Viruses involved in chickpea stunt

Promotor	:	Dr. R.W. Goldbach, Hoogleraar in de Virologie,
Co-promotor	:	Dr. Ir. L. Bos, Viroloog, IPO-DLO.

Nico M. Horn

Viruses involved in chickpea stunt

Proefschrift
ter verkrijging van de graad van doctor
in de Landbouw- en Milieuwetenschappen
op gezag van de rector magnificus,
Dr. C.M. Karssen,
in het openbaar te verdedigen
op dinsdag 31 mei 1994
des namiddags te vier uur in de Aula
van de Landbouwuniversiteit
te Wageningen.

Cover: Upper insets. Electron micrographs of virus particles of chickpea chlorotic dwarf geminivirus (left), a luteovirus from chickpea (middle), and faba bean necrotic yellows virus (right). Photographs. ICRISAT (left and middle), Dr L. Katul, BBA, Braunschweig, Germany (right), magnification 150,000 x.
Lower inset. Healthy chickpea plant (left) and chickpea plant affected by chickpea stunt (right).

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Stellingen

Behorende bij het proefschrift van N.M. Horn getiteld 'Viruses involved in chickpea stunt'.

1. Meer overeenstemming tussen veredelaars en virologen over het gebruik van de termen 'virusresistentie' en 'ziekteresistentie', en het aanpassen van de toetsmethoden aan deze twee vormen van resistentie, zou het effect van veredeling op resistentie tegen virusziekten zeer ten goede komen.

   Dit proefschrift.

2. De voorzichtigheid die Katul *et al.* (1993) bij het publiceren over 'faba bean necrotic yellows virus ' als veroorzaker van de door hen beschreven ziekte betrachten, omdat de postulaten van Koch niet volledig vervuld zijn, moet een voorbeeld zijn voor menig ander viroloog.

   Katul, L., Vetten, H.J., Maiss, E., Makkouk, K.M., Leseman, D.-E. & Casper, R., 1993. Characterisation and serology of virus-like particles associated with faba bean necrotic yellows. Annals of Applied Biology 123: 629-647.

3. De grote variatie binnen 'beet western yellows virus' en binnen 'bean leafroll virus' geeft de dringende noodzaak aan om de soorten binnen de luteovirusgroep beter te omschrijven.

   Duffus, J.W., Falk, B.W. & Johnstone, G.R., 1990. Luteoviruses - one system, many variation. pp. 86-104. In: Burnett, P.A. (Ed.), World perspectives on barley yellow dwarf.

   Van den Heuvel, J.F.J.M., Verbeek, M. & Peters, D., 1993. The relationship between aphid-transmissibility of potato leafroll virus and surface epitopes of the viral capsid. Phytopathology 83: 1125-1129.

   Dit proefschrift.

4. De conclusie van Fortass (1993) dat het één luteovirusisolaat is dat serologisch reageert als BWYV en in DNA-hybridisatieproeven als BLRV is voorbarig.

   Fortass, M., 1993. Viruses of faba bean (*Vicia faba* L.) in Morocco; surveying, identification, and ecological aspects. Thesis, Wageningen Agricultural University.123 pp.

5. De ontdekking dat een AIDS-vaccin wel laboratoriumstammen maar geen veldisolaten van het HIV neutraliseert, illustreert de noodzaak om bij toegepast virusonderzoek niet slechts met een laboratoriumisolaat te werken.

   Cohen, J., 1993. Jitters jeopardize AIDS vaccine trials. Science 262: 980-981.

6. Het onderzoek naar het virus dat verantwoordelijk was voor het sterven van 26 Navajo-indianen begin 1993 in de Verenigde Staten laat zien dat een combinatie van moleculaire biologie en epidemiologie een synergistisch effect kan hebben.
   Marshall, E., 1993. Hantavirus outbreak yields to PCR. Science 262: 832 - 836.

7. Met het vaststellen van de nucleotidenvolgorde van het genetisch materiaal van een virusisolaat, is dat isolaat wel geïdentificeerd, maar nog niet gekarakteriseerd.

8. Net zoals de Duitse GTZ (Gesellschaft für Technische Zusammenarbeit) en de Britse ODA (Overseas Development Administration) zou het Nederlandse DGIS (Directoraat Generaal Internationale Samenwerking) virologisch onderzoek ten behoeve van ontwikkelingslanden moeten financiëren, ook al vindt dat onderzoek in Nederland plaats.

9. Groeiend inzicht in levensprocessen maakt God niet kleiner maar juist groter.

10. De bureaucratie in Nederland is hoogstens anders maar zeker niet minder dan in ontwikkelingslanden.

11. De naam 'kikkererwten' heeft niets met kikkers te maken, net zo min als de naam 'kekererwten' iets met kekers van doen heeft.

12. Rijk zijn wordt in India bepaald door inkomen, in Nederland door levenshouding.

13. Een promovendus die niet zelfstandig in staat is zijn stellingen te verzamelen, is tijdens zijn onderzoek te éénzijdig bezig geweest.

PREFACE

My research at ICRISAT, Hyderabad, India, and this thesis would not have been possible without help from many people. I would like to thank all those who assisted in whatever way, either directly, or indirectly by making me and my family feel at home during our stay in Hyderabad from July 1989 till June 1992.

Special thanks and appreciation are due to Dr L. Bos (IPO-DLO, Wageningen, The Netherlands) and Dr D.V.R. Reddy (ICRISAT, India) for their continuous supervision, guidance, support and encouragement. Dr Bos introduced me to tropical legume virology during a short, preparative stay at IPO-DLO in 1985 prior to my assignment in Indonesia, helped initiating my thesis work at ICRISAT, advised me from a distance during the investigations, and contributed greatly during the writing of this thesis. Dr Reddy was responsible for the more daily guidance during the investigations in India, but allowed much freedom in performing the research. I would also like to thank Dr R.W. Goldbach, who contributed in a friendly, but effective, way, especially during finalization of my thesis.

Several members of the ICRISAT community have contributed to my research: Drs L.D. Swindale and J.G. Ryan (then consecutive Directors General of ICRISAT), Dr Y.L. Nene, (Deputy Director General) and Dr D. McDonald (Program Leader Legumes) provided mental and material support, B.S. Rao and S.V. Reddy (Research Associates) cooperated during field and laboratory work, all members of the Virology Unit of ICRISAT provided a stimulating atmosphere for daily work and assisted in various technical ways, and A.K. Murthy helped with the electron microscopy. During my final stay at IPO-DLO, Hans van den Heuvel and Martin Verbeek assisted in laboratory experiments and Chris Cuperus in text processing. My wife, Esther, never complained about my spending so much time in the field and laboratory.

I am grateful to DGIS for funding the first two years of my stay at ICRISAT, and to ICRISAT for funding my third year there and for providing research facilities, including travelling. Many thanks go to IPO-DLO for hospitality and collaboration during the final but essential stage of research and during the writing of this thesis. The Wageningen Agricultural University and its Department of Virology kindly contributed towards the costs of printing this thesis, and ICRISAT kindly printed the colour photographs, clearly illustrating the symptoms of chickpea stunt.

Nico Horn.

Contents

PRELUDE

1. Introduction

Chickpea, *Cicer arietinum* L., is the world's third legume crop. It is an important source of human food and animal feed, and also helps in managing soil fertility. The crop is grown annually on 9.6 million ha with a total production of 6.9 million tonnes of dry seeds. It is the most important pulse crop in South Asia, where 70% of the world production takes place. Other important production areas are West Asia and North Africa (Table 1).

Table 1. World chickpea production in 1990 (FAO, 1991).

	Area 1000 ha	yield kg/ha	production 1000 mt
WORLD	**9577**	**718**	**6876**
Africa	**454**	**664**	**301**
Ethiopia	130	962	125
N. Africa	191	617	118
North and Central America	**150**	**1133**	**170**
South America	**37**	**588**	**22**
Asia	**8740**	**709**	**6195**
Bangladesh	100	650	65
India	6495	652	4232
Iran	112	723	81
Myanmar	134	752	101
Nepal	28	590	17
Pakistan	1002	536	537
Syria	55	660	36
Turkey	800	1075	860
Europe	**101**	**802**	**81**
Australia	**94**	**1138**	**107**

Seeds of chickpea are a major component of the diet of many people in developing countries. Since its protein content is high, it is the principal protein source for millions of people, especially in the largely vegetarian diet in South Asia. A wide variety of foods can be prepared from this pulse. It can be eaten raw, boiled, baked or milled, as part of a meal or as a snack. It is also popular in other parts of the world. In western countries its consumption is increasing due to its high nutritional value and as a delicacy. Chickpea cultivation is extending in Australia, Mexico, the southern USA, and eastern Africa.

Fig 1. Chickpea *Cicer arietinum* L. a. branch with flowers, $^5/_6$ x; b. fruiting branch, $^5/_6$ x; c. seedling, $^5/_6$ x; d. seed, $2^1/_2$ x (Drawings: Department of Plant Taxonomy, Agricultural University, Wageningen, The Netherlands).

The species is a shrubby and pubescent annual plant, mostly with glandular hairs, which exude acid droplets. These droplets contain high concentrations of malic acid and can reach a pH as low as 1.3. Stems are branched, and roots are robust and long. The leaves are compound, and the flowers are white to purplish. Pods are acuminate and characteristically inflated. They therefore look short and thick. The pods are 1-3 cm long and contain one to three seeds each (Fig. 1 and 2).

A good description of the species is given by Van der Maesen (1972). The species can be divided in two major groups. The small-seeded types with coloured seed-coat and angular seed shape are generally called desi types. The larger-seeded types with cream-coloured seed-coat and ram-head seed shape are known as kabuli types. The latter normally do not contain anthocyanin and therefore have white flowers, whereas the former do contain anthocyanin and have purplish flowers. The desi types are mainly grown in South Asia, whereas the kabuli types are grown in West Asia and North Africa. The two groups are botanically similar and crossable. Taxonomically they are not clearly distinguishable.

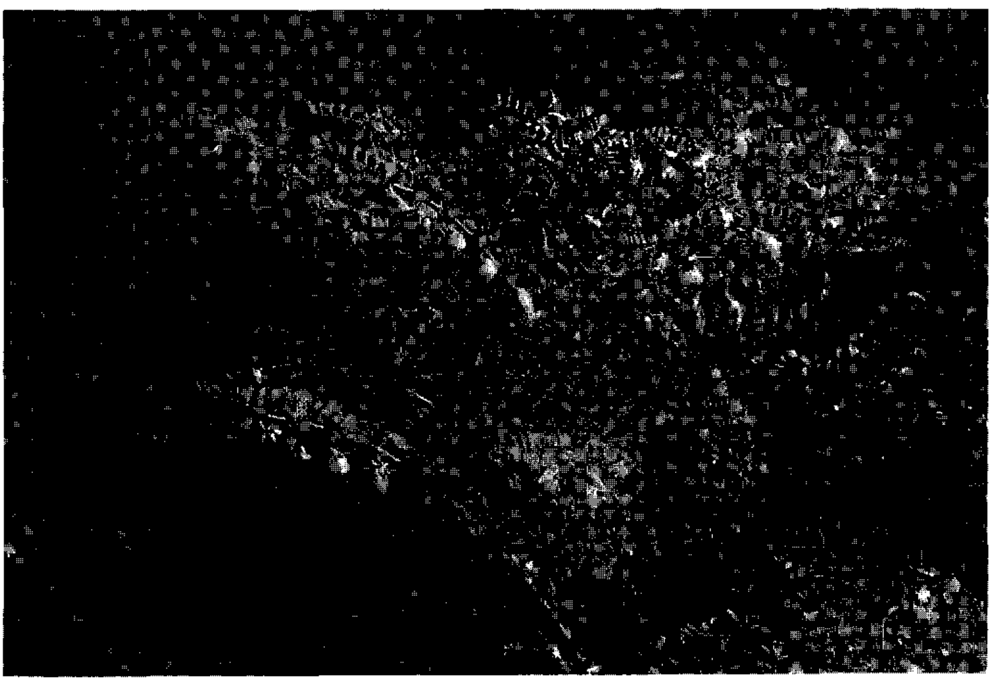

Fig. 2. Healthy field-grown chickpea plant with flowers and pods.

For more extensive information on its cultivation, taxonomy, distribution and importance, reference is made to Van der Maesen (1972) and Saxena and Singh (1987).

