chamber until the buffer reaches the level halfway between the smaller and longer plates.

(b) The remainder of the buffer is poured into the lower buffer chamber so that at least the bottom 1 cm of the gel is covered. Air bubble if is removed from the bottom of the gel so that good electric contact is achieved.

(c) The samples are loaded in the wells under the electrode buffer with a Hamilton syringe. In the first lane low mol. wt. standard (5 µl) is loaded. The pre-stained molecular weight marker from Biorad, consists of phosphorylase B (106,000 Da), Bovine serum albumin (80000 Da), Ovalbumin 49500 Da), Carbonic anhydrase (32500 Da), Soybean trypsin inhibitor (27500 Da) and Lysozyme (18500 Da) of low range.

3.9.1.3 Running of the Gel

The lid of the buffer chamber is placed on top of the lower buffer chamber to fully enclose the cell. The correct orientation is

made by matching the colours of the plugs on the lid with jacks on the inner cooling core.

(a) The electric leads are attached to 100 volts at 25 amp power supply and are run for 45 min.

3.9.1.4 Composition of Buffers used for SDS PAGE

(1) 0.5 ml Tris HCl buffer (pH 6.8) MW = 157.60

Tris HCl Base	7.8 gm
Distilled water	100 ml

(2) 0.5 ml Tris HCl buffer (pH 6.8) MW = 157.60

Tris HCl Base	23.6 gm
Distilled water	100 ml

3.9.1.5 Composition of chemicals used for SDS PAGE

(1) Laemmli buffer (Sample buffer)

Distilled water	4 ml
0.5 M Tris HCl buffer pH 8.8	1 ml
Glycerol	0.8 ml
10% SDS (w/v)	1.6 ml
2- Mercaptoethanol	0.4 ml
0.05% (w/v) Bromophenol	0.2 ml

(2) Seperating gel (12%)

Distilled water	6.70 ml
0.5 M Tris HCl buffer pH 8.8	5.00 ml
10% SDS (w/v)	200 µl
Acrylamide/ Bis stock	8.0 ml
10% APS	100 µl
TEMED	10 µl

(3) Stacking gel (Spacer gel) (4%)

Distilled water	6.1 ml
0.5 M Tris HCl buffer pH 8.8	2.5 ml
10% SDS (w/v)	100 µl
Acrylamide/Bis stock	1.3 ml
10% APS	50 µl
TEMED	10 µl

(4) Running buffer

5X electrode running Buffer (pH 8.3)	60ml
Distilled water	240 ml

(5) Coomassie Brillian6t blue R-450 (0.1%)

Coomassie blue4	100 mg
Methanol	50 ml
Acetic acid	10 ml
Distilled water	40 ml

Experimental Results

Four EXPERIMENTAL RESULTS

Experiments conducted to establish the "seedborne nature of urdbean leaf crinkle virus in urdbean" are being presented in the following paragraphs.

4.1 Detection of Seedborne Inoculum of Urdbean Leaf Crinkle Virus in Urdbean Seeds

The detection of urdbean leaf crinkle virus in urdbean seeds was determined by following three methods.

4.1.1 Dry Seed Examination

Seeds of urdbean plants with systemic infection of urdbean leaf crinkle virus (ULCV) were found to have certain morphological abnormalities. Therefore, the present experiment was conducted to find out the presence of shrivelled, oversized and off-coloured seeds in a seed lot collected from systemically ULCV infected plants of urdbean. Such categorized seeds were used for growing on test in order to observe the extent of seed transmission of urdbean leaf crinkle virus. The presence of virus in such seeds was also confirmed by DAC-ELISA as described under Materials and Methods.

4.1.1.1 Extent of Seed Abnormality

Data in Table 1 indicate that ULCV infection adversely affected the texture of urdbean seeds by increasing the percentage of shrivelled seeds (20.4 %) in systemically ULCV infected plants as compared to healthy plants in which only 1.9 per cent shrivelled seeds were recorded in urdbean variety PU 19.

Further, the ULCV infection enhanced the production of off-coloured seeds where the seed colour changes from normal black to light brown. Such off-coloured seeds were recorded up to 13.2 per cent in systemically ULCV infected plants, as compared to 2.9 per cent off-coloured seeds from healthy plants. Interestingly, ULCV infection caused higher production of oversized seeds. In systemically ULCV infected plants there were 17.5 per cent oversized seeds as compared to 1.3 per cent oversized seeds produced in healthy plants in variety PU 19. A small percentage of 5.6 of undersized seeds was also recorded in seeds collected from systemically ULCV infected plants (Plate 2).

4.1.1.2 Extent of Seed Transmission by Abnormal Seeds

Seed transmission studies were carried out using morphologically abnormal seeds for growing on test. The ULCV infection affected the seedling emergence in various categories of

Table 1. Extent of seed abnormality in PU 19 urdbean as revealed by dry seed examination

Seed collected from	Shrivelled seeds (%)	Off-coloured seeds (%)	Oversized seeds (%)	Undersized seeds (%)
Systemically ULCV infected plants	20.4	13.2	17.5	5.6
Healthy looking plants	1.9	2.9	1.3	1.2

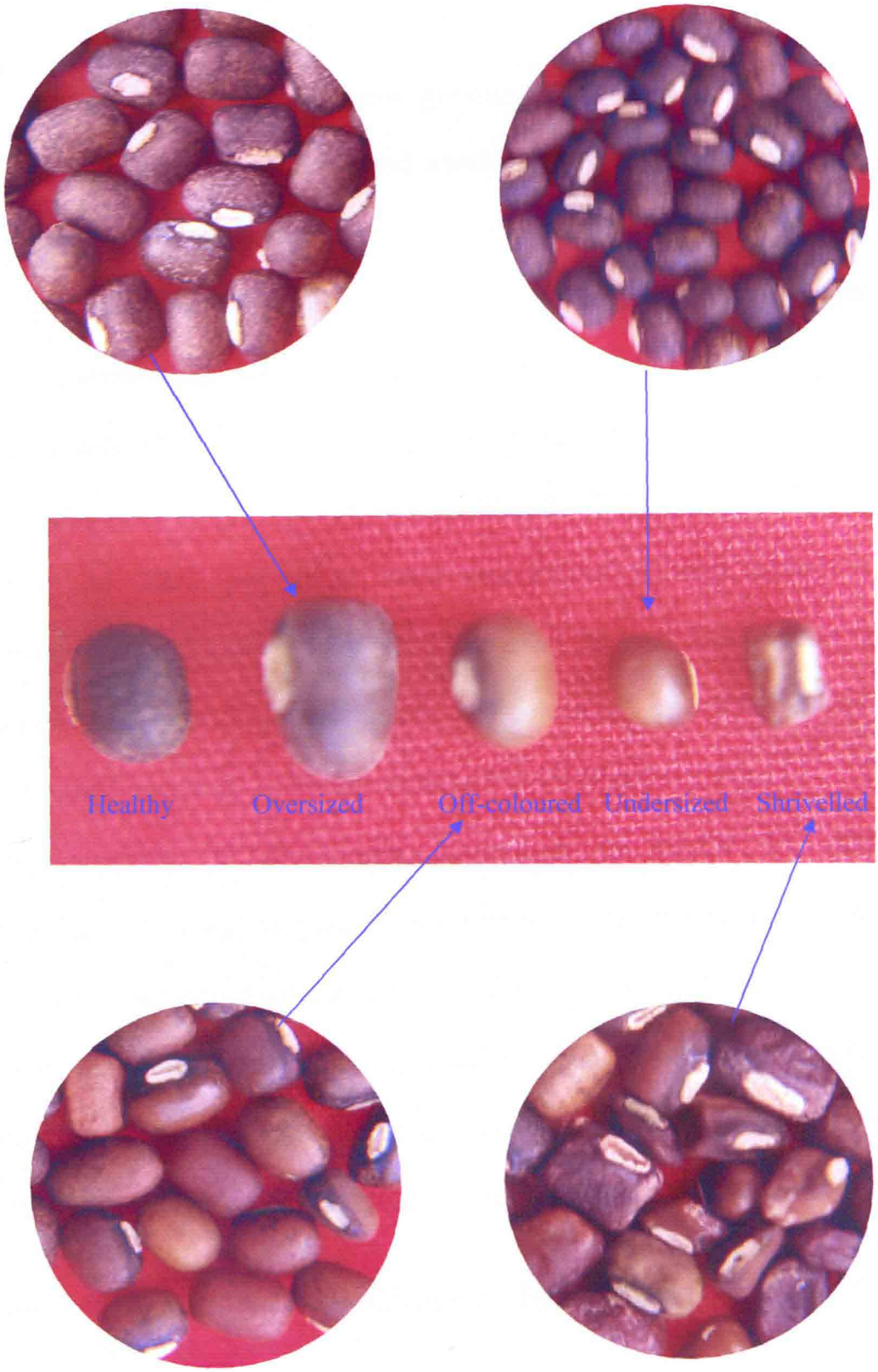


Plate 2. Morphological abnormalities in urdbean seeds collected from systemically ULCV infected plants

urdbean seeds showing morphological abnormalities. Shrivelled seeds exhibited 72.0 per cent germination followed by 88.0 per cent germination in undersized seeds and 92.0 and 94.0 per cent germination in oversized and off-coloured seeds, respectively. All the four categories of morphologically abnormal seed, collected from systemically ULCV infected plants yielded ULCV infection ranging from 9.0 to 22.2 per cent in the growing on test suggesting the transmission of seedborne inoculum of ULCV in urdbean. A highest of 22.2 per cent seed transmission was obtained in shrivelled seeds followed by 17.3 per cent in oversized seeds, 12.7 per cent in brown coloured seeds (off-coloured) and 9.0 per cent in undersized seeds of urdbean variety PU 19 (Table 2, Fig.1).

Presence of seedborne inoculum of ULCV in various categories of morphologically abnormal seeds in the same variety was also confirmed using DAC-ELISA test. Results indicate that shrivelled seeds gave strong positive reaction, while oversized seeds gave mild positive and undersized and off-coloured seeds gave vary mild positive reaction in DAC-ELISA test (Table 2).

4.2 Location of Seedborne Inoculum of ULCV in Urdbean Seeds

The indicator inoculation test was carried out to find out the association of urdbean leaf crinkle virus with different floral parts

Table 2. Extent of seed transmission of urdbean leaf crinkle virus through morphologically abnormal urdbean seeds as revealed by growing on and ELISA tests

Seed grade	No. of seeds sown	No. of seeds germinated	Seed germination (%)	No. of plants showing ULCV infection	Seed transmission (%)	Reaction on ELISA
Shrivelled	50	36	72.0	8	22.2	+++
Off-coloured	50	47	94.0	6	12.7	+
Oversized	50	46	92.0	8	17.3	++
Undersized	50	44	88.0	4	9.0	+

+++ = Strong reaction ++ = Mild reaction + = Very mild reaction

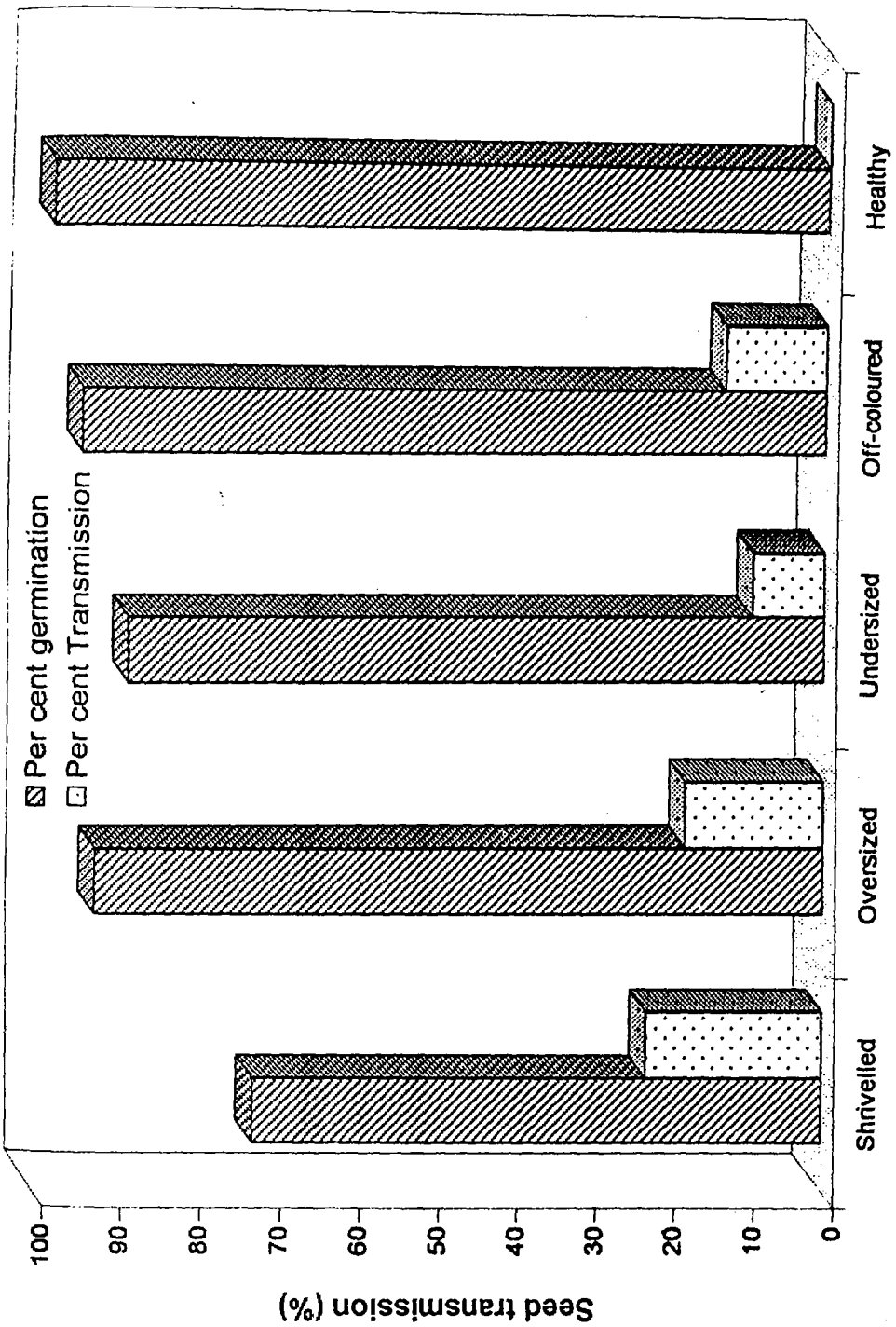


Fig.1 : Effect of morphological abnormalities on transmission of seedborne inoculum of ULCV

i.e. epicalyx, calyx, corolla, androecium and gynoecium and to detect the location of ULCV in different seed parts viz. seed coat, cotyledons and embryo to confirm the internally or externally seedborne nature of the virus. The association of ULCV with different parts of flower and seed was also confirmed by DAC-ELISA as described in Materials and Methods. Urdbean plants of variety PU 19 were used as test plants for indicator inoculation test under glass house conditions.