Chickpea is a cool-season crop of the semi-arid and arid tropics. It is mainly grown on residual moisture in winter and spring. It does best between 25 and 30^{0} latitude, but can be grown between 45^{0} N and 45^{0} S. Yields up to 4 t/ha have been obtained at research stations. This yield level, however, is rarely reached by farmers. The world's average productivity is only 0.7 t/ha. Chickpea is considered a marginal crop of small farmers which are using few or no inputs.

With the green revolution, based on high-yielding cultivars of wheat, areas originally under chickpea in North India were diverted to wheat. Chickpea was then shifted to more marginal soils. As irrigation and fertilizers came within the reach of farmers, more and more wheat was grown, especially on better soils. This partly explains the lack of progress in chickpea cultivation. In contrast to wheat, chickpea suffered from the green revolution, whereas it still plays an important role as protein source.

Improvements in chickpea production seem possible, for example, in the Mediterranean and in West Asia yields might be boosted by shifting from spring to winter planting. This would, however, require resistance to cold and *Ascochyta* blight. A slight increase in yield and more yield stability would make the crop competitive with wheat since its price is much higher than that of wheat.

The gap between the average yield on farmers' fields and the potential yield obtained at research stations is also due to a range of abiotic and biotic stresses: drought, low temperature, fungal and virus diseases. Insect pests are of minor importance in chickpea production, probably due to acid secretion by the plant (Saxena and Singh, 1987). The most important fungal diseases are *Ascochyta* blight (caused by *Ascochyta rabiei*), *Fusarium* wilt (caused by *Fusarium oxysporum* f.sp. *ciceri*), dry root rot (caused by *Rhizoctonia bataticola*), and *Botrytis* gray mould (caused by *Botrytis cinerea*). Depending on prevailing climatic factors, such as humidity and temperature, and soil conditions (for *Fusarium* wilt and root rots), in each region, one or more of these diseases are important. They have been reviewed extensively by Nene and Reddy (1987). A recent overview of the viruses known to infect chickpea is given by Kaiser *et al.* (1990). At least 16 viruses have been identified as natural chickpea pathogens (Table 2). Most of them (13) are aphid transmitted, and amongst them the luteoviruses seem to be most widely distributed and of major importance (Kaiser *et al.*, 1990).

With seasonal shifts in chickpea production, like from spring to winter planting in the Mediterranean and in West Asia, or with crop intensification by irrigation and with higher inputs of fertilizers, the virus situation should be carefully monitored, since such cultural changes are known to favour certain virus diseases. For example, in California, a shift from summer to winter planting of chickpea increased the incidence of virus diseases considerably (Bosque-Perez and Buddenhagen, 1990).

Table 2. Viruses occurring naturally in chickpea (Kaiser *et al.*, 1990).

Virus group	Virus	Distribution	Transmission	
			Sap	Vector
Carlavirus	Pea streak	USA	+	Aphids
Cucumovirus	Cucumber mosaic	Many countries	+	Aphids
Ilarvirus	Tobacco streak	USA	+	Thrips
Luteovirus	Bean (pea) leafroll	Many countries	-	Aphids
	Legume yellows	USA	-	Aphids
	Beet western yellows	Australia/USA	-	Aphids
	Subterranean clover red leaf	Australia/USA	-	Aphids
Nepovirus	Tobacco ringspot	India	+	ND
Pea enation	Pea enation mosaic	Italy/USA	+	Aphids
Potyvirus	Bean yellow mosaic	India/Iran/USA	+	Aphids
	Chickpea bushy stunt	India	+	ND
	Chickpea distortion mosaic	India	+	Aphids
	Chickpea filiform	USA	+	Aphids
	Lettuce mosaic	USA	+	Aphids
Rhabdo virus	Lettuce necrotic yellows	Australia	+	Aphids

ND = no data available

In the study described in this thesis, largely performed at the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), India, chickpea stunt, one of the virus diseases with a dramatic effect on plant yield, was investigated. The study was crop- and disease-oriented, because the major and ultimate aim of this research, as of ICRISAT at large, is to increase the productivity of small farmers in the semi-arid tropics. However, basic knowledge of the causal virus(es) and its (their) vector(s) is essential for reaching this goal. It requires proper identification of the virus(es) causing the disease and development of reliable and efficient detection methods. For setting priorities for the development of strategies of control, the incidence and economic importance of the virus(es), and its (their) ecology have to be studied. Information on the identity of the virus(es) involved and which viruses are of actual or potential economic importance is also a prerequisite to breeding and screening for resistance, one of the most effective methods of virus control in developing countries.

In this thesis the disease will first be described, the lack of information on the causal virus(es) at the moment this study started will be discussed, and a short introduction will be given on the involvement of other viruses, including a new geminivirus, as found in the course of the investigations (Chapter 2). Then the surveys of chickpea for chickpea stunt and associated viruses in India, Pakistan, Syria, Turkey and Lebanon will be reported, to assess the relative importance of the viruses involved (Chapters 3 and 4). The viruses concerned are further identified and discussed in Chapter 5 (luteoviruses) and Chapters 6 to 9 (geminivirus). To obtain more information on the ecology of the latter, hitherto unknown virus, the relationships of this geminivirus with its vector was studied (Chapter 7). The yield loss caused by this virus was also assessed, and screening for resistance was carried out (Chapters 8 and 9). In Chapter 10 an overall discussion will be given of the present state of knowledge on chickpea stunt disease and the viruses involved, and an indication will be presented of further research required.

Acknowledgements

The author would like to thank Drs L. Bos (IPO-DLO, Wageningen), D.V.R. Reddy (ICRISAT, India), and R.W. Goldbach (LUW, Wageningen) for critical comments on the manuscript.

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2. Chickpea stunt disease

Severe growth reduction of chickpea was first reported by Kaiser (1971) in Iran. The name chickpea stunt was first used by Nene and Reddy (1976) in India. Nene *et al.* (1978) described its symptoms extensively. The disease is characterized by overall growth reduction resulting in internode shortening and plant stunting or dwarfing, by phloem discoloration in the collar region, leaf brittleness, leaf reddening in the case of desi types, and leaf yellowing in kabuli-type chickpeas. The similarity of the symptoms listed by Kaiser (1972) to those of stunt in India justifies the conclusion that they concern the same disease. The disease can be recognized by the above-described symptoms, which are the visible results of the internal reaction of the plant to the pathogen.

Characteristic symptoms of stunt have later also been observed in Algeria, Bangladesh, Lebanon, Morocco, New Zealand, Pakistan, Syria, Tunesia, Turkey (Nene and Reddy, 1987), Zambia (Kannaiyan and Hariwa, 1989), USA (Bosque-Perez and Buddenhagen, 1990), Sudan (Abdalla and Van Rheenen, 1991), Myanmar (the former Burma; Reddy *et al.*, 1991), Ethiopia (Woldeamlak Araya *et al.*, 1991), Kenya (Mutshiya *et al.*, 1991), and Spain (Carazo *et al.*, 1993). The disease thus occurs in most chickpea-growing countries, but it has not yet been reported from Australia.

Symptoms

In the field, leaf yellowing (in kabuli types; Fig. 1A, opposite page 20) or leaf reddening (in desi types; Fig. 1B) is the most prominent symptom. In desi types, leaf yellowing may also be present but it is then mostly dominated by the reddening, which varies in intensity depending on the plant genotype. Another very obvious symptom in the field is the general growth reduction, especially in the tip of the plant, which makes it look very compact. In case of infection at an early stage of crop development, the whole plant stays small and looks stunted (Fig. 1A and 1B).

When infection takes place later in the season, the growth reduction is only present in the tip of the plant. Phloem discoloration (Fig. 1C) is highly characteristic of chickpea stunt, as is leaf brittleness. The latter symptom is often difficult to observe under field conditions. Symptoms may vary, but more in intensity, especially the leaf reddening and the phloem discoloration, than in nature, according to chickpea genotype and time of infection, which is unknown under field conditions. The leaf reddening or yellowing and the plant stunting as such are not characteristic of virus infection only. They can also be caused by other types of stress, like insects, mechanical damage (Nene and Reddy, 1976), and drought.

Etiology

In Iran (Kaiser and Danesh, 1971) and India (Reddy *et al.*, 1979) the disease was ascribed to bean leafroll virus (BLRV, also referred to as pea leafroll virus), at that time one of the few viruses later designated luteoviruses. Kaiser and Danesh (1971) and Nene and Reddy (1976) reported the transmission of the virus from chickpea to chickpea by *Aphis craccivora*, the only aphid species that regularly colonizes chickpea plants. Colonizing aphids are very rarely found on chickpea in the field, but dead, winged aphids more regularly occur on chickpea plants (personal observations). This suggests that migrating aphids play a more important role in virus transmission in this crop than colonizing aphids. Non-colonizing aphids, such as *Myzus persicae*, can also transmit viruses to chickpea (Bosque-Perez and Buddenhagen, 1990). Since 13 aphid-transmitted viruses have been reported from chickpea, many occasionally visiting aphids must transmit viruses to chickpea plants. In California a disease with symptoms similar to those of stunt (I.W. Buddenhagen, personal communication, 1990) was associated with beet western yellows, legume yellows and subterranean clover red leaf luteoviruses (Bosque-Perez and Buddenhagen, 1990). In other areas, none of the viruses involved have been identified.

Much about the etiology of chickpea stunt therefore remains uncertain. At the start of this study in 1989, only BLRV was thought to be associated with the disease in India (Reddy *et al.*, 1979). However, indications soon emerged that more viruses might be involved. For example, chickpea genotypes that were found to be chickpea-stunt resistant in field trials in India, showed clear stunt symptoms, including phloem discoloration, finally leading to plant death, when field- and greenhouse-tested in the Netherlands with BLRV, a Dutch isolate originating from lucerne (Dr L. Bos, IPO Wageningen, correspondence with Dr Y.L. Nene, 1980; Plate 22 in: Bos, 1983). This isolate was very similar, if not identical, to pea leafroll virus, later renamed bean leafroll virus, first isolated and characterized biophysically by Ashby and Huttinga (1979). Unidentified, geminivirus-like particles were later observed in India by electron microscopy after efforts to purify virus from field-infected chickpea plants showing stunt symptoms (Dr D.V.R. Reddy, pers. comm., 1989). The present studies showed that different luteoviruses (Chapter 3, 4 and 5), a new geminivirus (chickpea chlorotic dwarf virus, CCDV; Chapter 6) and faba bean necrotic yellows virus (FBNYV; Chapter 4) are associated with chickpea stunt.

Pathogenesis

All these viruses, including the latter two non-luteoviruses, are known to be phloem-limited and to initiate disease by causing phloem degeneration often leading to, or including, phloem necrosis (Esau, 1957; Rasa and Esau, 1961; Jensen, 1972; Bos and Ashby, 1978; Waterhouse *et al.*, 1988). Except for the phloem and associated tissue, these viruses do not directly influence other tisues of the plant, since they are not present there. Symptoms in these tissues are, therefore, caused secondarily. Most symptoms result from disturbed carbohydrate translocation (Jensen, 1972). They consist of growth retardation, accumulation of starch in the leaves, chlorosis or yellowing, and they lead to or are associated with premature senescence, overall plant decline, and often premature plant death. The most likely course of events in chickpea stunt is the blockage of the phloem by infection, which disrupts the phloem transport, resulting in accumulation of carbohydrates in the leaves (causing leaf brittleness). The disrupted transport of assimilation products causes a shortage of nutrients in the roots, which then causes a shortage of nutrients in the aboveground parts of the plant, resulting in general growth retardation (with stunting as a result), chlorosis and reddening, and eventually the infected plant dies prematurely. Plant genotypes that can produce anthocyanin (the desi types) react on the carbohydrate accumulation in the leaves with the production of anthocyanin (also a carbohydrate) and therefore with reddening. Thus, the directly visible symptoms, except phloem discoloration, are caused only indirectly by disturbance of the phloem transport.

Naming

The name chickpea stunt for the disease fits well the above-described symptoms, irrespective of the causal pathogen and even when the causal pathogen is not known yet. Major symptoms of the disease are often reflected by the name of the pathogen. Plant stunting or dwarfing is expressed in names such as barley yellow dwarf virus (Rochow, 1970) and soybean dwarf virus (Tamada and Kojima, 1977), chlorosis and yellowing in names such as beet western yellows virus (Duffus, 1972) and barley yellow dwarf virus (Rochow, 1970), hence also the more recent group name luteoviruses (luteus = yellow). The plants can also change color into red, as indicated in carrot red leaf virus (Waterhouse and Murant, 1982) and subterranean clover red leaf virus (Kellock, 1971). Similar symptoms are also known of other phloem-limited pathogens such as tobacco yellow dwarf geminivirus (Thomas and Bowyer, 1984), wheat dwarf geminivirus (Lindsten, 1980), and even mycoplasmas, for example, pear decline (Schneider, 1977) and aster yellows (McCoy, 1979). Mycoplasmas are reported to cause yellows diseases (McCoy, 1979) like the luteoviruses do, indicating that mycoplasmas can cause symptoms similar to those caused by the luteoviruses (Bos, 1986). Pathogens from both groups block the phloem and thereby cause external symptoms indirectly. Mycoplasmas, however, differ in that they also cause hormonal imbalance mostly leading to morphological abnormalities, such as virescence and phyllody of flowers and witches' broom growth of plants, and these are more diagnostic of mycoplasma infection than the change in color.

Thus, the symptoms of chickpea stunt are not only characteristic of the luteoviruses but more of phloem-limited pathogens in general. This is understandable since the symptoms are caused indirectly by degeneration of the phloem and thereby disruption of the phloem transport. These pathogens cause physiological decline and early senescence of the plant.

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The author would like to thank Drs L. Bos (IPO-DLO, Wageningen), D.V.R. Reddy (ICRISAT, India), and R.W. Goldbach (LUW, Wageningen) for critical comments on the manuscript.

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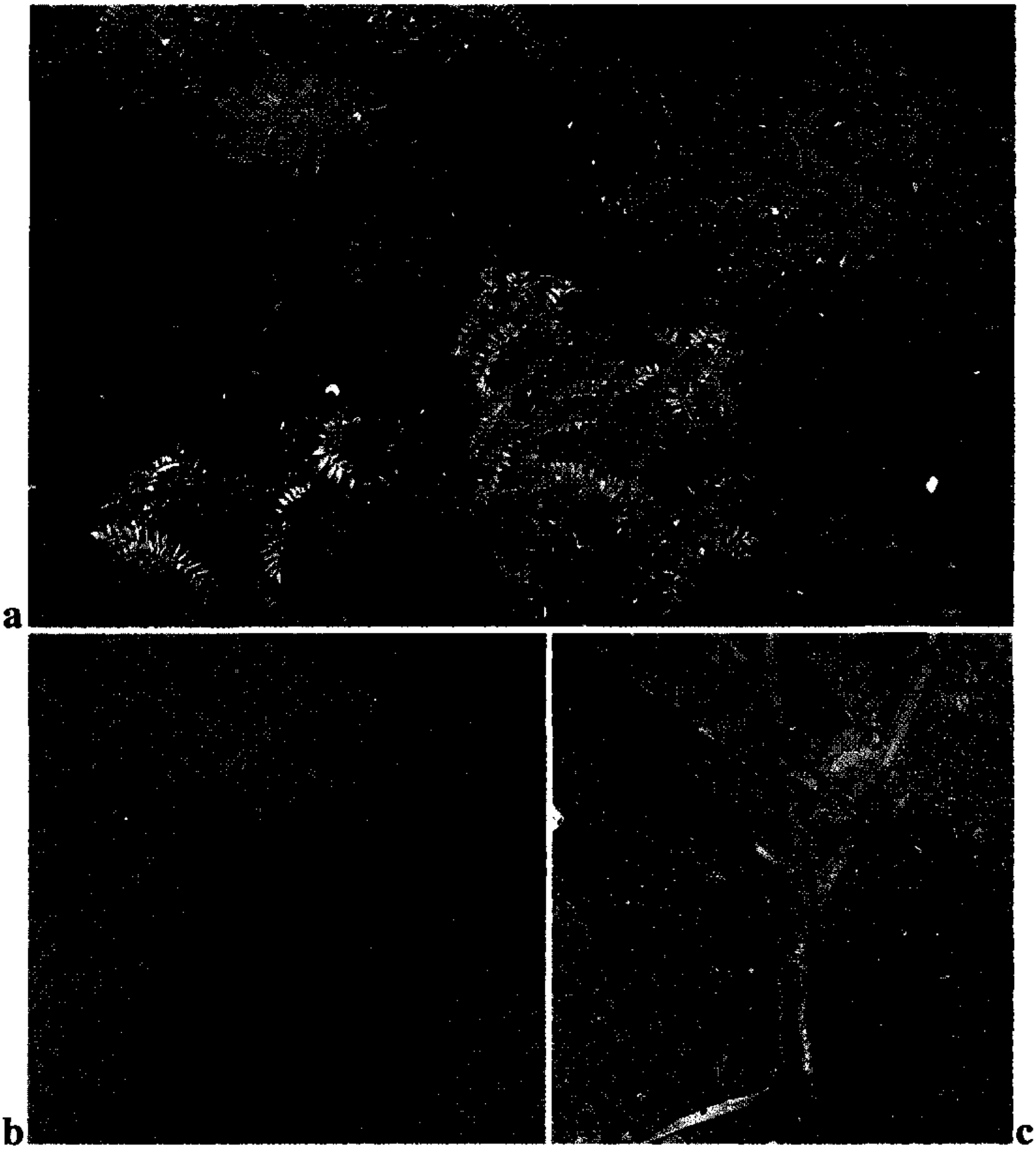

Figure 1. Symptoms of chickpea stunt. a. Stunting of kabuli-type plants with leaf yellowing and internode shortening. b. Stunting of desi-type plant with leaf reddening, reduction in size and downward curling of tip leaves, and internode shortening. c. Phloem discoloration at collar region of the stem, visible after removal of outer tissues.

S U R V E Y S

3. Survey of chickpea (*Cicer arietinum* L.) for chickpea stunt disease and associated viruses in India and Pakistan

N.M. HORN[a], S.V. REDDY[a], J.F.J.M. VAN DEN HEUVEL[b] and D.V.R. REDDY[a]

[a] International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, Andhra Pradesh 502 324, India

[b] DLO Research Institute for Plant Protection (IPO-DLO), P.O. Box 9060, 6700 GW Wageningen, The Netherlands

Summary

When during a survey in India and Pakistan 1804 plants with stunt-like symptoms were collected and tested with poly- and monoclonal antibodies, chickpea chlorotic dwarf geminivirus (CCDV), bean leafroll luteovirus (BLRV)-like isolates, and isolates reacting with an antiserum to a luteovirus isolate from chickpea, tentatively named chickpea luteovirus (CpLV), were found to be associated with chickpea stunt. Different viruses prevailed in different chickpea-growing areas. The reaction patterns of the luteovirus isolates with monoclonal antibodies (Mabs) differed from those of some known luteoviruses. In addition to CpLV other new luteoviruses and a so far unidentified, graft-transmissible agent may have been isolated. The BLRV-like isolates were of minor importance. CCDV and CpLV-like isolates were widely distributed. The etiology of chickpea stunt disease is more complex than initially thought.

Introduction

India is the largest chickpea-growing country in the world, producing 4 million tons annually on 6.5 million ha. Pakistan, ranking second, produces 0.5 million tons annually on 1 million ha. Due to many constraints, the production per ha is low. In order of importance, drought, fungi and viruses are major limiting factors in chickpea production (Saxena and Singh, 1987).

Chickpea stunt is the most important virus disease of chickpea. It is characterized by leaf reddening in the case of desi types, and yellowing in kabuli-type chickpeas. On either type, internode shortening, plant stunting and phloem browning in the collar region is observed (Nene *et al.*, 1978). These symptoms have also been reported from many other chickpea-growing areas in several countries (Jha *et al.*, 1981; Kaiser, 1972; Nene *et al.*, 1978; Reddy *et al.*, 1979; Saxena *et al.*, 1991). The disease can have a dramatic effect on production due to plant decline, ranging from poor performance to premature death. These symptoms result from phloem degeneration caused by the phloem-limited virus(es). Kaiser and Danesh (1971) reported 90-100% yield loss when chickpea plants were inoculated with bean leafroll luteovirus (BLRV), reported to cause chickpea stunt (Reddy *et al.*, 1979). Kotasthane and Gupta (1978) found 80-95% yield loss in chickpea by chickpea stunt. Chickpea chlorotic dwarf geminivirus (CCDV), also provoking symptoms characteristic of chickpea stunt (Horn *et al.*, 1993; Chapter 6), caused 75-100% yield loss depending on the time of infection (Chapter 8).

Chickpea stunt has been ascribed in India to BLRV (Reddy *et al.*, 1979), although at that time the identity of the virus, first isolated and characterized for its intrinsic properties in the Netherlands (Ashby and Huttinga, 1979), had not yet been established. In California other luteoviruses, viz. subterranean clover red leaf virus (SCRLV), legume yellows virus (LYV, probably a strain of BLRV) and beet western yellows virus (BWYV), have been shown to infect chickpea (Bosque-Perez and Buddenhagen, 1990) and to cause symptoms similar to those of chickpea stunt (Buddenhagen, pers. comm., 1990). BWYV and BLRV were also reported from Spain to infect chickpea (Carazo *et al.*, 1993). In India, the leafhopper-transmitted geminivirus CCDV was recently found to incite symptoms in chickpea similar to those described for chickpea stunt (Nene *et al.*, 1978; Horn *et al.*, 1993; Chapter 6). Thus, it appears that a geminivirus and a number of luteoviruses can cause similar, if not identical, symptoms in chickpea. No data are available on the viruses actually involved in chickpea stunt disease in farmers' fields and on their relative importance. Such information is essential for setting priorities for the development of control strategies.

Therefore surveys were conducted to study which viruses are associated with chickpea stunt and to assess their relative importance. This paper reports the results of these surveys in India and Pakistan during the 1991/1992 season. Results on the preliminary characterization of luteoviruses newly detected during the surveys, with poly- and monoclonal antibodies, are reported.

Materials and Methods

Areas surveyed

In India, surveys were conducted in the states of Rajasthan, Madhya Pradesh and Gujarat (Fig. 1) during January and February 1992. Chickpea fields were chosen with

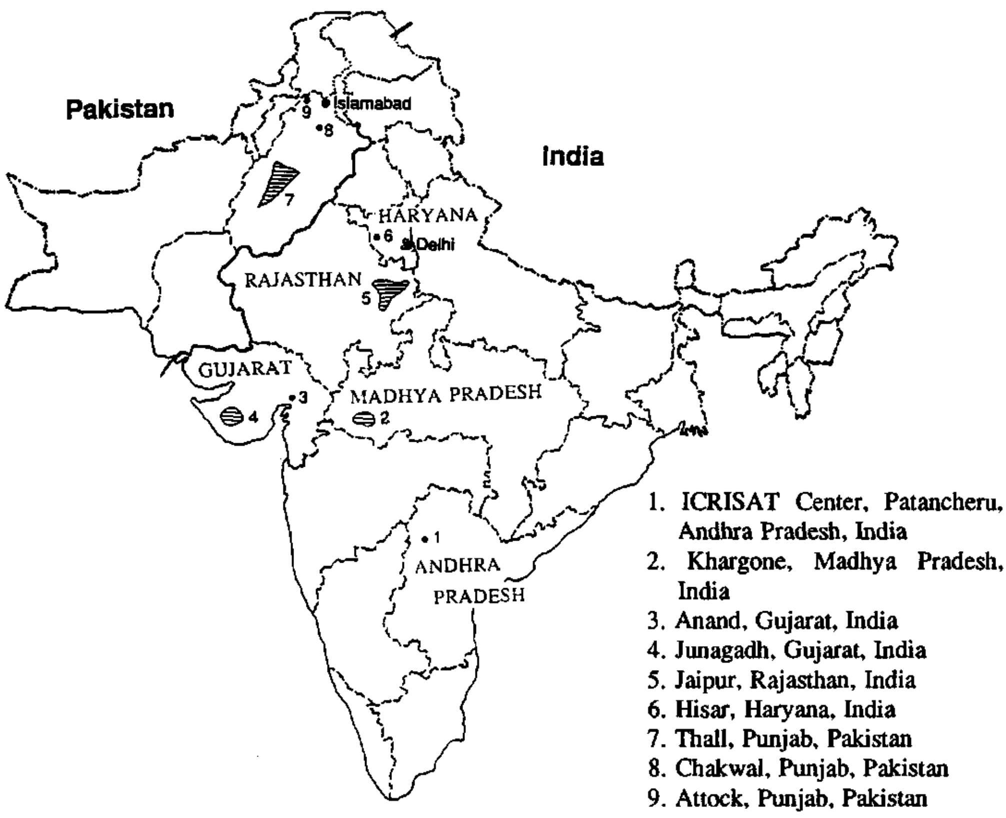

Fig. 1. The chickpea-growing areas surveyed for chickpea stunt in India and Pakistan during the 1991/1992 growing season.

the assistance of researchers familiar with chickpea production in these areas. Crops raised at research stations in the areas surveyed and at Anand (Gujarat), Hisar (Haryana), and Patancheru (ICRISAT Center, Andhra Pradesh), were also included (Fig. 1). In Pakistan, chickpea-growing areas in Punjab (Fig. 1) were visited during February 1992, the main area being the Thal, where 70% of Pakistan's chickpea production takes place (Khan *et al.*, 1991). The inspected fields were chosen systematically by making a stop after every five km during the trips, or at the nearest chickpea field thereafter.