4.2.1 In Flower Parts

The results of the assay of ULCV in different floral parts of urdbean viz. epicalyx, calyx, corolla, androecium, gynoecium as well as whole flower are given in Table 3 and Fig. 2. The results indicate that ULCV could be detected in the sap of all the floral parts. The artificial inoculation with sap extracted from different floral parts of urdbean exhibited a minimum of 6.6 per cent transmission of ULCV on inoculation with sap from epicalyx followed by 8.8 per cent each with sap from calyx and corolla and 15.5 per cent when inoculated with the sap extracted from androecium and gynoecium, respectively. Highest transmission of 28.8 per cent was recorded on inoculation with extracts from whole urdbean flower.

Table 3. Detection of urdbean leaf crinkle virus in different flower parts by indicator inoculation and ELISA tests

Flower part	Reaction on indicator plant	No. of plants inoculated	Average no. of plants showing ULCV symptom	ULCV transmission (%)	ELISA value
Epicalyx	✓	15	1.0	6.6	+
Calyx	✓	15	1.3	8.8	+
Corolla	✓	15	1.3	8.8	+
Androecium	✓	15	2.3	15.5	++
Gynoecium	✓	15	2.3	15.5	++
Whole flower	✓	15	4.3	28.8	+++
CD at 5%	-	-	1.39	9.27	-
Sem ±	-	-	.45	3.0	-

+++ = Strong reaction ++ = Mild reaction += Very mild reaction

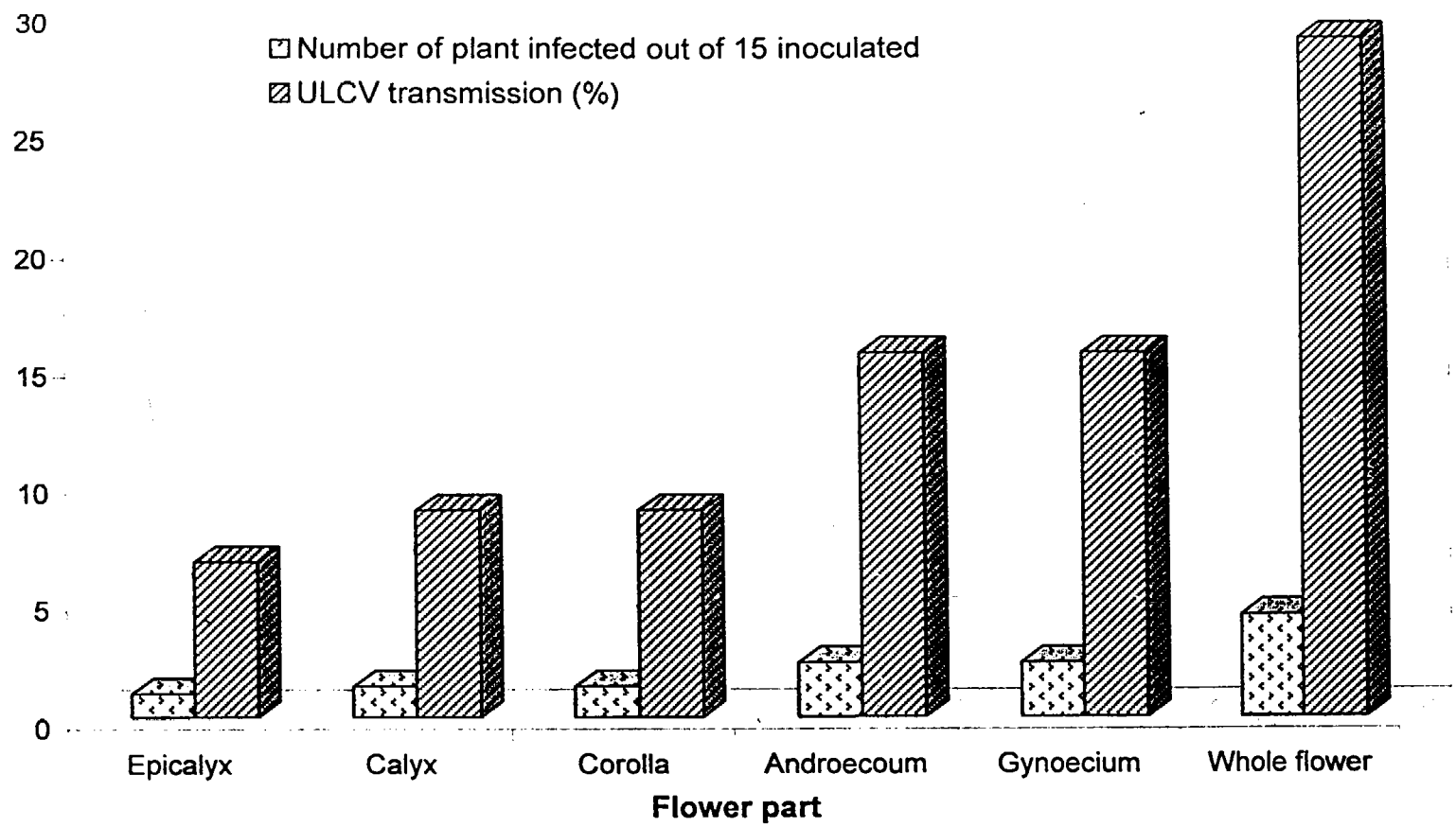


Fig.2 : Detection of ULCV in different flower parts of urdbean

The sap extracted from various floral parts also reported positive reaction for ULCV infection in DAC-ELISA test, thus indicating the possibilities of ULCV infection in all the flower parts (Table 3).

4.2.2 In Seed Parts

The results of the indicator inoculation test for locating of ULCV infection in different seed parts viz. seed coat, embryo and cotyledons are presented in Table 4. It is clear from the results that indicator plants on inoculation with sap of embryo and cotyledons separately of ULCV infected seeds gave a positive reaction under glass house conditions. Data also indicate that there was significant difference in transmission of ULCV when inoculated with sap of embryo and cotyledons, separately. The highest (20.0 %) transmission was recorded when plants were inoculated with sap of embryo, while transmission was 13.3 per cent when inoculated with sap of cotyledons. Inoculation of plants with sap from seed coat did not reveal any symptom of ULCV on plants (Fig. 3). The sap extracted from cotyledons and embryo part of urdbean seed, separately also responded positive reaction in DAC-ELISA test and showed very mild positive reaction (cotyledon) and mild positive reaction (embryo), indicating the presence of

Table 4. Detection of urdbean leaf crinkle virus by indicator inoculation and DAC-ELISA tests in different seed parts of urdbean

Seed part	Reaction on indicator plant	No. of plants inoculated	Average no. of plants showing ULCV symptom	ULCV transmission (%)	ELISA value
Seed coat	-	10	0.0	0.0	-
Cotyledons	✓	10	1.3	13.3	+
Embryo	✓	10	2.0	20.0	++
Seed surface washings	-	10	0.0	0.0	-
CD at 5%	-	-	1.08	10.86	-
Sem ±	-	-	.33	3.33	-

+++ = Strong reaction ++ = Mild reaction += Very mild reaction

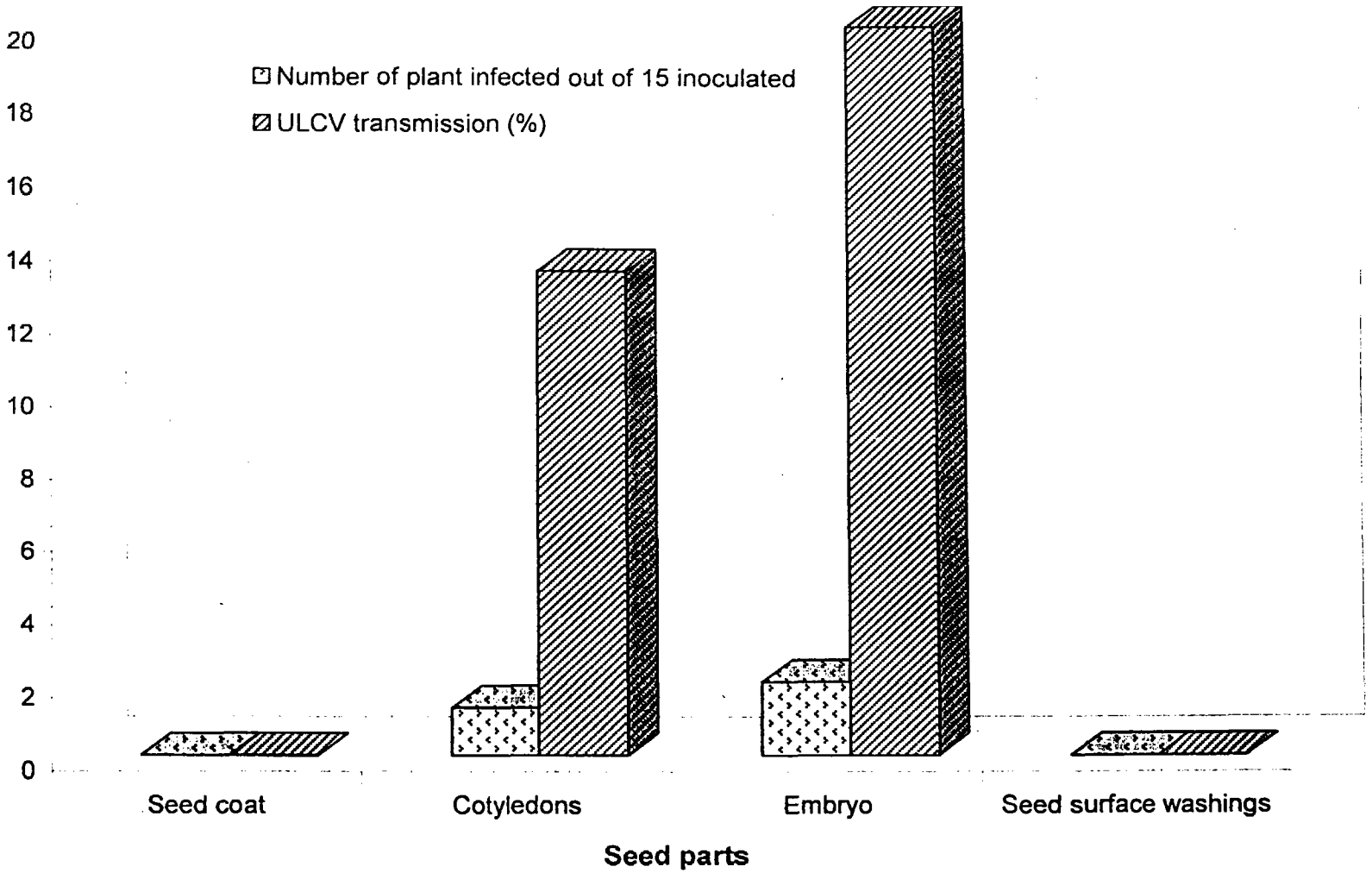


Fig.3 : Detection of ULCV in different seed parts of urdbean

virus in embryo and cotyledons of urdbean seeds. When seed surface washings were used for inoculating urdbean plants, no disease appeared, it shows that the ULCV is internally seedborne in nature.

4.2.3 Internal or External Nature of Urdbean Leaf Crinkle Virus

4.2.3.1. Seed Treatment with Sodium Triphosphate

Urdbean seeds collected from systemically ULCV infected plants and treated with 10 per cent sodium triphosphate (Na_3PO_4) prior to sowing exhibited 13.5 per cent seed transmission of ULCV as compared to 14.2 per cent seed transmission in untreated seeds collected from systemically ULCV infected plants (Table 5, Fig. 4).

4.2.3.2 Seed Surface Washings

It is clear from the results in Table 4 that inoculation of test plants with seed surface washings did not result in development of leaf crinkle symptoms at any growth stage of urdbean plants. Moreover, no reaction in DAC-ELISA was also observed with washings of urdbean seeds (Fig. 3).

4.2.3.3 Effect of Seed Coat Removal on Seed Transmission of ULCV

Results also indicate that removal of seed coat did not have any affect on seed transmission of ULCV in urdbean seeds as the

per cent transmission in seeds with and without seed coats was 16.6 and 15.5 per cent respectively (Table 6, Fig. 5). These results strongly indicate that urdbean leaf crinkle virus is not present as a surface contaminant on urdbean seeds, instead it is internally seedborne and located with in embryo of urdbean seeds.

4.3 Transmission of Seedborne Inoculum of ULCV in Urdbean Seeds

4.3.1 Expression of ULCV Symptoms at Different Stages of Plant Growth

To study the extent of natural transmission of ULCV and to determine the age of the plant up to which the plants could be examined for the expression of leaf crinkling at different stages of plant growth. Seeds both from healthy and systemically ULCV infected plants of variety PU 19 were sown @ 50 seeds/ category. The observations for symptom expression were started at primary leaf stage and continued up to 5th trifoliate leaf stage. The results are presented in Table 7.

A total of 96.0 and 98.0 per cent germination was observed in seeds collected from systemically ULCV infected and healthy urdbean plants, respectively. The results in Table 7 further indicate that a seed lot collected from systemically ULCV infected plants exhibited a total of 29.1 per cent seed transmission. Out of

Table 5. Effect of trisodium phosphate (10 %) treatment on transmission of ULCV through urdbean seeds

Treatment	No. of seeds sown	No. of seeds germinated	Seed germination (%)	No. of plants infected	Seed transmission of ULCV (%)
Treated	100	96	96.0	13	13.5
Untreated	100	98	98.0	14	14.2

Table 6. Effect of seed coat removal on transmission of urdbean leaf crinkle virus through seeds of PU 19 urdbean

Type of seed	No. of seeds sown	No. of seeds germinated	Seed germination (%)	No. of plants infected	Seed transmission of ULCV (%)
With out seed coat	50	45	90.0	7	15.5
With seed coat	50	48	96.0	8	16.6

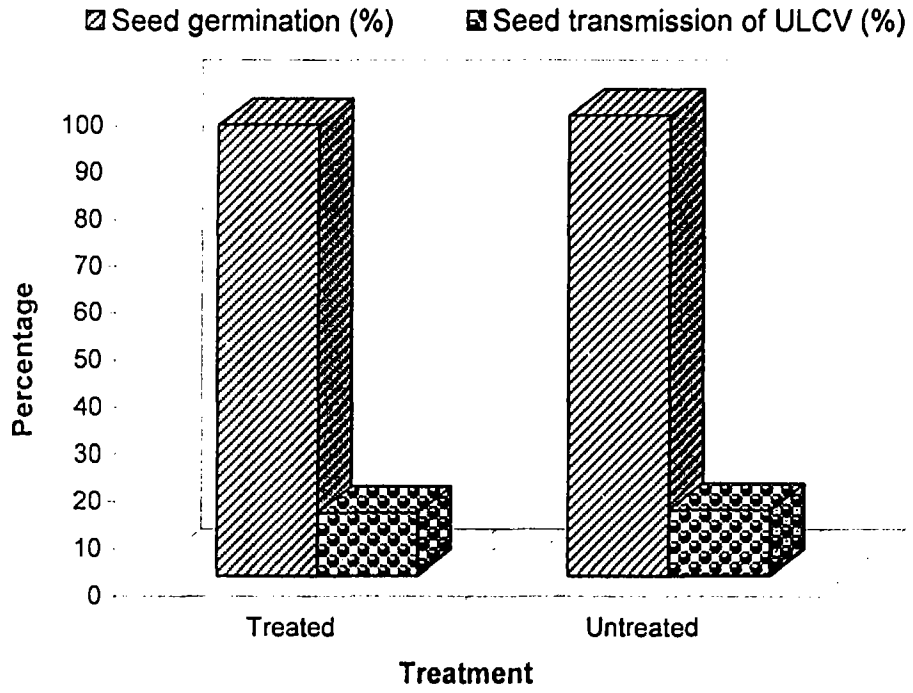


Fig. 4: Effect of trisodium phosphate treatment on ULCV transmission through urdbean seeds

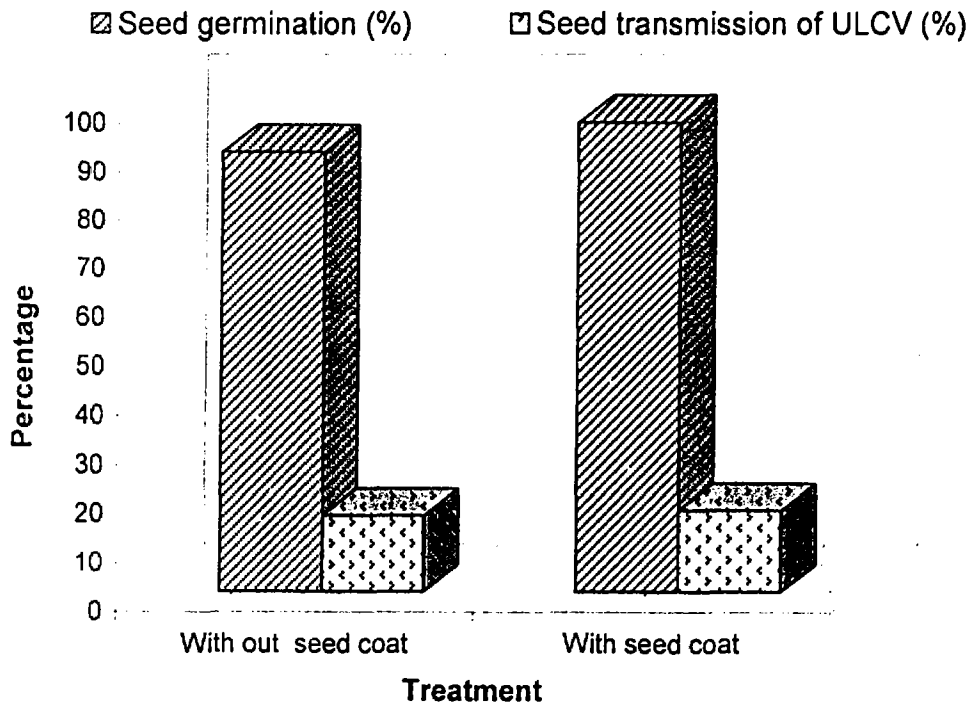


Fig.5. Effect of seed coat removal on transmission of seedborne inoculum of ULCV

Table 7. Expression of ULCV symptoms at different growth stages of PU 19 urdbean

Seed source	No. of seeds sown	No. of seeds germi-nated	Seed germi-nation (%)	Transmission at plant growth stage													
				Primary leaves		1st trifoliate		2nd trifoliate		3rd trifoliate		4th trifoliate		5th trifoliate		Total plants	
				Total	%	Total	%	Total	%	Total	%	Total	%	Total	%	Infected	Transmi-ssion (%)
Systemically ULCV infected plants	50	48	96.0	-	-	3	6.2	4	8.3	5	10.4	2	4.1	-	-	14	29.1
Healthy plants	50	49	98.0	-	-	-	-	-	-	-	-	-	-	-	-	-	-

these 6.2 per cent plants revealed infection at 1st trifoliate leaf stage, 8.3 per cent at 2nd trifoliate leaf stage, 10.4 per cent at 3rd trifoliate leaf stage and 4.1 per cent at 4th trifoliate leaf stage, respectively. No diseased symptoms were observed at primary leaf stage and at 5th trifoliate leaf stage or onwards, respectively. Also none of the plants raised from healthy urdbean seeds exhibited the ULCV infection.

4.3.2 Screening of Different Germplasms and Varieties of Urdbean for ULCV Infection

Nineteen different germplasms and varieties of urdbean were screened for per cent transmission of ULCV through seeds. A counted number of seeds were sown of each variety or germplasm and were observed regularly for seed transmission and subsequent symptom development of urdbean leaf crinkle virus under field conditions.

Seed transmission of ULCV in different germplasms and varieties varied from 3.3 to 20.3 per cent (Table 8). A highest of 20.3 per cent seed transmission of ULCV was recorded in VBG 73 followed by 16.5 per cent in KU 99-7, 14.28 in PU 19, 13.3 in KUG 15, 10.0 in IPU 2001, NDU 96-2, UPU 0031, LBG 623 and PU 35, 7.4 in IU 94-2, 7.1 in LBG 20 and OBG 4, 6.6 in Barabanki Local,

Table 8. Transmission of urdbean leaf crinkle virus through seeds of different germplasms and varieties of urdbean under field conditions

Name of germplasm/variety	No. of plants observed	No. of plants infected	Seed transmission of ULCV (%)
Barabanki Local	30	2	6.6
LBG 20	28	2	7.1
IPU 2001	28	3	10.0
TPU 4	28	2	6.6
NDU 96-2	28	3	10.0
VBG 73	28	6	20.3
KU 99-7	28	5	16.5
KUG 15	28	4	13.3
OBG 4	28	2	7.1
IU 83-5	28	1	3.3
IU 31-7	28	2	6.5
IU 94-2	27	2	7.4
UPU 0031	27	3	10.0
OBG 8	27	2	6.6
KU 321	25	1	4.0
LBG 623	25	3	10.0
Mash 1	25	1	3.3
PU 19	28	4	14.2
PU 35	28	3	10.0
CD at 5%	-	-	1.67
Sem ±	-	-	0.58

TPU 4, OBG 8, 6.5 in IU 31-7, 4.0 in KU 321 and a minimum of 3.3 per cent in IU 83-5 and Mash 1, under field conditions.

4.3.3 Transmission of ULCV through Seeds Obtained from Plants Infected at Different Stages of Plant Growth

Under field conditions, the urdbean plants showed symptoms of urdbean leaf crinkle virus at different stages of plant growth. Therefore, the present experiment was conducted to correlate the effect of plant age and the time of viral infection with seed quality, transmission of seedborne inoculum of ULCV and subsequent effect on yield contributing factors and yield in urdbean.

4.3.3.1 Effect of Plant Age on ULCV Infection and Incubation Period

Urdbean plants of variety PU 19 were sap inoculated at 7, 14, 21, 28, 35, 42 and 49 days after sowing (DAS) under glass house conditions. Table 9 and Fig. 6 reveal that the susceptibility of urdbean plants to ULCV infection significantly decreased, while the incubation period increased with increase in plant age. The ULCV infection ranged from 86.5-15 per cent in plants inoculated at weekly intervals up to seven weeks. A maximum of 86.5 per cent infection was recorded when plants were infected at 7 DAS,

Table 9. Effect of plant age at infection on urdbean leaf crinkle virus infection and incubation period in PU 19 urdbean

Plant age at inoculation	No. of plants inoculated	Average no. of plants infected	Percent ULCV infection	Incubation period (days)	
				Range	Average
7	20	17.3	86.5	12-15	13.3
14	20	16.6	83.3	15-20	17.6
21	20	10.0	50.0	18-22	20.0
28	20	7.3	36.6	24-25	24.3
35	20	4.6	23.3	25-28	26.6
42	20	4.6	23.3	30-35	32.6
49	20	3.0	15.0	35-39	38.0
CD at 5%	-	1.57	7.8	-	3.35
Sem ±	-	0.51	2.59	-	1.10

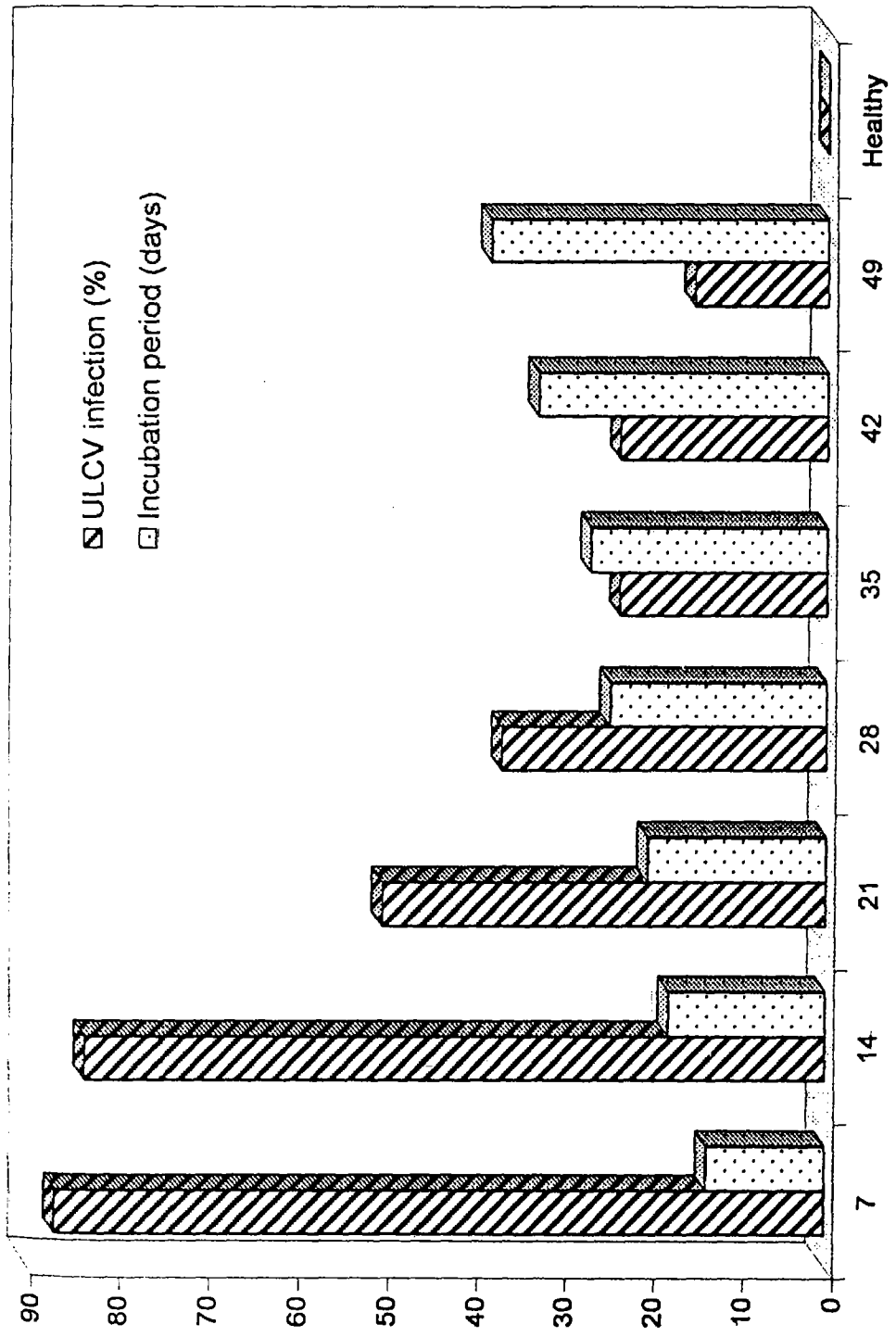


Fig.6 : Effect of plant age at infection on ULCV infection and incubation period

while the minimum 15.0 per cent infection was recorded in plants infected at 49 DAS. Incubation period ranged from 13 days to 38 days, and was minimum in plants infected at 7 DAS and maximum in plants infected at 49 DAS, respectively.

4.3.3.2 Effect of Plant Age at ULCV Infection on Texture, Colour and Size of Urdbean Seeds

The ULCV infection, significantly affected the texture of urdbean seeds by increasing the percentage of shrivelled seeds in ULCV infected plants. The urdbean plants infected at 7 DAS exhibited production of maximum number of shrivelled seeds (25.4 %), while in plants infected at 49 DAS, a total of 15.9 per cent seeds were found shrivelled. Any how seeds collected from healthy plants, also showed 5.3 percentage shrivelled seeds. The ULCV infection at different stages of plant growth changed the seed colour from natural black to light brown and such off-coloured seeds were significantly higher in plants infected early in the season than those infected late in the season. A maximum of 18.3 per cent off-coloured seeds were recorded from plants infected at 7 DAS, while plants infected at 49 DAS exhibited 5.0 per cent off-coloured seeds (Table 10, Fig. 7).