Observations and sample collection

At each field visited, the size of the field, stage of crop development, cropping pattern, crop density and stunt incidence were recorded. The incidence of stunt was assessed by counting the number of plants with stunt-like symptoms in five randomly-distributed groups of 100 plants each. If possible, samples from 10 to 15 plants

Fig. 2. Field symptoms of chickpea stunt in plants of chickpea. Healthy plants in the background.

with characteristic symptoms (Fig. 2) were collected in each field for further testing. Samples collected in India and Pakistan were processed at the ICRISAT Center and the National Agricultural Research Centre (NARC), Islamabad, respectively.

Serology

All plant samples were tested with polyclonal antisera in DAS-ELISA, as described by Clark and Adams (1977). BLRV polyclonal antiserum was used since this virus had been the only luteovirus reported from chickpea in India. In preliminary tests a luteovirus, which did not react with BLRV polyclonal antiserum, was found in many chickpea plants with symptoms of chickpea stunt at ICRISAT Center. Luteovirus-like particles were observed with the electron microscope. This virus was purified and a polyclonal antiserum was produced. In reciprocal tests in DAS-ELISA, this antiserum did not react with BLRV (Dutch isolate; Ashby and Huttinga, 1979), and BLRV antiserum did not react with the isolate from chickpea (data not shown). This isolate is thus serologically distinct from BLRV, and was tentatively named chickpea luteovirus (CpLV).

First screening of the samples collected during the surveys was done using polyclonal antisera to BLRV, CpLV and CCDV. In case hardly any of the samples collected in an area reacted with the antisera used, a number of the samples were also tested with polyclonal antisera to potato leafroll luteovirus (PLRV) and subterranean clover red leaf luteovirus (SCRLV).

Samples were ground in phosphate-buffered saline containing 0.05% Tween-20 and 2% polyvinylpyrrolidone (20 ml buffer per gram plant material). Samples reacting with one of the luteovirus polyclonal antisera were tested in a triple-antibody sandwich ELISA (TAS-ELISA) with the monoclonal antibodies (Mabs) mentioned below. In TAS-ELISA, coating was done with BLRV or CpLV polyclonal antiserum at a concentration of 2 µg/ml, and plant material treated as described above was used. The Mabs were used in the concentrations mentioned below and a goat-anti-mouse alkaline phosphatase conjugate was used.

The antiserum to BLRV (Ashby and Huttinga, 1979) was supplied by Dr L. Bos

(The Netherlands), to potato leafroll virus (PLRV) by Mr D.Z. Maat (The Netherlands), and to SCRLV by Dr G.R. Johnstone (Australia; Johnstone *et al.*, 1982). The antiserum to CCDV (Horn *et al.*, 1993; Chapter 6) and to CpLV had both been produced at ICRISAT Center. The Mabs to PLRV had been produced at the Wageningen Agricultural University (WAU), The Netherlands. The ones used in this study because of their differential reaction to a number of well-described luteoviruses (Van den Heuvel *et al.*, 1990; Table 5, lower part), and their dilutions (in brackets) were WAU-A2 (1,000 x), WAU-A6 (5,000x), WAU-A7 (5,000x), WAU-A12 (2,000x), WAU-A13 (1,000x), WAU-A24 (2,000x), WAU-A47 (2,500x), and WAU-B9 (1,000x). In addition, a Mab to barley yellow dwarf virus (BYDV), IL-1 (1,000x diluted), was used (D'Arcy *et al.*, 1989).

Results

In the areas surveyed, 90 farmers' fields and 10 research stations were visited. In total, 1804 chickpea plants, showing some or all of the symptoms characteristic of stunt, were collected and tested in ELISA.

Survey in India

Ten experimental chickpea fields at ICRISAT Center were repeatedly surveyed during the season. The incidence of stunt was always less than 1%. Of a total of 699 plants tested in ELISA, 396 reacted with CCDV antiserum, 36 with CpLV antiserum, and 2 with BLRV antiserum. None of the samples reacted with antisera to SCRLV or PLRV. The plants infected with CpLV were concentrated in some fields.

In Gujarat, 14 farmers' fields (of 0.2 - 3.0 ha) and experimental plots at the Junagadh and Anand Agricultural Research Station (Gujarat Agricultural University) were visited. Here, the crop was already at the pod-setting and -filling stage. The stunt incidence in farmers' fields ranged from 0 to 45% (average 12%; Table 1). Only 8 of the 217 samples tested reacted with CCDV antiserum, and 106 reacted weakly with CpLV antiserum. At either research station no CCDV was found, but a

Table 1. Results of the survey for chickpea stunt disease in Gujarat (India).

Location	Number of fields visited	Stunt incidence	Number of samples collected	Number (and percentage) of samples reacting with polyclonal antisera to		
				CCDV	CpLV	BLRV
Farmers' fields around Junagadh	14	0 - 45%	167	8 (5%)	79** (47%)	0
Research stations Junagadh	1	high	33	0	11** (33%)	0
Anand	1	high	17	0	16** (94%)	6 (35%)
Total			217	8 (4%)	106 (49%)	6 (3%)

* Average.
** Weak reaction with CpLV antiserum.

high proportion of the samples reacted (weakly) with CpLV antiserum. Besides, in Anand some of these samples also reacted with the BLRV antiserum.

In Haryana, only 1 experimental chickpea field at the Government Livestock Farm, at Hisar, was surveyed twice, once before flowering (December 1991) and once during flowering (February 1992). In total 308 plants were tested, of which 114 reacted with CCDV antiserum, 8 with CpLV antiserum, and none with BLRV antiserum. CpLV was not detected in plants collected during February.

In Madhya Pradesh, 16 farmers' fields, varying in size from 0.1 to 3.0 ha, and research plots of the Khargone Agricultural Research Station were visited. The crop was at the flowering or already at the pod-setting stage. The incidence of stunt ranged from 0 to 29%, the average being 4% (Table 2). At the research station, Table

Table 2. Results of the survey for chickpea stunt disease in Madhya Pradesh (India).

Location	Number of fields visited	Stunt incidence	Number of samples collected	Number (and percentage) of samples reacting with polyclonal antisera to		
				CCDV	CpLV PLRV	BLRV SCRLV
Farmers' fields	16	0 - 29%	111	5 (5%)	0	0
Research station Khargone	1	15%	99	3 (3%)	0	0
Total			210	8 (4%)	0	0

* Average.

Table 3. Results of the survey for chickpea stunt disease in Rajasthan (India).

Location	Number of fields visited	Stunt incidence	Number of samples collected	Number (and percentage) of samples reacting with polyclonal antisera to		
				CCDV	CpLV	BLRV
Farmers' fields	27	0 - 5.2%	119	105 (88%)	4 (3%)	2 (2%)
Research stations Diggi and Durgapura	2	<0.1%	47	47* (100%)	1* (2%)	0 (0%)
Total			166	152 (92%)	5 (3%)	2 (1%)

* One plant was infected with CCDV and CpLV.

incidence was 15%. Only 8 of the 210 samples collected reacted with CCDV antiserum, and none reacted with the four luteovirus antisera used.

In Rajasthan, 27 farmers' fields, varying in size from 0.25 to 2.5 ha, and research plots at Durgapura Agricultural Research Station and Diggi Agricultural Research Substation (Rajasthan Agricultural University) were visited when the crop was at early flowering. Stunt incidence was generally low (Table 3). In six fields not a single infected plant was found. Only in 3 fields more than 1% of the plants were affected, viz. 2.6, 3.8 and 5.2%. All plants collected from these three fields were infected with CCDV only. At the 2 research stations visited, stunt incidence was also low and all plants collected there were infected with CCDV. Plants infected with CpLV-like or BLRV-like isolates were found only in a few fields.

Survey in Pakistan

In the Thal area (Punjab), 28 farmers' fields were surveyed. The crop was at the flowering stage. Stunt incidence was generally low. Of the 163 samples collected, 84 reacted with CCDV antiserum, 11 with CpLV antiserum, and none with BLRV antiserum (Table 4). At a research station in Kallurkot in the western part of the Thal area, CCDV occurred at low incidence in local cultivars, whereas in exotic germplasm incidences up to 12% were recorded.

In the Attock and Chakwal districts (Punjab), 5 farmers' fields and 2 research stations were visited. In the farmers' fields only CCDV was found, whereas at the two research stations CpLV-like isolates were detected (Table 4).

Further testing with Mabs

From the above-mentioned areas, 38 representative samples of those that reacted with a polyclonal luteovirus-specific antiserum (CpLV or BLRV), were selected and tested further with the Mabs. Based on the reaction of the samples with the polyclonal antisera two groups could be distinguished, viz. BLRV- and CpLV-like isolates. The samples that reacted with CpLV polyclonal antiserum and not with BLRV polyclonal antiserum had been collected from ICRISAT Center, Rajasthan, Hisar and

Table 4. Results of the survey for chickpea stunt disease in Pakistan.

Location	Number of fields visited	Stunt incidence	Number of samples collected	Number (and percentage) of samples reacting with polyclonal antisera to		
				CCDV	CpLV	BLRV
Thal						
Farmers' fields	28	0 - 2.6%	148	74 (50%)	9 (6%)	0
Research station Kallurkot	2	0 - 12%*	15	10 (67%)	2 (13%)	0
Attock						
Farmers' fields	3	< 0.1%	12	5 (42%)	0	0
Research station	2	< 0.1%	9	0	5 (56%)	0
Chakwal						
Farmers' fields	2	< 0.1%	5	3 (60%)	0	0
Research station	1	< 0.1%	15	0	10 (67%)	0
Total			204	92 (45%)	26 (13%)	0

* Stunt incidence low in local varieties, high in some of the genotypes from other parts of Pakistan.

Pakistan. These isolates gave similar reaction patterns with the Mabs (Table 5). They reacted with one or more of the Mabs WAU-A12, WAU-A24 and WAU-B9 and not with the other Mabs used. The isolates from Rajasthan differed in that they reacted only with WAU-A12. The isolates from Junagadh reacted only weakly with CpLV polyclonal antiserum and reacted with the Mabs as the others in the first group, but their reaction with WAU-A24 was always strong. Such a strong reaction was not found with the other isolates in this group.

The samples that reacted only with BLRV polyclonal antiserum had been collected at ICRISAT Center and in Rajasthan. These isolates all reacted strongly with the BYDV Mab IL-1 and their reaction with the PLRV Mabs was different in

Table 5. Reaction of selected luteovirus isolates from India and Pakistan with polyclonal antisera and monoclonal antibodies, as compared with the reaction of described luteoviruses reported in literature.