Seeds from ULCV infected urdbean plants at different stages of their growth also showed varying percentage of the presence of

Table 10. Effect of plant age at urdbean leaf crinkle virus infection on texture and colour of urdbean seeds

Plant age at infection	Number of seeds (%)		
	Shrivelled	Off-coloured	Oversized
7	25.4	18.3	20.1
14	25.6	17.0	18.5
21	24.3	17.2	18.4
28	20.1	14.1	10.5
35	21.3	12.7	8.9
42	20.4	10.2	8.8
49	15.9	5.0	7.5
Healthy	5.3	1.0	2.1
CD at 5%	3.6	2.7	2.9
Sem ±	1.2	0.93	0.97

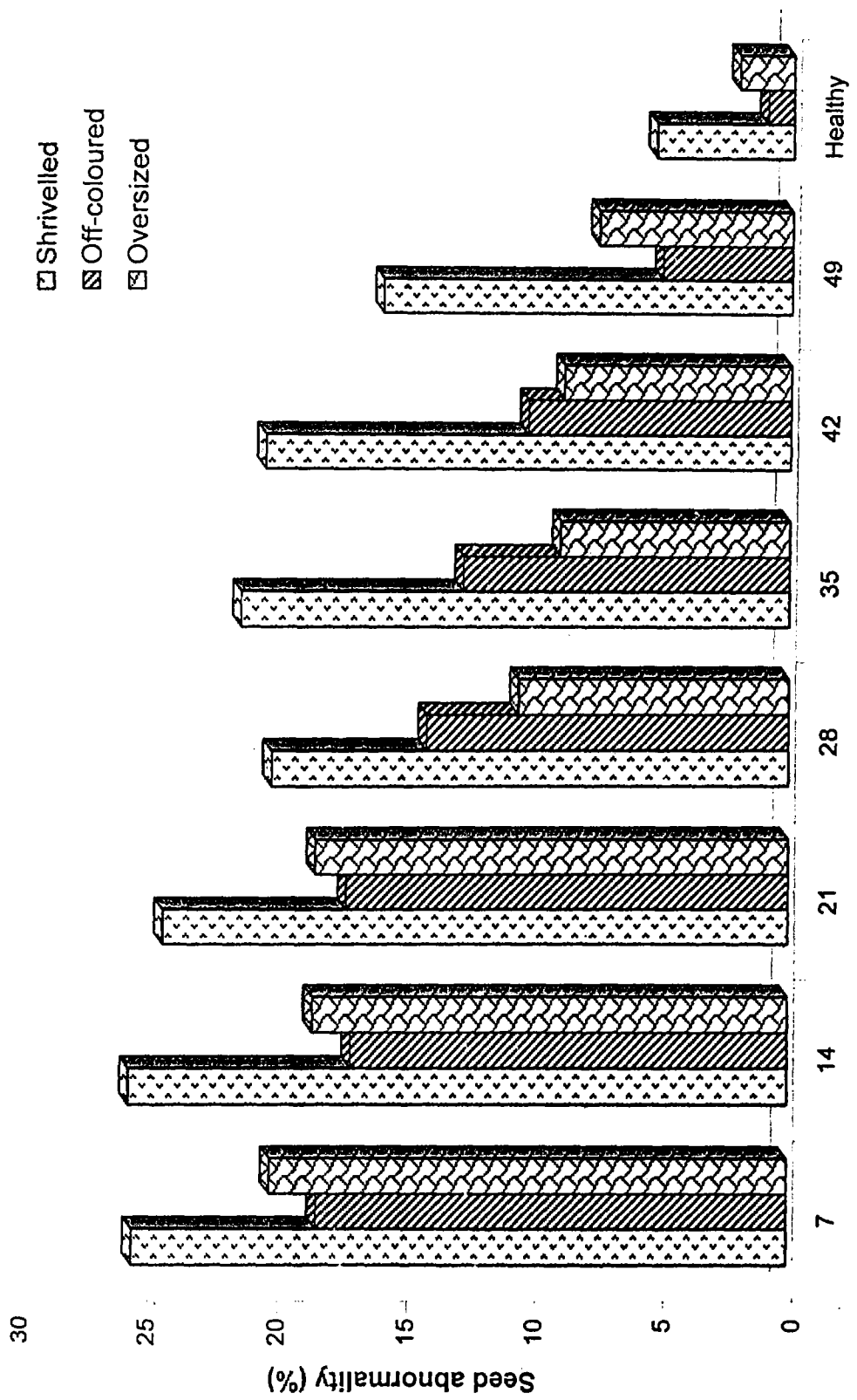


Fig.7 : Effect of plant age at ULCV infection on texture and colour of urdbean seeds

oversized seeds. Oversized seeds were recorded 20.1 per cent from plants infected at 7 DAS, while 7.5 per cent oversized seeds were produced in plants infected at 49 DAS. Thus it is clear from Table 10 that viral infection occurring in the early stages of plant growth caused higher percentage of oversized seeds than those plants where infection was occurring at late growth stages.

4.3.3.3 Seed Transmission of ULCV as Influenced by Plant Age

The germination of urdbean seeds was significantly affected as a result of ULCV infection. Seeds collected from plants infected at 7 DAS showed a minimum 76.0 per cent germination, while maximum 88.0 per cent germination was recorded in seeds collected from plants infected at 49 DAS which was significantly at par with germination in seeds collected from plants inoculated at 28 DAS. Seeds collected from plants inoculated at 7 DAS showed the transmission of ULCV to an extent of 26.2 per cent, while it was minimum (2.2 per cent) in seeds from plants infected at 49 DAS. Data in Table 11 indicates that transmission of ULCV through urdbean seeds was higher when plants were infected before 28 DAS (up to 15.9 % transmission) but thereafter percentage of seed transmission extensively reduced and was 2.2 per cent in seeds collected from urdbean plants infected at 49 DAS (Table 11, Fig. 8).

Table 11. Seed transmission of urdbean leaf crinkle virus in PU 19 as influenced by plant age at viral infection

Plant age at infection	No. of seeds from infected plants sown	No. of seeds germinated	Seed germination (%)	Total plants infected	Seed transmission (%)
7	50	38	76.0	10.0	26.2
14	50	41	82.0	9.0	21.9
21	50	42	84.0	6.0	15.9
28	50	44	88.0	7.0	14.3
35	50	42	84.0	2.0	4.7
42	50	42	84.0	2.0	4.7
49	50	44	88.0	1.0	2.2
CD at 5%	-	1.62	3.20	1.47	1.18
Sem ±	-	0.53	1.06	0.48	3.60

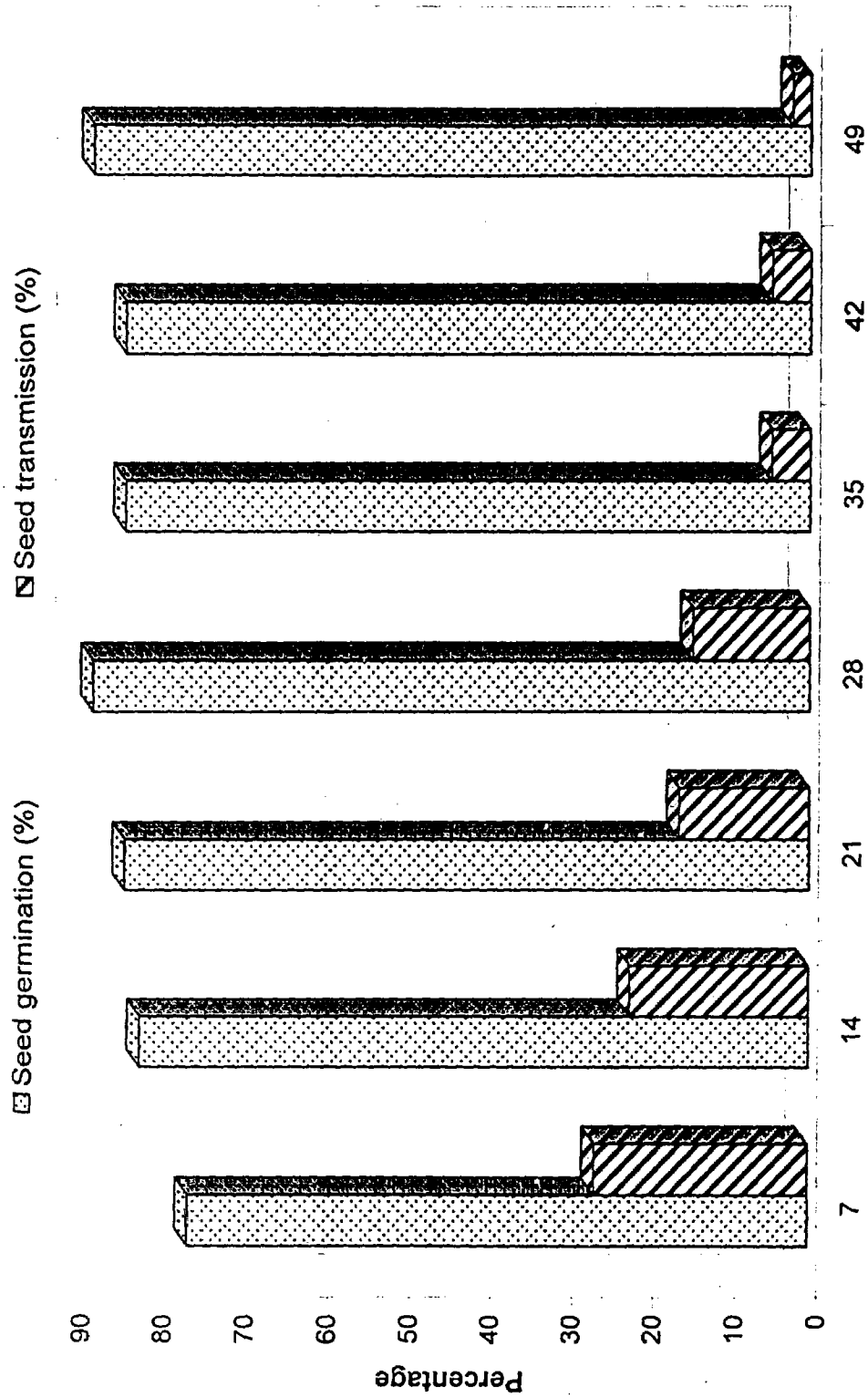


Fig. 8: Seed transmission of ULCV as influenced by plant age at the time of viral infection

4.3.3.4 ULCV Infection in Relation to Plant Age and Yield Contributing Factors and Yield in Urdbean

Seeds were collected from urdbean plants infected at weekly interval up to seven weeks separately and were sown under controlled glass house conditions as mentioned in Materials and Methods. At maturity of the crop observations were made on number of pods per plant, pod length, number of seeds per pod, 100 seed weight, yield per plant and per cent yield reduction, respectively.

Table 12 and Fig. 9 reveal that all the above-mentioned yield contributing factors were adversely affected as a result of ULCV infection. Average number of pods per plant was significantly lower in plant infections where inoculations were commencing from first week to seventh week as compared to healthy plants. A minimum average of 5.2 pods/ plant was found in plants inoculated at 7 DAS, while the maximum average number of pods was 21.6 pods/ plant at 49 DAS. In healthy plants an average of 25.5 pods/ plant was recorded. However, plants when inoculated after 28 days of planting did not reveal any significant decrease in pod length as compared to healthy plants. An average of 4 seeds per pod were recorded in case of plants infected at 7 DAS as compared to an average number of 5.8 and 5.9 seeds/ pod from

Table 12. Effect of ULCV on yield contributing factors and yield of PU 19 urdbean when infected at different interval after sowing

Plant age at infection	Average no. of pods per plant	Pod length	Average no. of seeds per pod	1000 seed weight	Yield	
					Per plant (gm)	Decrease over check (%)
7	5.2	3.7	4.0	24.9	1.9	71.7
14	6.7	3.8	4.2	26.7	2.3	65.4
21	8.3	3.9	4.6	29.3	2.7	59.5
28	11.9	3.9	5.1	30.2	3.5	47.2
35	14.4	4.1	5.5	32.1	4.5	33.5
42	19.2	4.2	5.8	38.9	4.7	29.5
49	21.6	4.7	5.8	37.4	5.6	16.4
Healthy	25.5	4.9	5.9	39.8	6.0	-
CD at 5%	1.47	.56	1.07	1.69	.82	2.58
Sem ±	.49	.18	.35	.56	.27	.86

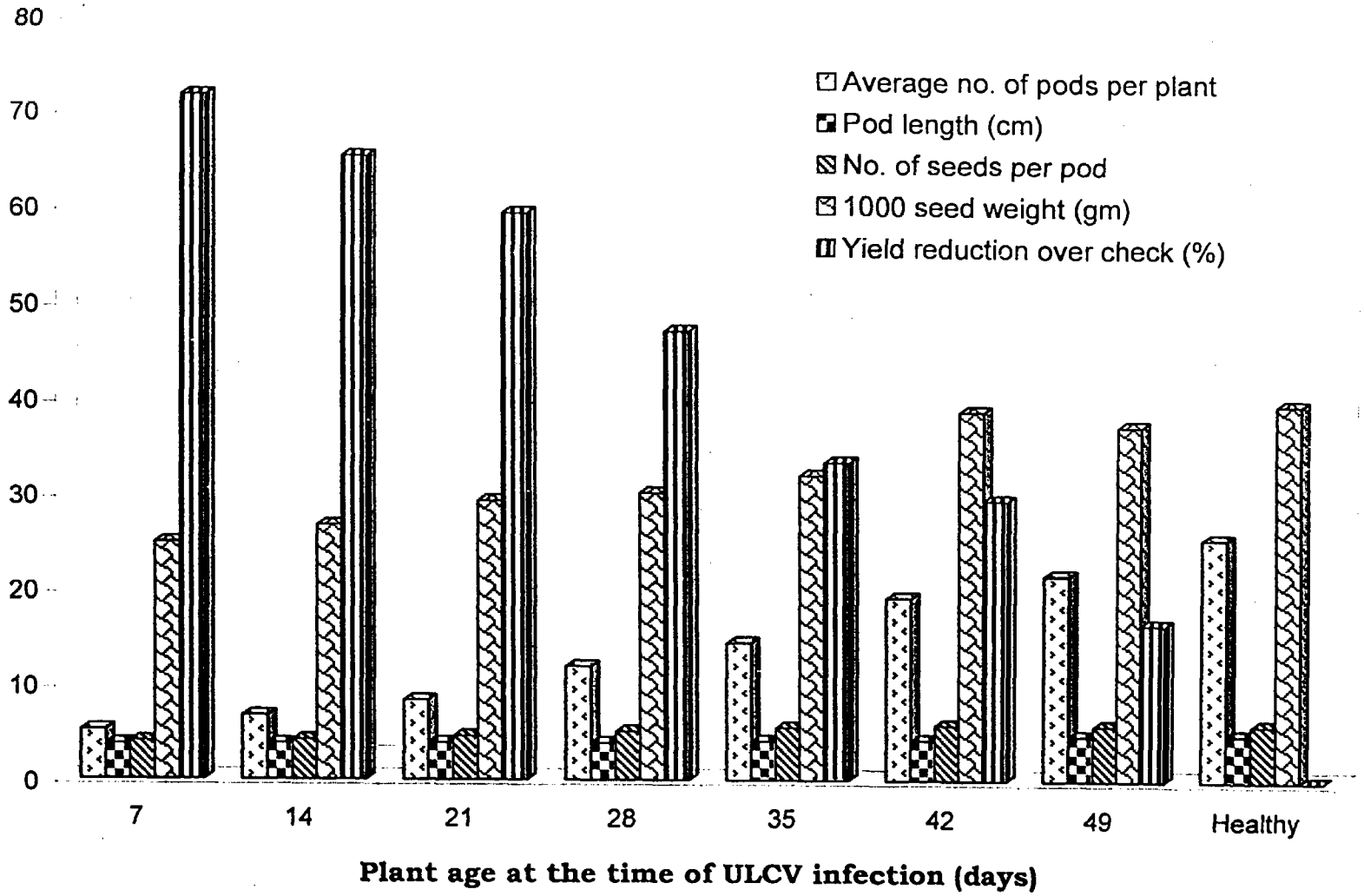


Fig. 9 : Effect of plant age at ULCV infection on yield contributing factors and yield of urdbean

plants infected at 49 DAS and healthy plants, respectively. Thousand seed weight was 24.9 gm in plants infected 7 DAS, 37.4 gm in plants infected at 49 DAS and was 39.8 gm when collected from healthy plants. Further, the ULCV infection at different stages of plant growth, significantly, affected the seed yield. Maximum yield reduction of 71.7 per cent was found when plant infection occurred at 7 DAS, which was significantly reduced with the delay in infection at 49 DAS where it caused only 16.4 per cent loss in yield. Thus, the yield loss ranged from 16.4 to 71.5 per cent in urdbean plants of variety PU 19 depending upon the plant age at the time of infection (Fig. 9).

4.3.4.1 Effect of Different Categories of Plant Infection on Seed Transmission of ULCV under Field Conditions

This experiment was conducted to study the effect of different categories of ULCV infection on seed germination, seedling mortality and seed transmission of ULCV infection in variety PU 19 under field conditions. Urdbean seeds were categorized as seeds collected from plants showing systemic ULCV infection (T_1), seeds from plants showing symptoms at later stages (T_2) and seeds collected from apparently healthy looking plants (T_3). All categories of seeds were separately sown in an insect proof screen house under field conditions as described in experiment number A (Plate 3). As

A- At initial stage

B- At maturity

C- Seed transmission of ULCV infection in naturally infected seeds of urdbean variety PU 19.

Plate 3. Use of insect proof cages for seed transmission study of ULCV under field conditions

revealed by Table 13 and Fig. 10, a lowest of 74.6 per cent germination was recorded in seeds collected from systemically ULCV infected plants followed by 88.3 and 94.0 per cent germination in seeds collected from plants showing symptoms at later stages and apparently healthy looking plants, respectively. Mortality was highest (18.4 per cent) in seeds collected from systemically ULCV infected plants, while it was minimum 3.5 per cent in seeds collected from apparently healthy looking plants. Findings in Table 13 further indicate that ULCV transmission through urdbean seeds was highest (30.8 per cent) in seeds collected from systemically ULCV infected plants followed by 18.1 per cent transmission through seeds of plants showing symptoms at late stage and only 4.0 per cent in seeds from apparently healthy looking plants, respectively. At maturity, seeds were collected from all the three treatments separately and sown again in next cropping season in experiment number B. Seed germination was 80.2, 85.4 and 96.0 per cent in T_1, T_2 and T_3, respectively. Similarly seedling mortality was 17.2, 10.4 and 4.2 per cent in all the three categories (T_1-T_3), respectively. Data in Table 13 clearly indicate an increase in seed transmission of ULCV up to 32.3 per cent in case of T_1, while seed transmission of ULCV was 19.8 in T_2 and 4.6 per cent in T_3 category of urdbean seeds, respectively.

Table 13. Effect of different categories of ULCV infection on seed germination, mortality, transmission of seedborne inoculum of ULCV and yield in PU 19 urdbean under field conditions

Experiment no.	Seed collected from systemically ULCV infected plants (T_1)					Seeds from plants showing symptoms at later stage (T_2)					Seeds from apparently healthy looking plants (T_3)				
	Germ. (%)	Mortality (%)	Infected plants (%)	1000 seed weight	Yield reduction (%)	Germ. (%)	Mortality (%)	infected plants (%)	1000 seed weight	Yield reduction (%)	Germ. (%)	Mortality (%)	infected plants (%)	1000 seed weight	Yield reduction (%)
A	74.6	18.4	30.8	33.1	21.2	88.3	12.0	18.1	36.2	13.9	94.0	3.5	4.0	42.1	-
B	80.2	17.2	32.3	31.6	23.5	85.4	10.4	19.8	34.1	17.4	96.0	4.2	4.6	41.3	-

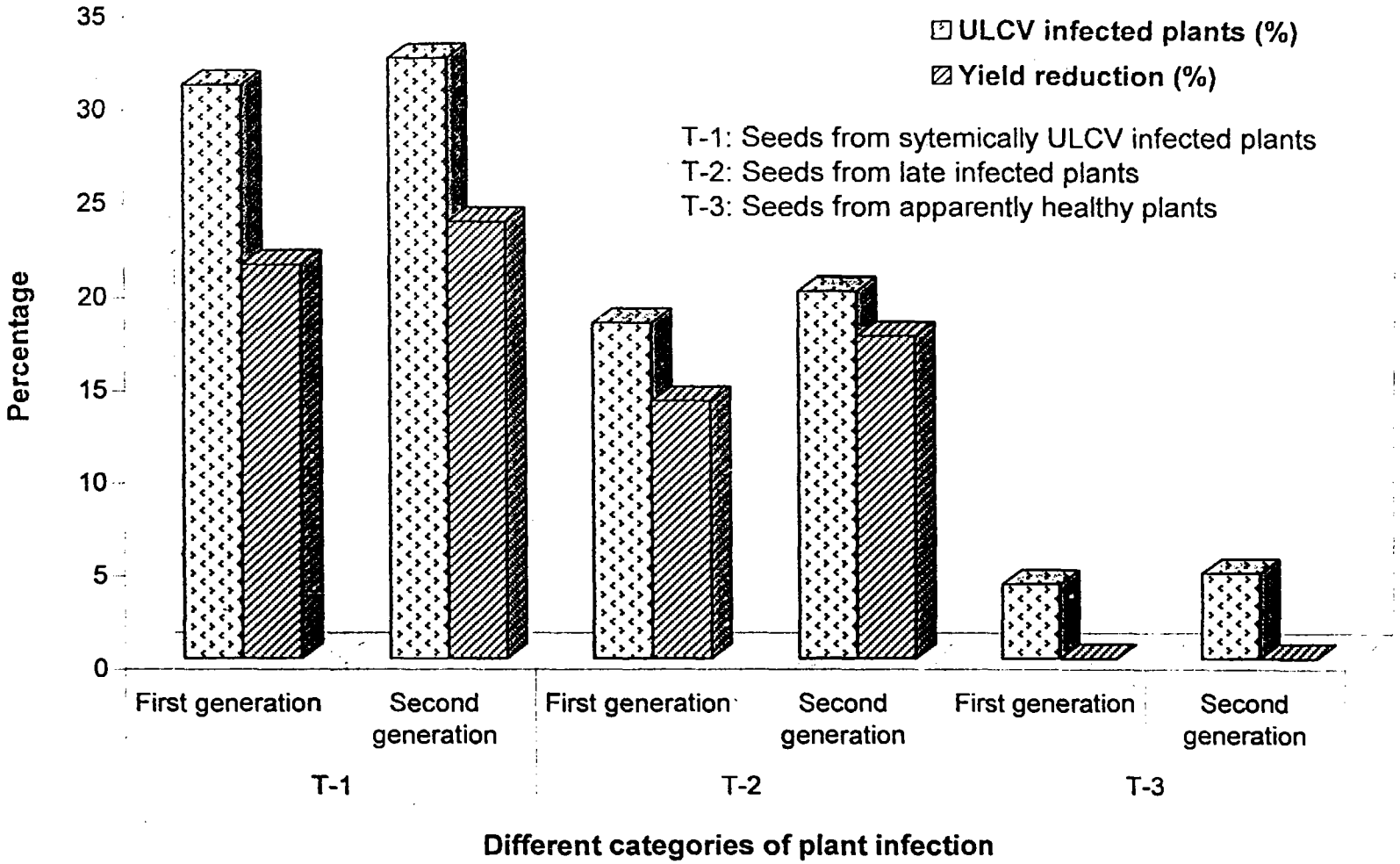


Fig.10 : Effect of different categories of plant infection on transmission of seedborne inoculum of ULCV and yield of urdbean

4.3.4.2 Effect of Different Categories of ULCV Infection on Yield of Urdbean

This experiment was conducted to study the extent of yield reduction due to seedborne inoculum of ULCV in urdbean plants showing different degree of ULCV infection under field conditions. Results presented in Table 13 indicate that yield reduction varied from 21.2 to 23.5 per cent in systemically ULCV infected plants, while it ranged from 13.9 to 17.4 per cent in plants showing ULCV infection at later stages of plant growth, respectively in both the above-mentioned experiments (Fig. 10). Thousand seed weight in apparently healthy looking plants was considered as standard for healthy plants.

4.3.5 Effect of Seed Maturation on Transmission of Seedborne Inoculum of ULCV

This experiment was aimed to find out the per cent transmission of ULCV in immature and mature seeds of urdbean plants of variety PU 19, as there may be possibility of inactivation of ULCV during seed developmental stages in urdbean. 100 each of physiologically mature but undried and equal number of physiologically mature but dried seeds were separately collected from systemically ULCV infected urdbean plants from field. The growing on test as described under Materials and Methods was followed for the estimation of per cent seeds transmission of urdbean leaf crinkle virus. Table 14 and Fig. 11 reveal that seed germination was 90.0 and

97.0 per cent in immature and mature urdbean seeds, respectively while per cent ULCV transmission in undried seeds and in dried seeds was 16.6 and 19.5 per cent, respectively.

4.4 Influence of Mother Plant Infection with ULCV on Flower Organelles

From the literature it appears that in some cases mother plant infection with the virus causes certain adverse effects on the normal functioning of flower organelles and seed quality. The present experiment was, therefore conducted to find out the influence of systemic ULCV infection in PU 19 urdbean plants on the flower formation and pollen viability, respectively.

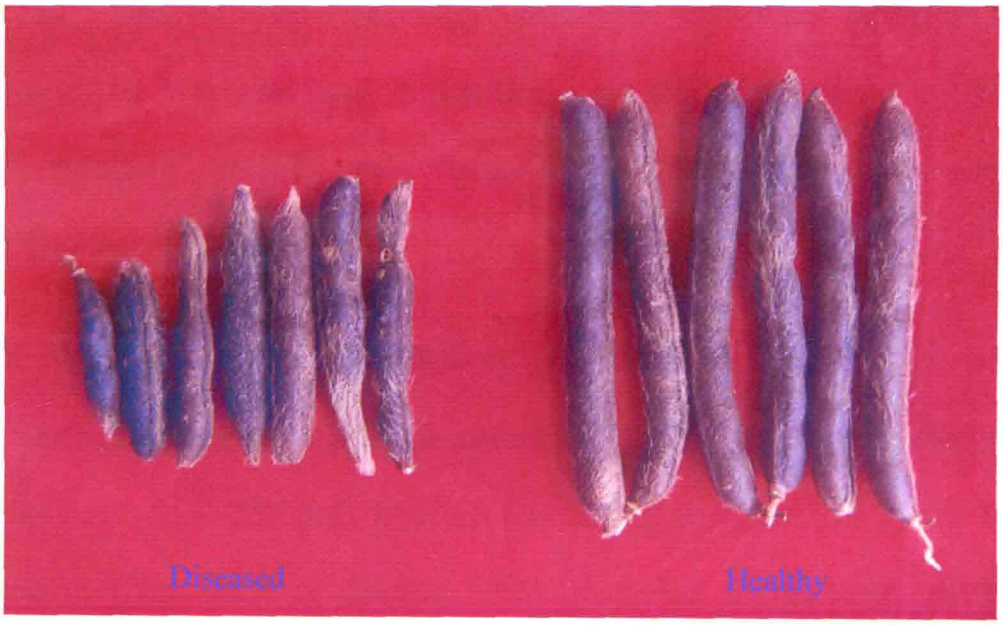
4.4.1 Flower Morphology

Observations made in systemically ULCV infected PU 19 urdbean showed the transformation of whole inflorescence into a bushy appearance due to presence of numerous small sized flower buds. This resulted in complete loss of yield in such plants. In the inflorescence of less affected plants, total number of flowers was drastically reduced (Plate 4A).

4.4.2. Pollen Viability

The viability of the pollen grains obtained from healthy and systemically ULCV infected plants was determined with the help of

A. Malformed inflorescence in ULCV infected urdbean plant



B. Effect of ULCV on pod development

Plate 4.

acetocarmine stain (1.5 %). Fifty flower buds from healthy and systemically infected PU 19 urdbean plants were analyzed separately. The pollen grains which did not take the stain were regarded as sterile (Plate 5). The results are presented in Table 15. A total of 20 per cent flower buds in systemically ULCV infected plants were found completely sterile, while pollen sterility was 24.5 per cent in rest of the flower buds. None of the flower buds collected from healthy plants showed complete sterility, however a small number (1.5 per cent) of pollen grains in flower buds of healthy plants were observed to be sterile (Fig. 12).

4.5.1 Purification of Urdbean Leaf Crinkle Virus

The ULCV was partially purified by the protocol as mentioned in Material and Methods. A single light scattering zone (white opaque band) was observed after density gradient centrifugation (Plate 6).