Origin of isolates	Polyclonal antibodies	Monoclonal antibodies									Number of isolates
		PLRV WAU								BYDV	
		A 2	A 6	A 7	A 12	A 13	A 24	A 47	B 9	IL1	
CpLV-like isolates											
ICRISAT	CpLV	-	-	-	S	-	M	-	S	-	3
	CpLV	-	-	-	S	-	M	-	W	-	3
	CpLV	-	-	-	S	-	W	-	M	-	1
Rajasthan	CpLV	-	-	-	S	-	-	-	-	-	5
Hisar	CpLV	-	-	-	S	-	M	-	M	-	3
Pakistan	CpLV	-	-	-	S	-	M	-	W	-	6
Junagadh	CpLV*	-	-	-	S	-	S	-	W	-	7
BLRV-like isolates											
ICRISAT	BLRV	-	-	-	-	-	W	-	W	S	2
Rajasthan	BLRV	-	-	-	W	S	-	-	-	S	2
Anand	CpLV	-	-	-	S	-	S	-	M	S	6
Described luteoviruses (according to Van den Heuvel *et al.*, 1990; D'Arcy *et al.*, 1989)											
BLRV		-	-	-	-	-	S	-	-	S	
BWYV		-	-	-	S	W	M	-	-	-	
BMYV		-	-	-	S	S	M	-	-	-	
PLRV		S	S	S	S	S	S	S	S	-	

* Polyclonal antiserum with which the isolates reacted in DAS-ELISA. This antiserum was used in TAS-ELISA for coating.

** Reactions in ELISA: S = strong (OD > 0.6), M = medium (0.6 > OD > 0.3), W = weak (0.3 > OD > 0.1) after 1 - 2 hr of substrate development at room temperature.

spectrum and intensity for the two different areas. Only in one case luteovirus isolates, from Anand, had been recognized by both the BLRV and the CpLV polyclonal antiserum. It is not clear whether a double infection with a BLRV-like and a CpLV-like virus was the case or one luteovirus was present that reacted with both polyclonal antisera.

Discussion

Surveying chickpea for the viruses involved in chickpea stunt turned out to be far from simple, as is the etiology of the disease. At different places different viruses, causing identical symptoms, were found to be involved or to prevail (Table 6). While serologically testing many samples, in addition to BLRV and CCDV, earlier detected at ICRISAT during studies of chickpea stunt (Horn *et al.*, 1993: Chapter 6), a number of luteoviruses dissimilar to any of the known legume luteoviruses appeared to occur. So, the surveying was not merely a matter of routine detection of viruses with a standard range of antisera to known viruses. It gradually led to the detection of a number of new viruses and to their tentative characterization, providing information that will facilitate future surveying.

Of the 1804 samples with symptoms tested with polyclonal antisera, 42% reacted with CCDV antiserum, 10% with CpLV antiserum, and 0.6% with BLRV antiserum. CCDV turned out to be the predominant virus in India in Rajasthan, at Hisar, and at ICRISAT Center, and in Pakistan. With respect to the luteoviruses, the surveys reported here have now clearly shown that BLRV is not the only virus of this group involved in chickpea stunt. Nearly all samples reacting with antiserum to CpLV or to BLRV, did not react with the other one, and this corroborates the discrimination of CpLV as a serologically distinct luteovirus. CpLV-like isolates appeared to be widely distributed in India and Pakistan, although often at low incidence. BLRV-like isolates were only found at two locations in India and at low incidences only. Thus, at least two distinct luteoviruses, viz. a BLRV-like and a CpLV-like virus were present. Another luteovirus or luteovirus strain may have been involved in Gujarat (Table 3).

Table 6. Summary of the results of surveys for chickpea stunt disease during the 1991/1992 season.

Country, Area	Stunt incidence	Prevailing virus	Minor viruses
India			
ICRISAT (Andhra Pradesh)	Low	CCDV	CpLV-like BLRV-like
Gujarat: Junagadh	High	a luteovirus*	CCDV
Anand	High	a luteovirus*	-
Hisar (Haryana)	Low-High**	CCDV	CpLV-like
Madhya Pradesh	High	Unknown agent	CCDV
Rajasthan	Low	CCDV	CpLV-like BLRV-like
Pakistan	Low	CCDV	CpLV-like

* Weak reaction with CpLV antiserum.
** Stunt incidence high in stunt nursery ICRISAT, low in other fields.

The samples from that area reacted only weakly with CpLV polyclonal antiserum, and their reaction with the monoclonal antibody WAU-A24 was strong.

Still other (luteo)viruses must also have been present since nearly 50% of the samples with stunt symptoms did not react with any of the antisera to luteoviruses used here nor with CCDV antiserum. For example, only 8 of the 210 samples collected in Madhya Pradesh (Table 2) reacted with antiserum to CCDV, and none with antisera to four luteoviruses. Purification and electron microscopy from a number of such plants did not reveal any virus particle, sap- and aphid-transmission experiments were unsuccessful, but stunt-like symptoms could be reproduced by graft-transmission (data not shown). Also non-viral factors could have caused some of the symptoms characteristic of chickpea stunt. Leaf reddening may be induced by several types of stress, either biotic or abiotic (Nene *et al.*, 1978; Bos, 1983).

BLRV-like isolates appeared to be of minor actual importance and they reacted with the BYDV Mab Il-1 as did BLRV (D'Arcy *et al.*, 1989), but in their reaction with the PLRV Mabs they differed clearly from the type isolate of BLRV (Table 5 lower part; Van den Heuvel *et al.*, 1990). This raises the question whether these isolates really are strains of BLRV or separate luteoviruses related to BLRV. The reaction patterns of all isolates tested with the Mabs (Table 5, upper part) are different from those of known luteoviruses (Table 5, lower part). Thus, new luteoviruses may well have been detected here. The luteovirus isolates cannot be fully identified on the basis of these data. Further tests, including host range and vector specificity, are definitely needed to identify luteovirus isolates as distinct viruses or strains of them. For example, a number of PLRV isolates from the U.K. reacted similarly with a panel of Mabs, but differed considerably in severity of the symptoms they caused on potato (Massalski and Harrison, 1987). The reactions with the Mabs of the BLRV-like isolates of this study showed quite some variation. Apparently, considerable variation is present among the luteovirus isolates studied, as was also found for luteoviruses from faba bean (Fortass, 1993). It is not known whether such variation in reaction with Mabs can occur within a single luteovirus.

Low stunt incidences in local cultivars (land races) and high stunt incidences in introduced genotypes at the research station in Kallurkot (Pakistan), show the potential threat of CCDV. New genotypes must therefore be screened for resistance to CCDV prior to introduction into areas where CCDV is indigenous. The much lower stunt incidence in local genotypes suggests that these have already genetically adapted to the virus probably during a long-time association with this virus in the area, during which selection has occurred in the local genotypes.

Some observations on the epidemiology of luteoviruses were done during the surveys. The occurrence of these viruses in a few fields suggests that their spread was limited or that their sources of infection were localized. Other observations (data not shown) indicated effects of cropping pattern on the incidence of luteoviruses. They seemed to occur most at sub-optimal plant densities, and more in mono than in mixed cropping. Since these epidemiological aspects may be important for disease control (Bos, 1983; Tresh, 1982), they require more extensive observations.

CCDV and CpLV-like isolates have shown to be a potential threat to chickpea production. Further surveys are necessary and should be done at different times throughout the chickpea-growing season. The increase in incidence and shift in relative importance of viruses can then be monitored. The luteoviruses occurring in chickpea are currently being characterized and assessed for their importance.

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4. Survey of chickpea (*Cicer arietinum* L.) for chickpea stunt disease and associated viruses in Syria, Turkey and Lebanon

N.M. HORN[a], K.M. MAKKOUK[b], S.G. KUMARI[b],
J.F.J.M. VAN DEN HEUVEL[c] and D.V.R. REDDY[a]

[a] International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, Andhra Pradesh 502 324, India.

[b] International Center for Agricultural Research in the Dry Areas (ICARDA), P.O. Box 5466, Aleppo, Syria.

[c] DLO Research Institute for Plant Protection (IPO-DLO), P.O. Box 9060, 6700 GW Wageningen, The Netherlands.

Summary

Faba bean necrotic yellows virus (FBNYV) was found to be the most important virus associated with chickpea stunt in Syria, Turkey and Lebanon. More than 50% of the 313 plant samples collected during May 1992 reacted with FBNYV antiserum. Moreover, luteovirus isolates resembling bean leafroll luteovirus, beet western yellows luteovirus and chickpea luteovirus were detected in ELISA using poly- and monoclonal antibodies. Chickpea chlorotic dwarf geminivirus was not detected in chickpea. The reaction patterns of the luteovirus isolates with monoclonal antibodies differed from those of some known luteoviruses. The relative importance of these viruses was different for the areas visited. Data presented in this study suggested that the etiology of chickpea stunt disease is more complex than originally thought.

Introduction

Chickpea stunt is the most important virus disease of chickpea in several countries. It is characterized by leaf reddening of desi types, and yellowing of kabuli-type chickpeas, and by internode shortening, plant stunting, and phloem browning in the collar region in both types (Nene *et al.*, 1978). These symptoms have been reported from chickpea-growing areas in Iran (Kaiser, 1972), India (Reddy *et al.*, 1979; Nene *et al.*, 1978), California (Bosque-Perez and Buddenhagen, 1990), Morocco, Tunisia and Algeria (ICRISAT, 1980; Reddy *et al.*, 1980), and Zambia (Kannaiyan and Hariwa, 1989). These symptoms result from phloem degeneration caused by the phloem-limited viruses. They are often followed by progressive plant decline and premature plant death. This disease therefore has the potential to cause severe losses to chickpea crops (Kaiser and Danesh, 1971; Kotasthane and Gupta, 1978; Chapter 8).

In Iran, bean leafroll virus (BLRV, earlier named pea leafroll virus) was reported to cause these symptoms (Kaiser, 1972). In India, the disease was ascribed to BLRV (Reddy *et al.*, 1979). It is now known that several luteoviruses can cause stunt-like symptoms in chickpea in India. At least two luteoviruses or distinct luteovirus strains are involved. Some of them are BLRV-like and others are clearly distinct from BLRV (Chapter 3). In California, subterranean clover red leaf (SCRLV), legume yellows (LYV, probably a strain of BLRV) and beet western yellows luteoviruses (BWYV) have been shown to cause symptoms in chickpea similar to chickpea stunt (Bosque-Perez and Buddenhagen, 1990; pers. comm. Buddenhagen, 1990). A leafhopper-transmitted geminivirus, chickpea chlorotic dwarf virus (CCDV), widely distributed in India and Pakistan (Chapter 3), can also cause the symptoms characteristic of chickpea stunt (Horn *et al.*, 1993; Chapter 6).

No reports are available on the incidence of chickpea stunt disease in Syria, Turkey and Lebanon, and on the viruses associated with this disease in these areas, but ICARDA and ICRISAT scientists regularly visiting these countries do observe chickpea plants with stunt symptoms. Luteoviruses are already known to occur in other leguminous crops in the area (Makkouk *et al.*, 1988; 1992). Recently, faba bean necrotic yellows virus (FBNYV), a new, not yet grouped, virus, has been described from faba bean in Syria, (Katul *et al.*, 1993), and been reported to also infect lentil and chickpea (Makkouk *et al.*, 1992; Katul *et al.*, 1993). CCDV was never looked for in this region since it was only recently described from India. The vector of this virus, *Orosius orientalis*, has been reported from Turkey (Lodos and Kalhadelen, 1985) and from Egypt (Habib *et al.*, 1976; El-Nahal *et al.*, 1989).

Therefore CCDV could occur also in this region.

To set priorities for disease control it is important to know which viruses are involved and to have information on their distribution and incidence. In this paper we report on collaborative ICARDA/ICRISAT surveys of chickpea for chickpea stunt and associated viruses in Syria, Turkey and Lebanon during May 1992. Results of the preliminary identification of luteoviruses with poly- and monoclonal antibodies are reported.