4.5.2 Molecular Weight of Coat Protein of ULCV

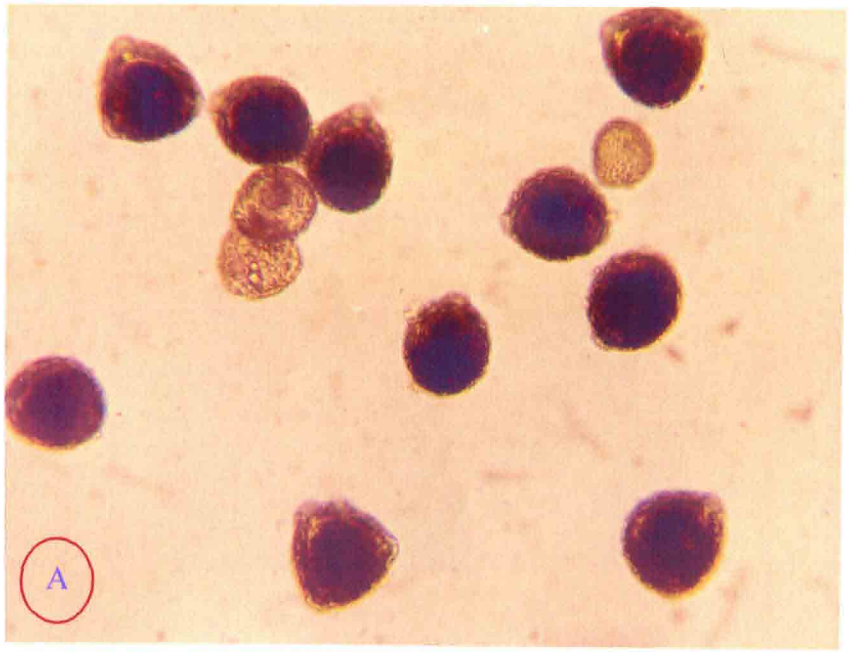
Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) of ULCV coat protein has been depicted in Plate 7. It is evident that lane 2 loaded with purified virus coat protein sample showed only one band of protein of approximately 31 kDa molecular weight when stained with commassie blue, suggesting the molecular weight of coat protein of purified ULCV to be 31 kDa.

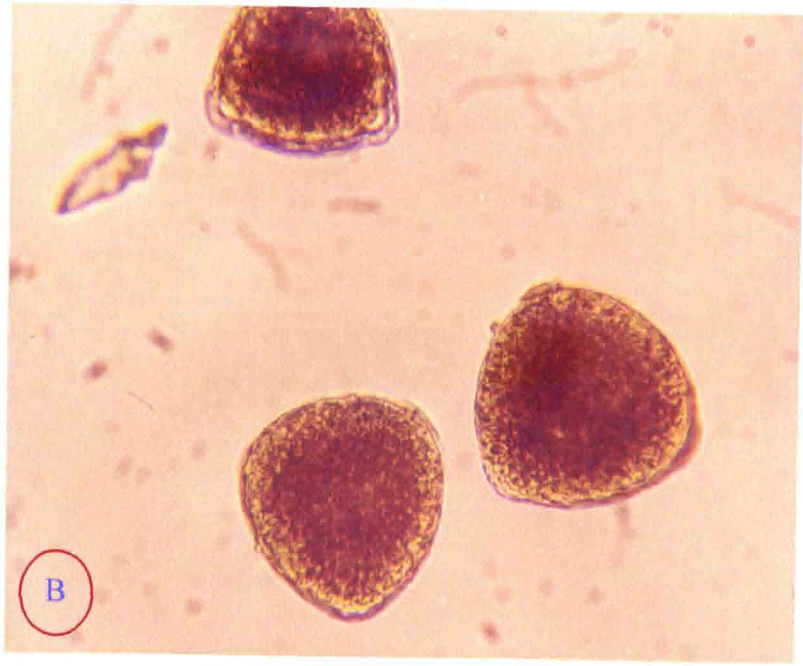
Table 14. Effect of seed maturation on transmission of urdbean leaf crinkle virus through seeds of PU 19 urdbean

Type of seeds	Total seeds sown	Total seeds germinated	Germination (%)	Total plants infected	Seed transmission of ULCV (%)
Immature	100	90	90.0	15	16.6
Mature	100	97	97.0	19	19.5

Table 15. Pollen sterility as influenced by systemic urdbean leaf crinkle virus infection in PU 19 urdbean

Flower buds collected from	Total flower buds examined	Completely sterile buds (%)	Pollen sterility (%)
Systemically ULCV infected plants	50	20.0	24.5
Healthy plants	50	Nil	1.5





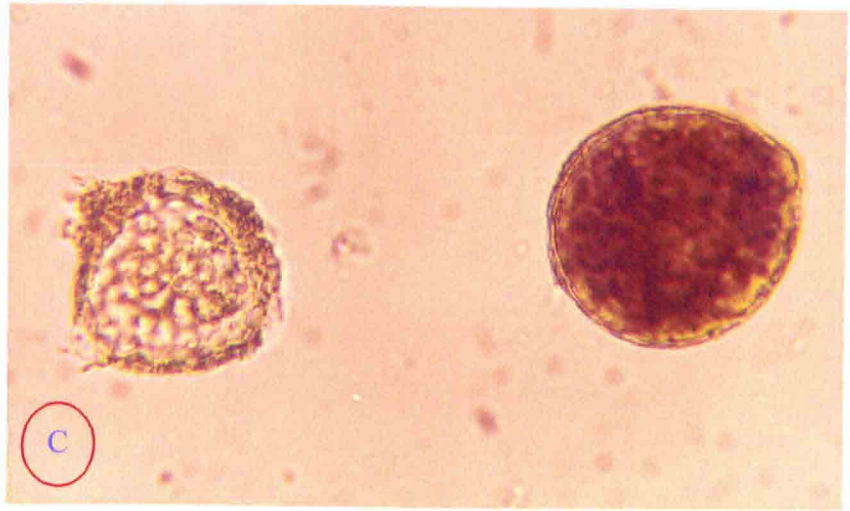


Plate 5: Test for pollen viability in floral buds of ULCV infected plants

A- Sterile and viable pollens
B- Viable pollens
C- Enlarge view of viable and non viable pollen

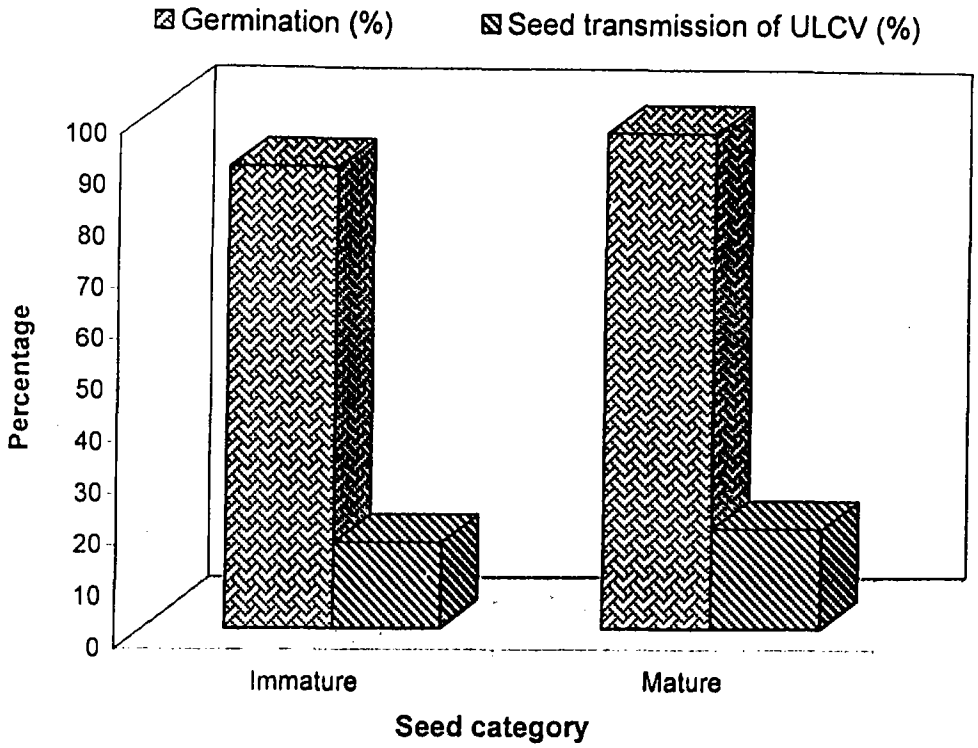


Fig.11 : Effect of seed maturation on transmission of seedborne inoculum of ULCV

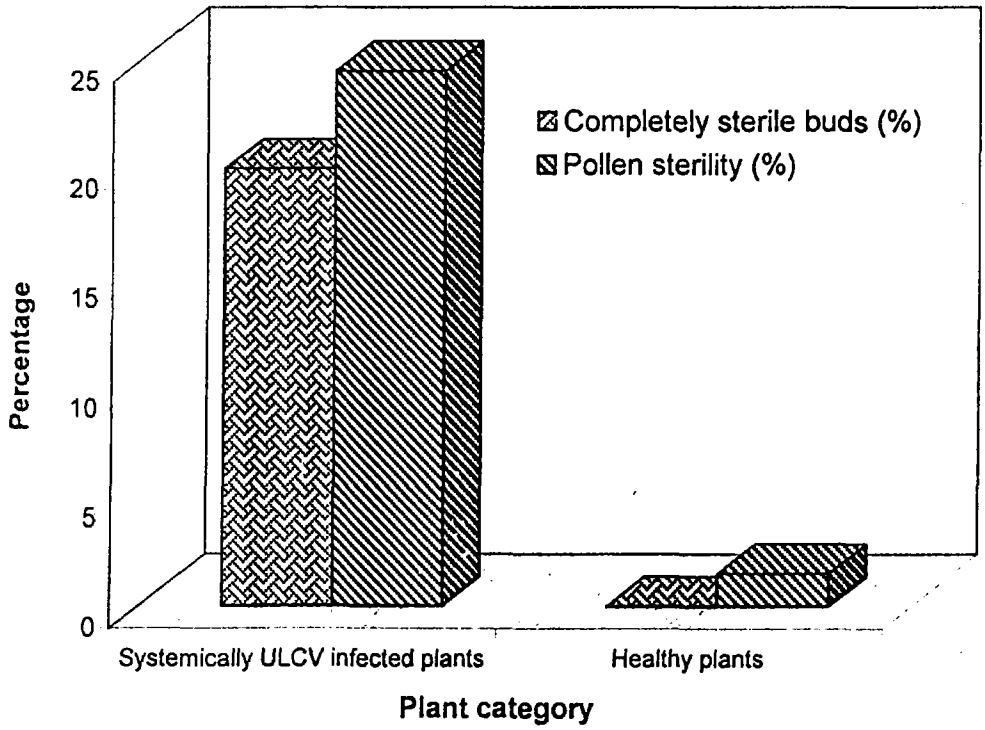


Fig.12 : Pollen sterility in flower buds obtained from ULCV infected plants

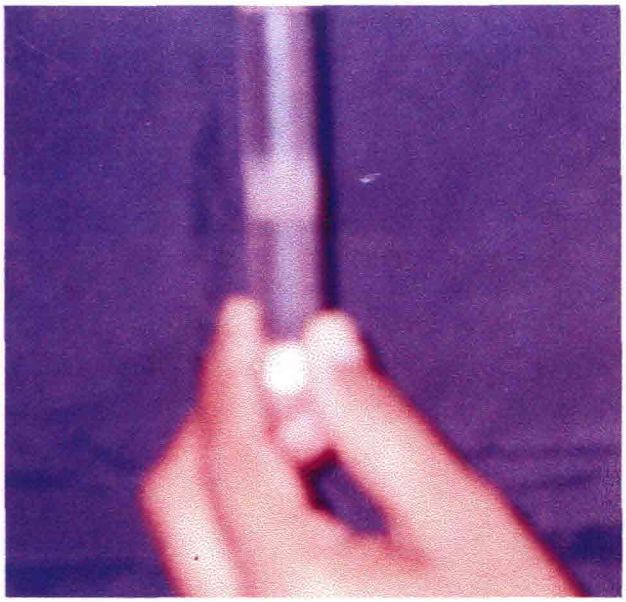


Plate 6: Purified band of ULCV on density gradient

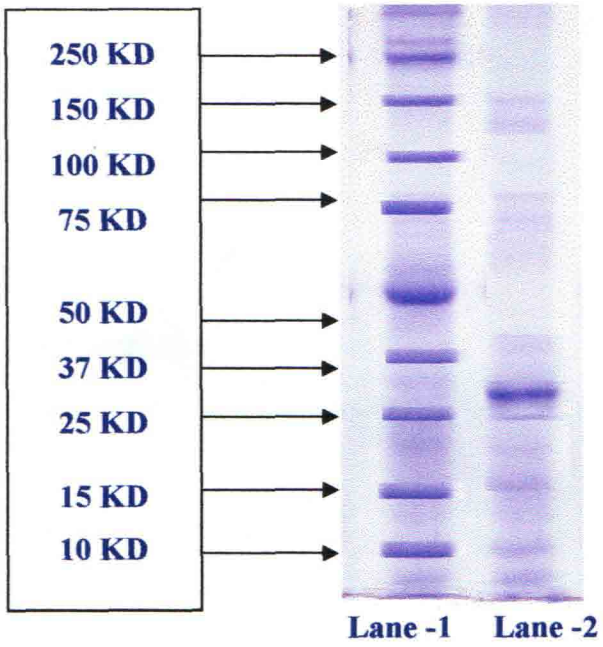


Plate 7: Molecular Weight and purity of the coat protein of ULCV using SDS-PAGE

Lane -1 Standard Molecular Marker (From 10-250 KD)

Lane -2 Coat protein of purified ULCV

Discussion

Five DISCUSSION

Among the various pulses grown in India, urdbean or black gram (*Vigna mungo* L. Hepper) is one of the most important pulse crops. In India, it has been cultivated since ancient times. There are several biotic and abiotic stresses which are responsible for the low production and productivity of urdbean. Among these, diseases caused by viruses and fungi are the major limiting factors in the cultivation of urdbean. Of the several diseases infecting urdbean, leaf crinkle virus has become a potential threat to the cultivation of urdbean, as most of the high yielding cultivars of urdbean are susceptible to urdbean leaf crinkle virus (ULCV). The disease under natural conditions was first reported by Williams *et al.*, (1968) from Delhi and by Nene (1968) from *tarai* of Uttar Pradesh. Since then incidence of ULCV has been reported from different states of India (Kolte and Nene, 1970; Khatri *et al.*, 1971; Gupta, 1974; Dhingra, 1975; Narayanasamy and Jaganathan, 1975; Beniwal and Chaubey, 1979; Singh *et al.*, 1979; Kadian, 1980; Dubey and Sharma, 1985; Brar and Rataul, 1986; Mishra *et al.*, 1994; Mahajan and Joi, 1999). The disease has also been reported from neighbouring countries like Sri Lanka

(Shivanathan, 1980) and Pakistan (Bashir *et al.*, 1991). Consequently, a lot of informations were generated on symptomatology, host range, epidemiology, characterization of the virus etc. However, the role of insect vector in the secondary spread of the disease has not been conclusively established so far. Management of disease through cultural practices and application of insecticides has been tried with limited success. The production and distribution of good quality seed emphasizes to pay more attention to workout different parameters affecting the seed quality due to this disease.

Therefore, present investigation was aimed at gaining the better knowledge of urdbean leaf crinkle virus, detection of seedborne inoculum of urdbean leaf crinkle virus through dry seed examination, growing on test, indicator inoculation test and also by Enzyme linked immunosorbent assays (ELISA) technique, to study the location of seedborne inoculum of ULCV in different floral and seed parts, to establish the seedborne nature of viral inoculum; to study the influence of mother plant infection on flower organelles and seed quality; detailed studies regarding the seed transmission of ULCV; yield losses due to the seedborne inoculum of ULCV and finally an attempt for purification and

molecular weight study of coat protein of urdbean leaf crinkle virus.

5.1 Detection of Seedborne Inoculum of Urdbean Leaf Crinkle Virus in Urdbean Seeds

The morphologically abnormal seeds showing reduced seed size, seed coat mottling and shrivelling have been known to cause a higher percentage of transmission of certain viruses. Seeds of variety PU 19 of urdbean with systemic infection of ULCV showed certain adverse affect on seed morphology. A close and careful visual observation of seeds collected from systemically ULCV infected seeds of variety PU 19 revealed the presence of shrivelled, oversized, undersized and off-coloured seeds. These observations are also made by Beniwal and Chaubey (1979) and Beniwal *et al.*, (1984) who also mentioned the morphological seed abnormality in ULCV infected plants. Production of shrivelled and off-coloured seeds has also been reported in other viral diseases such as mungbean yellow mosaic virus (Nene, 1969) and mungbean mosaic virus (Phatak, 1974), urdbean plants infected with bean common mosaic virus (Agarwal, *et al.*, 1977, 1979a) and urdbean plants infected with mungbean yellow mosaic virus (Vohra and Beniwal, 1979) etc. Further, the ULCV infection changed the seed

colour from natural black to light brown and the number of such off-coloured seeds was significantly higher in systemically ULCV infected plants.

All the above-mentioned categories of morphologically abnormal seeds viz. shrivelled, off-coloured, oversized and undersized seeds obtained from systemically ULCV infected plants yielded ULCV infection varying from 22.2 per cent, 12.7 per cent, 17.3 per cent and 9.0 per cent, respectively in the growing on test (Table 2). The presence of ULCV in different seed categories was also confirmed by DAC-ELISA test and it was observed that shrivelled seeds yielded strong positive reaction, while oversized seeds gave moderately positive and off-coloured and undersized seeds gave mild positive reaction in DAC-ELISA.

These results also indicate that the seed abnormalities in urdbean seed lots may be due to ULCV infection. It was also observed that presence of oversized seed is the most characteristic feature of a seed lot infected with ULCV. Therefore, on the basis of seed morphology, the presence of oversized seeds in urdbean seed lots may be indicative of the presence of ULCV in the urdbean seeds.

Anyhow, morphologically abnormal seeds infected with ULCV affected the emergence of urdbean seeds but to a less extent

and the germination percentage of seeds in different categories of abnormal seeds viz., shrivelled, undersized, oversized and off-coloured varied from 72.0 per cent to 94.0 per cent. Narayanasamy and Jaganathan, (1973), however, reported a drastic reduction in seed germination in ULCV infected seeds up to 44.0 per cent. The drastic reduction in seed germination may be due to the infection of plants at most susceptible stage of seed setting.

5.2 Location of Seedborne Inoculum of ULCV in Urdbean Seeds

Indicator inoculation test and serological test (DAC-ELISA) were performed to study the location of ULCV infection in urdbean seed. The indicator plants when sap inoculated at primary leaf stage with the extracts prepared from different flower and seed parts resulted in the transmission of ULCV infection. The assay results of different floral parts i.e. epicalyx, calyx, corolla, androecium and gynoecium obtained from systemically ULCV infected plants showed the presence of ULCV in all floral parts (Table 3). The presence of virus in the various seed components viz., seed coat, cotyledons and embryo was also detected in indicator inoculation test (Table 4). Symptoms of ULCV started

appearing on the second trifoliate leaf stage onwards. The third trifoliate, generally showed light green colour and increased size followed by conspicuous crinkling of the upper surface. The crinkling usually became evident at 13 days after inoculation. Subsequently produced trifoliate also showed similar symptoms. The indicator inoculation test has also been performed to find out the presence of ULCV in different parts of urdbean seed by Beniwal and Chaubey (1984) and Patel *et al.*, (1999).

In assay test, however it is difficult to estimate the per cent seed infection, unless individual seed extract is tested for assay. This method would be useful specially for quarantine purpose where it may be important to know only the presence or absence of the virus with the seeds. It may, therefore, be inferred on the basis of indicator inoculation test that the ULCV was recoverable from the infected floral and seed parts in quantities sufficient to perform assay. There is further need to workout the dilution end point (DIP) for ULCV infection from different floral or seed parts.

To investigate whether the virus was present on seed surface as a contaminant, seeds of variety PU 19 of urdbean from systemically ULCV infected plants were used. Seeds treated with trisodium phosphate gave almost similar seed transmission of ULCV as compared to untreated seeds (13.5 and 14.2 %) (Table 5).

The location of the ULCV in urdbean seeds has further been supported by experiment on seed surface washings. The test plants when inoculated with seed washings did not develop disease symptoms at any growth stage of the plant indicating the absence of virus in seed washings. DAC-ELISA using seed surface washings as antigen also did not give any indication of the presence of virus in such washings. Moreover, seeds with or without seed coat, when planted also showed more or less the same transmission (16.6 and 15.5 %), thus indicating that the virus is internally present in the urdbean seeds (Table 6). All the three tests when considered together strongly indicate that ULCV is internally seedborne, located within the embryo and cotyledons of the urdbean seeds and it is not present as surface contaminant. Similar results have been shown with pea seedborne mosaic virus (Stevenson and Hagedorn, 1973), cowpea aphid borne mosaic virus (Phatak, 1974), bean common mosaic virus in French bean (Ekpo and Saettler, 1974) and urdbean (Agarwal *et al.*, 1979b), and urdbean leaf crinkle virus (Beniwal *et al.*, 1984).

5.3. Transmission of Seedborne Inoculum of ULCV in Urdbean Seeds

This study was undertaken to find out the transmission (1) of seedborne inoculum of ULCV in different germplasms and

varieties of urdbean, (2) in seeds obtained from plants infected at different stages of plant growth, (3) in seeds collected from naturally ULCV infected plants viz. (i) systemically infected, (ii) infected late in season and (iii) apparently healthy looking plants, and (4) in seeds exhibiting morphological abnormalities.

Studies were carried out to find the extent of natural transmission of ULCV at different stages of plant growth and further to determine the plant stage up to which the plants should be examined for complete recovery of seedborne infection. The experiment conducted showed that out of total 29.1 per cent seed transmission, 6.2, 8.3, 10.4 and 4.1 per cent seed transmissions was observed at first, second, third and fourth trifoliate leaf stages, respectively (Table 7). On the basis of results it could be inferred that the disease symptoms of ULCV, on plants raised from naturally infected seeds are expressed up to 4th trifoliate leaf stage. Pushpalatha *et al.*, (1999) also performed the growing on test for ULCV detection in urdbean seeds and observed the appearance of leaf crinkling up to 3rd trifoliate leaf stage. There are no other viruses reported to produce typical crinkling in urdbean similar to those of ULCV.

The growing on test revealed the presence of ULCV in seeds of all the nineteen germplasm and varieties of urdbean

investigated under field conditions, though the percentage transmission varied from 3.3 to 20.3 per cent (Table 8). VBG 73, an urdbean cultivar showed highest percentage transmission (20.3) of seedborne inoculum of ULCV, while it was minimum (3.3%) in IU 83-5 and Mash 1, respectively. The possible reason for this variation in per cent transmission of seedborne inoculum could be (1) either difference in relative susceptibility of different germplasms and varieties, (2) the relative persistence of the virus within the seeds during seed developmental stages, (3) relative capabilities of host virus combination for successful transmission or (4) the environmental conditions during seed developmental stages. The presence of ULCV in all the 19 germplasms and varieties of urdbean indicates the great potential of the virus to survive within the seeds of urdbean grown under field conditions.

To study the effect of plant age at the time of viral infection on (1) ULCV expression and incubation period, (2) texture, colour and size of urdbean seeds, (3) seed transmission, (4) yield contributing factors and yield of urdbean, plants were grown under glass house conditions. Seedlings raised from urdbean plants of variety PU 19 were sap inoculated at 7, 14, 21, 28, 35, 42 and 49 days after sowing. The ULCV infection ranged from 86.5 to 15.0 per cent in plants inoculated 7 DAS and 49 DAS,

respectively. As revealed in Table 9, the susceptibility of urdbean plants to ULCV reduced with increase in the age of the plant, while the incubation period for disease expression increased with increase in plant age. Incubation period ranged from 13 to 38 days depending upon the age of the plants while inoculating.

Seeds collected from each category of inoculated plants (1st to 7th week) were collected and examined for the presence of shrivelled, oversized and off-coloured seeds. It was observed that seed texture was significantly affected as a result of ULCV infection. Data in Table 10 also clearly indicates that the proportion of shrivelled seeds was more in plants inoculated at earlier developmental stages as compared to plants inoculated late. Seed colour also changed from normal black to light brown. Such results are in accordance with those reported by Beniwal and Chaubey, (1979). Interestingly, ULCV infection caused more production of oversized seeds. Infections occurring in the early stages of plant growth caused higher percentage of oversized seeds than those occurring in late plant growth stages (Table 10). Expansion of leaf lamina has been reported by Kolte and Nene, (1972, 1979). Bhaktavatsalam *et al.*, (1982) mentioned that an increase in auxin concentration in ULCV infected plants might be responsible for expansion of leaf lamina. The higher number of

oversized seeds in ULCV infected plants may be one of the reasons of higher auxin production in infected plants. This needs further studies. Presence of shrivelled seeds in ULCV infected plants was also observed. However, the average size of seeds from ULCV infected plants was not affected. Beniwal and Chaubey, (1979) and Beniwal *et al.*, (1983a) also observed the increased amount of shrivelled, off-coloured and oversized seeds in the urdbean plants infected at earlier stages of their development.

When urdbean seeds collected, separately from plants inoculated at weekly interval (7DAS-49DAS) were sown under glass house conditions, the seed germination was significantly reduced as a result of ULCV infection. However, seeds collected from plants inoculated at 7 DAS showed minimum 76.0 per cent germination, while the maximum germination percentage (88.0 %) was recorded in seeds collected from plants inoculated at 28 and 49 DAS, respectively (Table 11). Narayanasamy and Jaganathan, (1975), Beniwal and Chaubey, (1979), Beniwal *et al.*, (1983a, 1984), Kadian (1983), Patel, *et al.*, (1999) also reported reduced seed germination as a result of ULCV infection.

Seed transmission of ULCV, as influenced by the plant age at the time of viral infection clearly indicates that plant age at time of viral infection, does affect the transmission of urdbean leaf

crinkle virus through urdbean seeds. Generally, the transmission of ULCV in seeds obtained from plants inoculated at 7 DAS was 26.2 per cent, which went on decreasing and at 28 DAS, it was 14.3 per cent. But thereafter percentage of seed transmission reduces drastically and it was only 2.2 per cent in plants infected at 49 DAS (Table 11). These results are in agreement with Narayanasamy and Jaganathan, (1975) and Beniwal, *et al.*, (1983a), who also observed lower percentage of seed transmission of ULCV in urdbean plants infected at later stages. According to Beniwal *et al.*, (1979), ULCV infection in early infected plants has its origin in the seed. It is possible that plants infected prior to flowering carried more viral infection in their seeds as compared to those which got infection just at flowering or when flowering was over. It seems that early stages of plant infection facilitated the movement of virus to reach the floral parts and seeds.

A number of parameters such as pods per plant, pod length, number of seeds per pod, 1000 seed weight, yield per plant and yield reduction were studied in PU 19 variety of urdbean. As revealed in Table 12, deterioration of all the yield contributing factors and yield in ULCV infected plants was observed as compared to healthy plants. A minimum average of 5.2 pods/

plant was found in plants inoculated at 7 DAS, while the maximum average number of pods was 21.6 pods/ plant at 49 DAS. In healthy plants an average of 25.5 pods/ plant was recorded. An average of 4 seeds per pod were recorded in case of plants infected at 7 DAS as compared to an average number of 5.8 and 5.9 seeds/ pod from plants infected at 49 DAP and healthy plants, respectively. Thousand seed weight was 24.9 gm in plants infected 7 DAS, 37.4 gm in plants infected at 49 DAS and was 39.8 gm when collected from healthy plants. Earlier Kolte and Nene, (1979); Beniwal and Chaubey, (1979); Kadian, (1983); Brar and Rataul, (1989); Bashir *et al.*, (1991); Mishra *et al.*, (1994) and Patel *et al.*, (1999) also reported a considerable reduction in number of pods per plant, pod length and number of seeds per plant. Further, the ULCV infection at different stages of plant growth also adversely affected the seed yield significantly. Maximum yield reduction of 71.7 per cent was found in plants infected at 7 DAS, which went on reducing significantly with the delay in infection up to 49 DAS where only 16.4 per cent yield loss in PU 19 variety of urdbean was recorded. Critical analysis of results indicates that the reduction in number of pods per plant is the most important single factor which affected the yield adversely. Pods per plant were reduced because the ULCV

infection has been shown to cause considerable malformation of the inflorescence and pollen sterility (Kolte and Nene, 1972, 1979). It seems that 1000 seed weight was not a significant factor for the reduction in yield due to ULCV infection as there was not significant difference between 1000 seed weight of the seeds collected from diseased and healthy plants, separately. Various workers have also reported the effect of urdbean leaf crinkle virus on yield contributing parameters and yield (Kolte, 1971; Nene, 1973; Kolte and Nene, 1979; Kadian, 1983; Bashir *et al.*, 1991; Mishra *et al.*, 1994). The variation in yield losses reported by different workers may be due to different environmental conditions at their work place, stage of infection and presence of insect vectors in an area causing secondary spread.