Materials and Methods

Areas surveyed

The major chickpea-growing areas in Syria and southeastern Turkey (Fig. 1) were surveyed during May 1992. In Turkey, assistance was obtained from the Turkish Directorate for Agriculture. In the areas surveyed, research stations were also

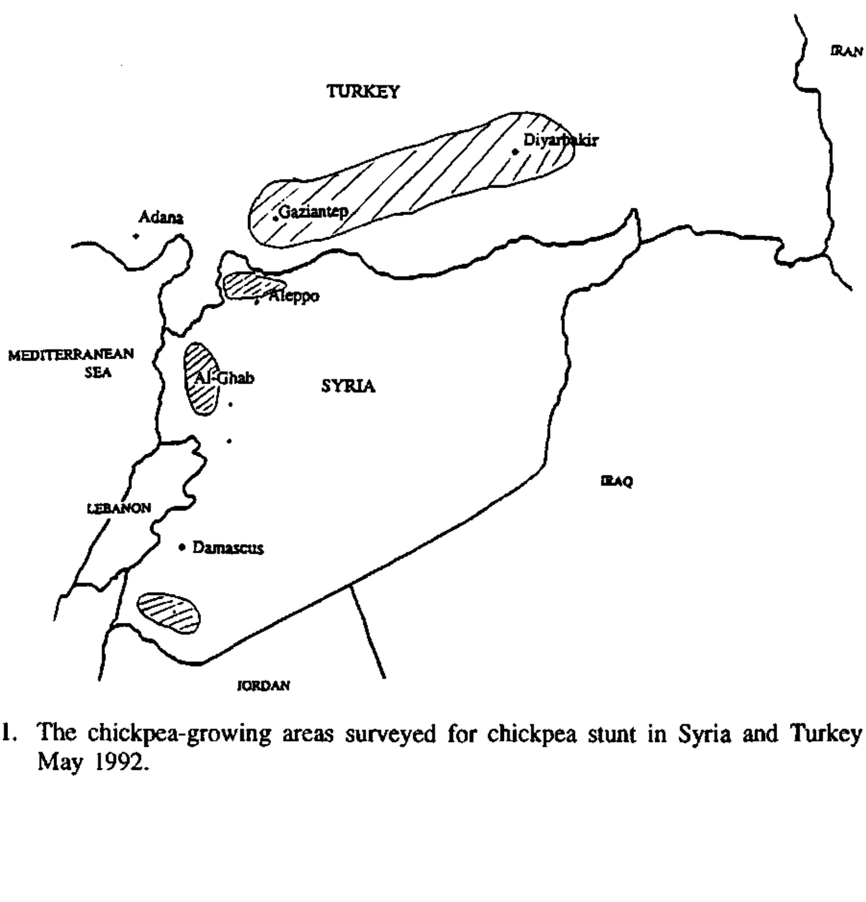

Fig. 1. The chickpea-growing areas surveyed for chickpea stunt in Syria and Turkey during May 1992.

included. Additionally, an ICARDA sub-station in Lebanon (Terbol) was visited. The inspected fields were chosen systematically by making a stop every five km during the trips, or at the nearest chickpea field thereafter.

Observations and sample collection

At each field visited, the size of the field, stage of crop development, cropping pattern, crop density, and stunt incidence were recorded. The incidence of stunt was assessed by counting the number of plants with stunt-like symptoms in five randomly-distributed groups of 100 plants each. If possible, samples from 10-15 plants with characteristic symptoms (Fig. 2) were collected in each field and brought to ICARDA (Aleppo, Syria) for testing.

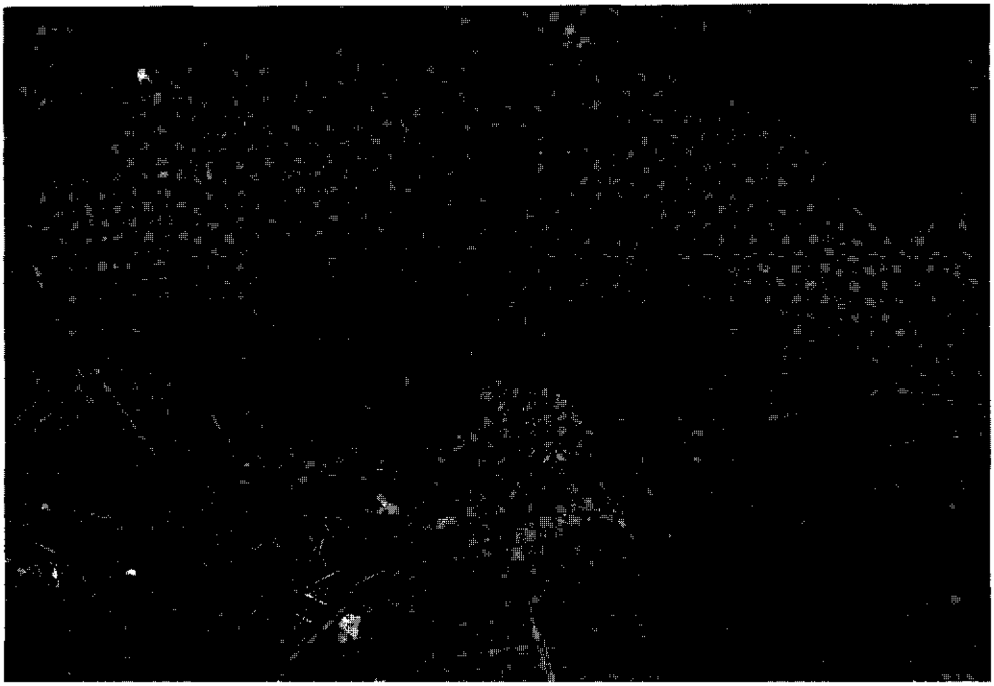

Fig. 2. Field symptoms of chickpea stunt. Healthy plants in the background.

Serology

All plant samples were tested with polyclonal antisera in DAS-ELISA, as described by Clark and Adams (1977). BLRV polyclonal antiserum was used since this virus had been reported from chickpea in Iran and India (Kaiser and Danesh, 1971; Reddy et al., 1979). In preliminary tests a luteovirus, which did not react with BLRV polyclonal antiserum, was found in many chickpea plants with symptoms of chickpea stunt at ICRISAT Center. Luteovirus-like particles were observed with the electron microscope. This virus was purified and a polyclonal antiserum was produced. In reciprocal tests in DAS-ELISA, this antiserum did not react with BLRV (Dutch isolate; Ashby and Huttinga, 1979), and BLRV antiserum did not react with the isolate from chickpea (data not shown). This isolate is thus serologically distinct from BLRV, and was tentatively named chickpea luteovirus (CpLV). Screening of the samples collected during the surveys was done using BLRV, BWYV, CpLV, FBNYV and CCDV polyclonal antisera.

Samples were ground in phosphate-buffered saline containing 0.05% Tween-20 and 2% polyvinylpyrrolidone (20 ml buffer per gram plant material). Samples reacting with one of the luteovirus polyclonal antisera were retested in a triple-antibody sandwich ELISA (TAS-ELISA) with the monoclonal antibodies (Mabs) mentioned below. In TAS-ELISA, coating was done with BLRV, BWYV or CpLV polyclonal antiserum at a concentration of 2 µg/ml, and plant material treated as described above was used. The Mabs were used in the concentrations mentioned below and a goat-anti-mouse alkaline phosphatase conjugate was used.

The antiserum to BLRV (Ashby and Huttinga, 1979) was supplied by Dr L. Bos (The Netherlands), to BWYV by Dr R. Casper (Germany) and to FBNYV by Dr L. Katul (Germany). The antisera to CCDV (Horn *et al.*, 1993; Chapter 6) and to CpLV had both been produced at ICRISAT. The Mabs to PLRV had been produced at the Wageningen Agricultural University (WAU), The Netherlands. The ones used in this study because of their differential reaction to a number of well-described luteoviruses (Van den Heuvel *et al.*, 1990; Table 4, lower part) and their dilutions (in brackets) were WAU-A2 (1,000x), WAU-A6 (5,000x), WAU-A7 (5,000x), WAU-A12 (2,000x), WAU-A13 (1,000 x), WAU-A24 (2,000x), WAU-A47 (2,500 x), and WAU-B9 (1,000x).

Results

In the areas surveyed, 50 farmers' fields and fields at 5 research stations were inspected. From all fields visited, 313 plants, showing some or all of the symptoms characteristic of stunt, were collected and tested in ELISA.

Survey in Syria

In southern Syria, 10 farmers' fields (of 1 - 3 ha) and experimental plots at the Jileen and Ezra Research Stations were visited when the crop was at early flowering.

Table 1. Results of the survey in Syria.

Location	Number of fields visited	Stunt incidence	Number of samples collected	Number of samples reacting with polyclonal antisera to				
				FBNYV	CCDV	CpLV	BLRV	BWYV
Southern Syria								
Farmers' fields	10	<0.1%	23	8	0	7	4	7
Research stations								
Ezra	1	<0.1%	6	4	0	1	1	1
Jileen	1	<0.1%	18	6	0	10	10	11
Al-Ghab area								
Farmers' fields	9	0-9.4%	65	34	0	18	25	28
Northern Syria								
Farmers' fields	9	<0.1%	17	11	0	3	3	2
Research station								
Jinderess	1	<0.1%	6	4	0	1	1	0
Total	31		135	67	0	40	44	49

Most of the samples that reacted with luteovirus antisera did so with more than one of them.

Stunt incidence was low (Table 1). Both FBNYV and luteoviruses were detected. At the research station in Ezra, mainly FBNYV was present, whereas at the station in Jileen more luteovirus was found.

In Al-Ghab (northwestern Syria), 9 farmers' fields (of 0.5 - 3 ha) were surveyed when the crop was at the flowering or pod-setting stage. Stunt incidence was low except in one field where it was 9.4% (Table 1). In the field with the high stunt incidence, luteovirus(es) that reacted with BWYV and/or BLRV antisera prevailed. In the area, FBNYV appeared to be as important as the luteoviruses.

In northern Syria, 9 farmers' fields (of 1 - 5 ha) and experimental plots at an ICARDA substation in Jinderess were inspected when the crop was at the flowering or pod-setting stage, and stunt incidence was low (Table 1). In this area, mainly FBNYV occurred. Out of 23 samples, 15 contained FBNYV and 5 were infected with luteovirus(es).

Survey in Turkey

In southeastern Turkey (between and around Gaziantep and Diyarbakir) 22 farmers' fields (of 1 - 3 ha) and experimental plots at one research station, the Southeastern Anatolia Agricultural Research Station in Diyarbakir, were visited. Six fields were surveyed around Gaziantep, 6 around Diyarbakir, and 10 between Gaziantep and Diyarbakir. In most of the fields, the crop was not yet flowering. The stunt incidence was low (Table 2), whereas at the Diyarbakir Research Station no plants with stunt symptoms were found.

Around Gaziantep mainly FBNYV occurred, whereas around Diyarbakir the incidence was very low and it was difficult there to collect plants with symptoms. Two of the 8 plants collected reacted with FBNYV antiserum, and one reacted with luteovirus antisera. The other 5 samples collected did not react with any of the antisera used. Between Gaziantep and Diyarbakir, FBNYV prevailed.

Near Adana (southern Turkey), one field with high stunt incidence was accidentally noticed. Incidence was estimated to be 30%, and 23 samples were collected from this field. Fifteen reacted with FBNYV antiserum and 9 with luteovirus antisera, most of them with more than one luteovirus antiserum (Table 2).

Table 2. Results of the surveys in Turkey.

Location	Number of fields visited	Stunt incidence	Number of samples collected	Number of samples reacting with polyclonal antisera to				
				FBNYV	CCDV	CpLV	BLRV	BWYV
Around Gaziantep								
Farmers' fields	6	<0.1%	18	13	0	2	2	2
Around Diyarbakir								
Farmers' fields	6	<0.1%	8	2	0	1	1	1
Research station	1	0%	0	0	0	0	0	0
Gaziantep - Diyarakir								
Farmers' fields	10	<0.1%	94	63	0	6	9	8
Adana, southern Turkey								
Farmers' field	1	30%	23	15	0	9	3	8
Total	24		143	93	0	18	15	20

Survey in Lebanon

From the ICARDA substation in Terbol, 35 plants with stunt-like symptoms were collected for testing, but not all of them showed all characteristic stunt symptoms. A number of plants collected did not show phloem browning. In 20 of the collected plants no virus could be detected with the antisera used. Of the remaining plants, 8 reacted with FBNYV antiserum, 1 with BLRV antiserum, 4 with BWYV antiserum, and 1 with both FBNYV and the three luteovirus antisera used (Table 3).

Table 3. Summary of the ELISA results of all samples collected during the surveys in Syria, Turkey and Lebanon.