Studies on transmission of ULCV through naturally infected seeds of variety PU 19 of urdbean, carried out using seeds from plants (1) exhibiting systemic ULCV infection (T_1), (2) showing symptoms at later stage (T_2), and (3) apparently healthy looking (T_3), indicate a higher reduction in germination and increased mortality in seeds collected from systemically ULCV infected plants as compared to seeds obtained from plants showing symptoms at late in season and apparently healthy looking plants. As revealed by Table 13, the transmission of seedborne inoculum through seeds

collected from systemically ULCV infected plants was highest (32.3 %) in second generation as compared to 30.8 per cent transmission in first generation. Further, seed transmission ranged from 18.1 to 19.8 per cent and 4.0 to 4.6 per cent in seeds collected from urdbean plants showing leaf crinkling late in the season and from apparently healthy looking plants, respectively. Results also suggest that seed transmission of ULCV increased when infected seeds were reused for sowing purpose in next generations. This is indicative that ULCV infection prevails in seeds and expresses in next generation. Kadian, (1983) also observed an average 21.0 per cent seed transmission of ULCV when infected seeds were used for sowing purpose up to three generations.

An average, 22.3 per cent yield reduction was recorded when seeds from systemically ULCV infected plants were sown for two generations, while it was 15.7 per cent when seeds collected from urdbean plants showing symptoms later in season were used for sowing purpose.

5.4 Effect of Seed Maturation on Transmission of Seedborne Inoculum of ULCV

Results on effect of seed maturation on transmission of seedborne inoculum of ULCV in urdbean are depicted in Table 14.

Results reveal a total of 16.6 and 19.5 per cent transmission of ULCV through seeds collected before and after physiological maturity, respectively. Low percentage transmission of seed borne inoculum has been attributed to a number of factors, of which one is the inactivation of virus during seed development stages. However, ULCV in urdbean does not seem to be inactivated during seed development as the physiologically mature but undried seeds revealed almost the same percentage of seed transmission of ULCV as those from physiologically mature and dried seeds.

5.5 Influence of Mother Plant Infection with ULCV on Flower Organelles

The urdbean leaf crinkle virus was found in all the floral and seed parts (cotyledons and embryo) of systemically ULCV infected urdbean plants. Under natural conditions as well as by sap inoculation, crinkled surface of lamina was the characteristic morphological change observed on infected trifoliate. Observations on morphology of the inflorescence clearly showed that floral buds become thick, greener with bushy appearance than the normal ones. This may probably be due to the overall effect of the systemic infection on the plant due to which the growth was found to be reduced. Such abnormalities resulted in the pollen sterility leading to few pod setting, thereby causing severe reduction in

yield contributing parameters. Such observations were also made by Sharma and Dubey, (1985), Patel (1999). Out of total 50 flower buds collected from systemically ULCV infected plants, 20.0 per cent of the buds possess all the sterile pollens and the remaining 80.0 per cent buds possess 24.5 per cent sterile pollens (Table 15). This therefore, indicates that ULCV adversely affects the pollen viability and this might be one of the reasons for low pod setting on the diseased plants. Khatri *et al.*, (1971); Kolte and Nene, (1972) and Patel (1999) also reported the pollen sterility as a result of ULCV infection.

5.7 Purification and Molecular Weight Study of Coat Protein of ULCV

For detailed serodiagnostic and molecular study, it is important to purify the virus from its host. A single light scattering zone (white opaque band) was observed after density gradient centrifugation (Plate 6). The purity of this was confirmed by SDS-PAGE (a single band of coat protein). Bhaktavatsalam (1976) and Patel (1999) also tried to purify the ULCV by polyethylene glycol method, using different combinations of PEG and NaCl, respectively.

The molecular weight of coat protein of partially purified ULCV was determined by sodium dodecyl sulphate polyacrylamide

gel electrophoresis (SDS-PAGE). In the SDS-PAGE a dark band was observed with approximately 31 kDa molecular weight of virus coat protein from purified as well as crude sap preparation (Plate 7). Patel (1999) observed the molecular weight of ULCV coat protein up to 28 kDa.

From these investigations sufficient light has been thrown on seedborne nature of urdbean leaf crinkle virus. Since seedborne nature is proved, the initial source of infection under field conditions may be the infected seeds. Further, infection at an early stage of crop growth is known to cause sterility of plants, leading to heavy crop loss. Thus, the use of virus free seeds would definitely help in the management of leaf crinkle disease of urdbean.

Summary

SUMMARY

Investigations were carried out on detection techniques, location and transmission of the seedborne inoculum of urdbean leaf crinkle virus (ULCV), which has now become a potential threat to the cultivation of urdbean (*Vigna mungo* L. Hepper) in India. The results obtained in the present studies have been summarized below.

1. Dry seed examination exhibited the presence of higher number of shrivelled, oversized, undersized and off-coloured seeds in seed lots collected from systemically ULCV infected urdbean plants. The growing on test revealed the transmission of seedborne inoculum of ULCV which varied in different categories of morphologically abnormal seeds. DAC-ELISA test also confirmed the presence of ULCV in morphologically abnormal seeds.
2. The entire floral parts of ULCV infected plants viz. epicalyx, calyx, corolla, androecium and gynoecium revealed the presence of ULCV in infectivity as well as in ELISA test. However, highest percentage of transmission (28.8 %) was observed when the sap of whole flower was used as source of

inoculum. Among the various seed parts, cotyledons and embryo of infected seeds exhibited the presence of virus when the sap from these parts was used for infectivity test, while the sap from seed coat gave negative results in infectivity test. In ELISA test too, the different seed components (embryo and cotyledons) showed positive reaction, while the seed coat negative for the presence of virus. The higher 20.0 per cent transmission of the virus was observed when sap inoculation from embryo portion of the seeds, collected from systemically ULCV infected urdbean plants was used for infectivity test.

3. Seeds when washed with Na_3PO_4 and tap water, separately, showed a similar amount of seed transmission of ULCV in growing on test. Naturally infected seeds with or without seed coat on planting showed almost the same percentage of ULCV transmission (16.6 and 15.5 %). Assays of surface washings of seeds obtained from systemically ULCV infected plants in indicator plant test and in ELISA test showed absence of the ULCV infection. All these observations strongly support that ULCV is internally seedborne, located in the embryo of the urdbean seeds and is not present as surface contaminant.

4. Seed transmission studies in naturally infected seeds revealed that naturally infected seeds of ULCV expressed the systemic transmission up to fourth trifoliate leaf stage in growing on test. Out of a total 29.1 per cent seed transmission of ULCV in naturally infected seeds of variety PU 19 of urdbean, 6.2 per cent plants exhibited diseased symptoms at first trifoliate, 8.3 per cent at second trifoliate, 10.4 at third trifoliate and 4.1 per cent at fourth trifoliate leaf stages, respectively.

5. A total nineteen germplasms and varieties of urdbean when screened for transmission of ULCV infection through seeds, the seed transmission ranged from 3.3 to 20.3 per cent. A maximum of 20.3 per cent seed transmission of ULCV was observed in variety VBG 73, while it was a minimum of 3.3 per cent in varieties IU 83-5 and Mash 1.

6. The ULCV infection ranged from 86.5 to 15.0 per cent in plants inoculated at 7 and 49 DAS, respectively. The susceptibility of urdbean plants to ULCV decreased with an increase in plant age, while the incubation period increased with the increase in plant age at the time of inoculation. Incubation period varied from 13 to 38 days in plants

inoculated at weekly interval up to 7 weeks after sowing and was a minimum of 13 days when plants were inoculated at 7 DAS, while it was 38 days when plants were inoculated at 49 DAS.

7. Young urdbean plants when were sap inoculated with ULCV infection at different dates of sowing at weekly intervals up to 7 weeks exhibited the significant effect of ULCV infection on texture, colour and size of urdbean seeds, and number of shrivelled, off-coloured, oversized and undersized seeds were observed in seed lots collected from plants inoculated with ULCV infection at different intervals through infected sap.

8. Seeds, separately collected from plants, inoculated at weekly intervals showed varying percentage of reduction in seed germination and transmission of seedborne inoculum of ULCV. A maximum of 26.2 per cent transmission was found in seeds collected from plants inoculated at 7 DAS. Generally, the transmission of ULCV was higher in seeds obtained from plants inoculated at 14, 21 and 28 DAS but thereafter percentage of seed transmission drastically reduced and was 2.2 per cent in seeds procured from plants inoculated at 49 DAS.

9. The ULCV infection at different plant growth stages also adversely affected the yield contributing factors viz. number of pods per plant, pod length, number of seeds per pod, 1000 seed weight as well as seed yield of urdbean. Maximum yield reduction of 71.7 per cent was recorded in ULCV infection occurring at 7 DAS, which went on reducing with delay in ULCV infection of plants and in plants infected at 49 DAS, 16.4 per cent yield loss was recorded.

10. Natural seed transmission of ULCV through seeds collected from systemically ULCV infected plants (T_1) was highest (32.3 %) in second generation as compared to 30.8 per cent in first generation. Seed transmission of ULCV in T_2 category of seeds collected from plants showing leaf crinkling late in season was 18.1 per cent and 19.5 per cent and was 4.0 per cent and 4.6 per cent in T_3 category of seeds during kharif 2002 and kharif 2003 from apparently healthy looking plants, respectively in first and second generations.

11. On an average, 22.3 per cent yield reduction was recorded when seeds collected from systemically ULCV infected plants (T_1) were sown for two successive generations, while it was 15.7 per cent when seeds collected from plants showing

symptoms late in season (T_2) were used for sowing as compared to the yield in apparently healthy looking seeds.

12. Urdbean seeds that were physiologically mature but undried and the seeds which were physiologically mature but dried revealed a total of 16.6 and 19.5 per cent transmission of urdbean leaf crinkle virus.
13. Out of total 50 floral buds collected from systemically ULCV infected plants, 20.0 per cent buds had all the sterile pollens and the remaining 80.0 per cent buds yielded 24.5 per cent pollen sterility.
14. For purification of ULCV, chloroform was used for initial clarification for removing chlorophyll, while PEG and NaCl were used to facilitate the precipitation of virus and final clarification was done by cesium sulphate-sucrose density gradient centrifugation. A single light scattering zone (white opaque band) was observed after density gradient centrifugation and its purity was confirmed by SDS-PAGE.
15. In SDS-PAGE, virus coat protein was observed as one dark band of approximately 31 kDa molecular weight.

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ABSTRACT

The present investigations were carried out on detection techniques, location and transmission of the seedborne inoculum of urdbean leaf crinkle virus (ULCV), which has now become a potential threat to the cultivation of urdbean (*Vigna mungo* L. Hepper) in India and is responsible for substantial yield losses.

Dry seed examination of urdbean seeds collected from ULCV infected plants exhibited the shrivelled, oversized, undersized and off- coloured seeds. The growing on test and ELISA test revealed the transmission of seedborne inoculum of ULCV through morphologically abnormal seeds. All the floral parts viz. epicalyx, calyx, corolla, androecium, gynoecium and seed parts such as embryo and cotyledons revealed the ULCV infection both in infectivity and ELISA test. Seed coat gave negative reaction for ULCV infection. Seeds washing with Na_3PO_4, removal of seed coat and assays of seed surface washings of ULCV infected seeds on indicator plant strongly supported that ULCV is internally seedborne, located in embryo of urdbean seeds and is not present as surface contaminant.

Out of total 29.1 per cent seedborne infection of ULCV, 6.2 per cent was expressed at first trifoliate, 8.3 per cent at second trifoliate, 10.4 at third trifoliate and 4.1per cent at fourth triofoliate leaf stages, respectively. Seed transmission ranged from 3.3 to 20.3 per cent in nineteen urdbean germplasms and varieties screened for transmission of ULCV. A maximum of 20.3 per cent seed transmission of ULCV was observed in variety VBG 73, while it was minimum 3.3 per cent in varieties IU 83-5 and Mash 1. The ULCV infection ranged from 86.5 to 15.0 per cent in plants inoculated at 7 days after sowing and 49 DAS, respectively. ULCV infection significantly affected the texture, colour and size of urdbean seeds, as more number of shrivelled, off-coloured, oversized and undersized seeds observed in seed lots collected from plants inoculated at different intervals. A maximum of 26.2 per cent transmission was found in seeds collected from plants inoculated at 7 DAS. Maximum yield reduction of 71.7 per cent was recorded in ULCV infection occurring at 7 DAS, while it was only 16.4 per cent in plants infected at 49 DAS. Natural seed transmission of ULCV through seeds collected from systemically ULCV infected plants (T_1) was highest (32.3 %) in second generation as compared to 30.8 per cent in first generation, while seed transmission in T_2 category of seeds collected from plants showing leaf crinkling late in season was 18.1 per cent and 19.8 per cent and was 4.0 per cent and 4.6 per cent in T_3 category of seeds during kharif 2002 and kharif 2003 from apparently healthy looking plants, respectively in first and second generations. An average 22.3 per cent yield reduction was recorded in T_1, while it was 15.6 per cent in T_2 category of ULCV infected plants.

Urdbean seeds both physiologically mature but undried and physiologically mature but dried revealed a total of 16.6 and 19.5 per cent transmission of ULCV. Out of total 50 floral buds collected from systemically ULCV infected plants, 20.0 per cent buds had all the sterile pollens and the remaining 80.0 per cent buds yielded 24.5 per cent sterile pollens. On purification of ULCV, a single light scattering zone (white opaque band) was observed after density gradient centrifugation and its purity was confirmed by SDS-PAGE. In SDS-PAGE one dark band was observed with approximately 31 kDa molecular weight of virus coat protein.

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