Country	Number of samples collected	Number of samples reacting in ELISA with polyclonal antisera			
		FBNYV only	luteovirus only	FBNYV and luteovirus	negative to all antisera used
Syria	135	59 (44%)	54 (40%)	8 (6%)	14 (10%)
Turkey	143	91 (64%)	21 (15%)	2 (1%)	29 (20%)
Lebanon	35	8 (23%)	5 (14%)	1 (3%)	20 (57%)
Total	313	161 (51%)	81 (26%)	10 (3%)	61 (19%)

Further testing with Mabs

From the above-mentioned areas, 28 representative samples of those that reacted with one or more of the luteovirus-specific polyclonal antisera (CpLV, BLRV, or BWYV), were selected and tested further with the Mabs. Based on the polyclonal antiserum with which the samples reacted strongest, three groups could be distinguished, viz. CpLV-like, BLRV-like and BWYV-like isolates (Table 4). The reactions of the CpLV-like isolates from Syria and Turkey with the Mabs are similar to those of the CpLV-like isolates from India and Pakistan (Chapter 3). These isolates reacted with one or more of the Mabs WAU-A12, WAU-A24 and WAU-B9. The reactions of the BLRV-like isolates from Syria and Turkey with the Mabs were as variable as those of the BLRV-like isolates from India and Pakistan (Chapter 3). Apparently, the group of BLRV-like isolates is quite heterogeneous, at least in its serological reactions with the selected Mabs.

Table 4. Reactions of selected luteovirus isolates from Syria and Turkey with polyclonal Table antisera and monoclonal antibodies, as compared with the reaction of described luteoviruses.

Origin	Polyclonal antibodies	Monoclonal antibodies WAU								Number of isolates
		A 2	A 6	A 7	A 12	A 13	A 24	A 47	B 9	
Sy/Tu*	CpLV	-	-	-	S**	-	M/W	-	-	8
Sy/Tu	CpLV	-	-	-	S	-	-	-	-	3
Tu	CpLV	-	-	-	S	-	M/W	-	M/	2
Sy	BLRV	-	-	-	S	-	W	-	W	1
Sy/Tu	BLRV	-	-	-	W/-	-	M	-	W/	3
Sy/Tu	BLRV	-	M	-	-	-	M/-	-	-	2
Tu	BLRV	W	-	-	W	-	-	-	-	1
Tu	BLRV	W	M	-	M	M	-	-	-	1
Sy	BLRV	-	W	-	S	S	M	-	W	1
Sy/Tu	BWYV	-	-	-	S	-	M/W	-	-	6
Sy	BWYV	-	-	-	S	S	S	-	W	1
Sy	BWYV	-	-	-	S	W	W	W	W	1
Described viruses for comparison (Van den Heuvel *et al.*, 1990)										
BLRV		-	-	-	-	-	S	-	-	
BWYV		-	-	-	S	W	M	-	-	
BMYV		-	-	-	S	S	M	-	-	
PLRV		S	S	S	S	S	S	S	S	

* Origin of the samples, Sy = Syria, Tu = Turkey.

** Reaction in TAS-ELISA after 1 -2 hour substrate development at room temperature, S = strong (OD > 0.6), M = medium (0.6 > OD > 0.3), W = weak (0.3 > OD > 0.1), - = no reaction (OD < 0.1).

Most of the BWYV-like isolates reacted with WAU-A12 and WAU-A24. These reactions are more or less similar to those of the CpLV-like isolates. Only two BWYV-like isolates reacted differently with the Mabs, but one of them may have been

Discussion

For final discussion, all results of the surveys in Syria, Turkey and Lebanon are summarized in Table 3. More than 50% of the 313 samples collected reacted with FBNYV antiserum only, and very few (3%) reacted with antisera to both FBNYV and luteoviruses. FBNYV thus appeared to be an important virus in chickpea in the three countries surveyed. Earlier laboratory tests had demonstrated that this virus could cause stunt and chlorosis in chickpea (Katul *et al.*, 1992), but then the plants were not examined for phloem browning (L. Katul; pers. comm., 1992). Since FBNYV was the only virus detected in our field survey in slightly over 50% of the chickpea plants with the characteristic stunt symptoms including phloem browning, it is most likely that in these cases the symptoms were caused by this virus alone. FBNYV is also phloem limited and induces external symptoms secondarily, as luteoviruses do (Waterhouse *et al.*, 1988). Information on actual incidence of this virus in chickpea is still limited. In Syria, it was recently found to occur at high incidences in lentil (Makkouk *et al.*, 1992). Since in that country chickpea is often grown along with lentils, and the virus was found to occur in many of the chickpea plants with stunt symptoms, the virus may well be or become economically important there.

Although all collected samples were also tested with CCDV antiserum, this virus, widely distributed in the Indian subcontinent (Chapter 3), was not observed during our present survey. Of the 313 samples tested, 92 reacted with one or more luteovirus polyclonal antiserum (BWYV, BLRV, CpLV). Twenty of them reacted with only one of the three antisera, 30 with two, and 42 with all three antisera. Heterologous reactions are common among luteoviruses (Waterhouse *et al.*, 1988). The multiple reactions with luteovirus polyclonal antisera, as found now, cannot all be attributed to cross reactions, since some luteoviruses involved here reacted with one of the three antisera only. These luteoviruses are likely to be present also in the plants that reacted with more than one luteovirus antiserum, and thus to occur together with other luteoviruses in multiple infections. Recent tests at ICRISAT Center with field-collected samples from India, frequently revealed the simultaneous presence in single plants of more than one luteovirus, identified by monoclonal antibodies produced to individual luteoviruses

(A.S. Ratna, S.V. Reddy, R.A. Naidu and D.V.R. Reddy, unpublished data).

Our serological tests with polyclonal antisera only point to the involvement of BWYV-like, BLRV-like and CpLV-like isolates in chickpea stunt. When producing CpLV antiserum, this virus was found to cross react with BWYV antiserum. A number of CpLV-like isolates found in our survey did not react with BWYV polyclonal antiserum. This suggests variation within the group of CpLV-like isolates. The reactions of most of the BWYV-like isolates were very similar to those of the CpLV-like isolates, indicating their close relationship. One of the BWYV-like isolates reacted with the Mabs as earlier found by Van den Heuvel *et al.* (1990) for beet mild yellowing virus (BMYV). The reactions of our BLRV-like isolates with the Mabs showed quite some variation, and they were different from those of a Dutch isolate of BLRV (Ashby and Huttinga, 1979; Van den Heuvel *et al.*, 1990). Since the affinities of most isolates towards the Mabs (Table 4, upper part) differed from those of known luteoviruses (Table 4, lower part), we may well have traced new luteoviruses here. The luteovirus isolates studied here vary considerably, as was also found for luteoviruses from faba bean in Morocco (Fortass, 1993). Until sequence information of part of the genomes of these viruses is available, and their host range and vector specificity are determined, no valid conclusions can be drawn about their relationships with other well characterized luteoviruses.

The surveys of chickpea in Syria, Turkey and Lebanon for chickpea stunt and its associated viruses revealed a number of viruses, causing identical symptoms, as was found in India and Pakistan (Chapter 3). However, their distribution and relative importance differed in different areas. Also a virus not found in India and Pakistan (FBNYV) was involved in Syria, Turkey and Lebanon. This virus had not yet been encountered in the disease before, and this information adds to our knowledge of the complexity of chickpea stunt (Chapter 3). Breeding for resistance to chickpea stunt should be adjusted to these findings. Further surveying and diagnosis, and better characterization of deviant isolates, remains imperative. The chickpea luteoviruses as such require further study. Diversity in these luteoviruses is already under investigation at ICRISAT and IPO-DLO.

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LUTEOVIRUSES

5. Luteovirus isolates from a single stunted plant of chickpea (*Cicer arietinum* L.) in India and their possible role in the etiology of chickpea stunt

N.M. HORN and J.F.J.M. VAN DEN HEUVEL

DLO Research Institute for Plant Protection (IPO-DLO), P.O. Box 9060,
6700 GW Wageningen, The Netherlands

Summary

In an orientational experiment with a single field-infected chickpea plant with symptoms characteristic of chickpea stunt three luteovirus isolates were obtained. One isolate was characterized by host range, ELISA and Western blot analysis, and identified as a distinct strain of beet western yellows virus (BWYV). Another isolate appeared to be potato leafroll virus-like (PLRV-like) by its reactions with poly- and monoclonal antibodies. Both isolates failed to cause chickpea stunt on their own. However, when the PLRV-like isolate was transferred from *Physalis floridana* to chickpea, it appeared that these *P. floridana* plants contained also another isolate, which was serologically similar to the BWYV isolate characterized here. However, it differed in that it could produce all symptoms of chickpea stunt. Apparently, the behaviour of luteoviruses in chickpea and their involvement in the etiology of chickpea stunt is complex.

Several viruses have so far been isolated from chickpea plants (*Cicer arietinum* L.) with the stunt syndrome, viz. chickpea chlorotic dwarf geminivirus (Horn *et al.*, 1993; Chapter 6), and a number of luteoviruses: bean leafroll virus (BLRV; Kaiser, 1972; Reddy *et al.*, 1979), subterranean clover red leaf virus (SCRLV) and beet western yellows virus (BWYV; Bosque-Perez and Buddenhagen, 1990; Carazo *et al.*, 1993). They were all reported to cause symptoms similar, if not identical, to those described by Reddy *et al.* (1979) for the disease earlier ascribed to BLRV alone, but the postulates of Koch have not been applied, so that their actual role in the etiology of chickpea stunt remained uncertain. Recent surveys in India and ELISA identification with poly- and monoclonal antibodies revealed the presence of a possibly new luteovirus in chickpea (CpLV) and of BLRV-like and CpLV-like luteovirus isolates (Chapter 3).

In this study, three luteovirus isolates from a single chickpea plant naturally infected in the field in India, and their involvement in chickpea stunt were studied. One of them was characterized biologically and physico-chemically, using host-range studies, ELISA, SDS-PAGE and Western blot analysis. This isolate did not cause stunt disease. Therefore, more isolates from the original chickpea plant were studied and identified by their reactions with poly- and monoclonal antibodies. One of them was found to be associated with the symptoms of chickpea stunt under laboratory conditions.

The virus isolates were obtained from a chickpea plant collected at ICRISAT Center, Patancheru, near Hyderabad, India, in December 1990. This plant showed the characteristic stunt symptoms including phloem browning, and reacted strongly with CpLV polyclonal antiserum, whereas it did not react with CCDV polyclonal antiserum. For virus maintenance and transmission 1-day-old nymphs were allowed a 1-day acquisition access period (AAP), followed by a 3-day inoculation access period (IAP). For comparison a Dutch isolate of BWYV from lettuce and potato leafroll luteovirus (PLRV), both maintained in *Physalis floridana* by regular transfers with *M. persicae* (Van den Heuvel *et al.*, 1990), and a BLRV isolate, from and maintained by vegetative propagation in *Medicago sativa*, were used.

The first isolate was transferred with *Myzus persicae* from the field-infected chickpea plant to pea cv. Onyx, and maintained and propagated in this cultivar. To study its host range, at least eight plants of several species were aphid inoculated using 10 nymphs per plant. The test plants were grown in a glasshouse at 20-25°C, observed visually for symptoms three weeks after inoculation, and then tested by double antibody sandwich (DAS-)ELISA for the presence of the virus. The isolate infected *Capsella bursa-pastoris* (shepherd's purse), *Crambe abyssinica, Raphanus* *sativum* (radish), and *Pisum sativum* (pea) cv. Onyx. It did not infect *Beta vulgaris* (sugarbeet) and *Lactuca sativa* (lettuce), even when up to 20 plants and up to 20 aphids per plant were used. For inoculation of chickpea, the IAP was reduced to one day, since most nymphs did not survive for longer periods of time on chickpea, and the AAP was increased to three days. The isolate only occasionally infected chickpea (2 out of 50 plants inoculated), but neither growth reduction nor the other characteristic stunt symptoms were observed. Chickpea was also inoculated with the reference isolates of BWYV and PLRV. BWYV infected chickpea efficiently (5 out of 6 plants inoculated) and caused the characteristic stunt symptoms, whereas many attempts to transfer PLRV to chickpea (42 plants) with *M. persicae* were unsuccessful.

The new virus isolate (first isolate) was successfully purified from pea cv. Onyx as described by Van den Heuvel *et al.* (1990). For antiserum production 200 µg purified virus was emulsified with Freund's incomplete adjuvant and injected into a rabbit subcutaneously, and this was followed three weeks later by another subcutaneous injection of 100 µg antigen. Starting three weeks after the second injection, the rabbit was bled at 3-week intervals. Double antibody sandwich (DAS)-ELISA and triple antibody sandwich (TAS)-ELISA were performed as described by Clark and Adams (1977) and Van den Heuvel *et al.* (1990), respectively. The BLRV antiserum was kindly supplied by Dr L. Katul (Braunschweig, Germany). The monoclonal antibodies (Mabs) had been produced at the Wageningen Agricultural University (WAU; Van den Heuvel *et al.*, 1990). The following Mabs were used at the dilutions indicated in brackets: WAU-A2 (500x), WAU-A6 (40,000x), WAU-A7 (40,000x), WAU-A12 (20,000x), WAU-A13 (500x), WAU-A24 (15,000x), WAU-A47 (10,000x) and WAU-B9 (5,000x). The reactions with the Mabs of the original chickpea plant and the isolate obtained after transfer to pea are given in Table 1. In DAS-ELISA, the isolate and BWYV reacted strongly in reciprocal tests. Both viruses could not be detected in DAS-ELISA with PLRV polyclonal antiserum. PLRV could not be detected in DAS-ELISA with BWYV polyclonal antiserum and the antiserum produced in this study. The luteovirus isolate and BWYV reacted with the Mabs WAU-A12, WAU-A13 and WAU-A24. The reaction of the field-infected plant with the Mabs was quite similar to that of the first isolate (Table 1). However, the original chickpea plant had a low affinity with WAU-A13, whereas the isolate had a high affinity with this Mab.

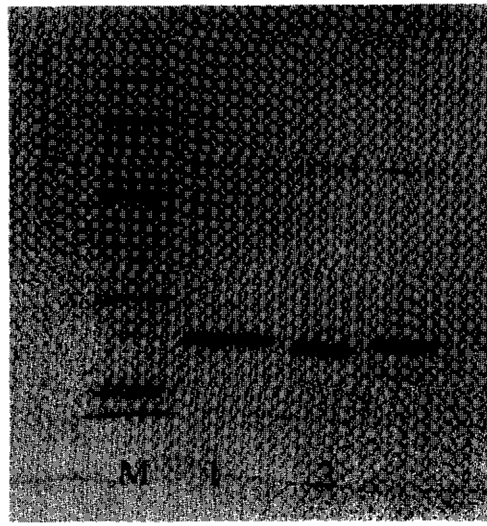
To study the size of the structural proteins, purified virus was electrophoresed under denaturing conditions on a 4% (w/v) polyacrylamide stacking gel and a 12% (w/v) separating gel. Then, proteins were either stained with Coomassie Brilliant blue

Table 1. The reactions with BWYV and PLRV polyclonal antisera and with the Mabs used of the original field-infected chickpea plant and of the isolates obtained from this plant.

	Polyclonal antisera		PLRV monoclonal antibodies WAU							
	BWYV	PLRV	A 2	A 6	A 7	A 12	A 13	A 24	A 47	B 9
Original field-infected plant										
chickpea			-	-	-	+	+/-	+	-	-
After transfer from the original chickpea plant to pea										
pea[1]	+	-	-	-	-	+	+	+	-	-
chickpea[1]	+	-	-	-	-	+	+	+	-	-
After transfer from the original chickpea plant to *Physalis floridana*										
Physalis floridana[2]	-	+	+	+	+	+	+	+	+	+
chickpea[3]	+	-	-	-	-	nt[4]	+	+	-	-
Reference viruses (Van den Heuvel *et al.*, 1990)										
BWYV	+	-	-	-	-	+	+	+	-	-
PLRV	-	+	+	+	+	+	+	+	+	+

[1] first isolate, [2] second isolate, [3] third isolate, [4] not tested.

Fig. 1. SDS-PAGE of the proteins of the chickpea isolate (1), BWYV (2), and markers (M).



or transferred onto nitrocellulose sheets. After blocking with 0.5% BSA the sheets were incubated with rabbit IgG alkaline phosphate conjugate in PBS-tween containing 0.05% BSA for 3 h at 20°C. Immobilized conjugate was visualized by a mixture of 5-bromo-4-chloro-3-indolylphosphate p-toluidine salt and nitroblue tetrazolium chloride in 0.1 M Tris-HCl buffer, pH 9.5, containing 0.1 M NaCl and 5 mM $MgCl_2$.

Two virion-associated proteins were revealed by SDS-PAGE (Fig. 1), the smaller protein of 26 kDa (relative molecular mass) probably is the major coat protein species, and a larger protein of 57 kDa, assumed to be the read-through product (Bahner *et al.*, 1990). In Western blot analysis, the antiserum against the isolate from chickpea reacted strongly with its homologous antigen and with BWYV, but only faintly with BLRV (Fig. 2). BWYV antiserum reacted strongly with the chickpea isolate and with BLRV (Fig. 2).

Based on the serological reactions in ELISA and a comparison of our data on the host range with literature data (Duffus, 1960), especially the infection of shepherd's purse, radish and *Crambe abyssinica,* with literature data (Duffus, 1960), the isolate appears to be related to BWYV. However, it differed from BWYV in that the coat protein was larger in size than that of BWYV, as found by coelectrophoresis of both the polypeptides (data not shown). Additionally, in Western blot analysis, the reactivity of their antisera with BLRV differed. Moreover, the isolate did not infect lettuce

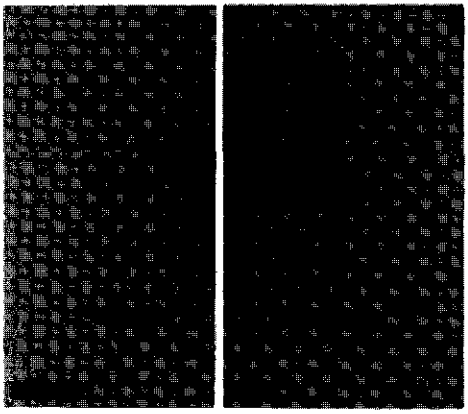


Fig. 2. Western blot analysis of virion-associated proteins from the chickpea isolate (1), BWYV (2), and BLRV (3) with the antiserum produced against the chickpea isolate (A) and with BWYV antiserum (B).

and sugarbeet, as was also found for other BWYV isolates by Duffus (1964). We therefore consider the luteovirus isolate from chickpea distinct from BWYV.

The isolate could not induce the characteristic stunt symptoms in chickpea, whereas BWYV did induce the symptoms under the same conditions. The original field-infected chickpea plant was therefore reinvestigated for the presence of other luteoviruses. A second luteovirus isolate was indeed obtained by transmission with *M. persicae* to *P. floridana*. The sap of infected plants of *P. floridana* reacted with PLRV polyclonal antiserum and with the Mabs produced for PLRV (Table 1), and therefore, the *P. floridana* plants contained a PLRV-like isolate. When virus was further transferred to chickpea with *M. persicae,* clear stunt symptoms, including phloem browning appeared. Virus was efficiently transferred to chickpea in six replications. In total 36 plants were inoculated, and 22 of them became stunted. Most strikingly, however, these chickpea plants did not react with PLRV polyclonal antiserum. The plants with symptoms reacted strongly with BWYV polyclonal antiserum, and with the Mabs WAU-A13 and WAU-A24 only (WAU-A12 was not tested), this resembled the reaction pattern of BWYV (Table 1), and indicated the involvement of a third isolate. In all six replications the reaction pattern was the same. This third isolate must have been present in the *Physalis floridana* plants below the detection level of the ELISA used, and have come forth after transfer to chickpea.

These orientational experiments suggest that the original chickpea plant from the field contained a mixture of luteovirus variants, of which three were isolated here. Mixtures of luteovirus variants have also been found in potato plants (Duffus, 1981). Our plants of *P. floridana* that contained the PLRV-like isolate contained a mixture of variants since the inoculated plants reacted with PLRV polyclonal antiserum and did not react with BWYV polyclonal antiserum, whereas the opposite was found after transfer to chickpea (Table 1). The BWYV-like isolate detected in this chickpea after transfer of the PLRV-like isolate to chickpea may have been the same as the isolate characterized here. Sequence information of the genome of the BWYV isolates, viz. the first and third isolate, is required to fully determine their identity and their relationships with BWYV. Our findings demonstrate the continuing problem of reliable identification of luteovirus isolates because the taxonomy of luteoviruses remains unclear. Thus, either a second BWYV-like isolate, causing chickpea stunt on its own, is involved here, or only one BWYV-like isolate, the one characterized here. This isolate then needs the PLRV-like isolate to efficiently infect chickpea and cause chickpea stunt. This indicates the complicated behaviour of the luteoviruses in chickpea, and the complexity of their involvement in the etiology of chickpea stunt.

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GEMINIVIRUS

6. Chickpea chlorotic dwarf virus, a new leafhopper-transmitted geminivirus of chickpea in India[1]

N.M. HORN[a], S.V. REDDY[a], I.M. ROBERTS[b] and D.V.R. REDDY[a]

[a] International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, Andhra Pradesh 502 324, India

[b] Scottish Crop Research Institute, Invergowrie, Dundee, DD2 5DA, U.K.

Summary

A disease of chickpea in India, characterized by chlorosis, severe stunting and phloem browning, was shown to be caused by a geminivirus. This virus was transmitted by the leafhopper *Orosius orientalis* from chickpea to chickpea and several other plant species. A method for purification of this virus was devised and a polyclonal antiserum produced. The majority of the purified particles were geminate. The size of the coat protein was shown to be 32 kD and the nucleic acid was shown to be circular ssDNA of 2900 nucleotides. By immunosorbent electron microscopy this virus was shown to be unrelated to the leafhopper-transmitted geminiviruses known to infect dicotyledons such as beet curly top, bean summer death and tobacco yellow dwarf viruses. On the basis of particle morphology, leafhopper transmission, host range and serology this virus was considered to be a new, hitherto undescribed, geminivirus and was named chickpea chlorotic dwarf virus.

Introduction

Stunt is an important viral disease of chickpea (*Cicer arietinum* L.) in many chickpea-growing countries. The characteristic symptoms are internode shortening, plant stunting or dwarfing, leaf reddening in the case of desi-type and yellowing in the case of kabuli-type chickpeas and phloem browning in the collar region (Nene *et al.*,

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1991; Nene and Reddy, 1987; Duffus, 1979). Bean leafroll luteovirus (BLRV) has been mentioned to be associated with the disease in India (Reddy *et al.*, 1979; Nene *et al.*, 1991) . Several other luteoviruses have been found to infect chickpea, viz. pea leafroll virus (= BLRV) in Iran (Kaiser and Danesh, 1971), subterranean clover red leaf (SCRLV), beet western yellows (BWYV) and legume yellows (LYV) viruses in California (Bosque-Perez and Buddenhagen, 1990; Duffus, 1979).

Several chickpea plants showing stunt symptoms were collected from different parts of India and processed, several plants together, for the isolation and purification of luteoviruses. Although luteoviruses were recovered from these field-infected plants, samples from several places were also found to contain a geminivirus, so far unknown for this plant species. It was therefore further investigated to know if it is different from other geminiviruses described so far. Since the vector of this virus was not known the initial study of this virus was difficult. Only after the discovery of its leafhopper vector could the virus be isolated and characterized and was found to be different from other leafhopper-transmitted geminiviruses. This paper reports the host range, transmission, and physico-chemical properties of this geminivirus.

Materials and Methods

Virus isolate

The virus isolate was obtained from field-infected chickpea plants ('WR 315') collected at Hisar, Haryana State (India), showing reddening of the leaflets, phloem browning and plant stunting.

Mechanical inoculation

Young chickpea leaflets showing typical symptoms were triturated in 50 mM potassium phosphate buffer (pH 7.0) containing 750 µl/litre thioglycerol (1 g tissue/ 9 ml buffer). The extract was used to inoculate manually carborundum-dusted leaves of chickpea, pea and tobacco plants.

The insects

A culture of the leafhopper *Orosius orientalis* Matsumura, previously named *O. albicinctus* Distant, was maintained on *Sesamum indicum* L. and *Crotalaria juncea* L. and a culture of the whitefly *Bemisia tabaci* was maintained on cotton (*Gossypium hirsutum* L.)

A partially purified virus preparation containing sucrose (150 g/litre) was used to feed *O. orientalis* and *B. tabaci* adults through a parafilm^R membrane. After a one-day acquisition-access period, the leafhoppers and whiteflies were allowed a two-day inoculation-access period on pea (*Pisum sativum* 'Bonneville') and chickpea ('WR 315') plants. The virus isolated from chickpea plants was maintained on pea plants ('Bonneville'). For this purpose *O. orientalis* was allowed a two-day acquisition-access period on infected pea plants followed by a two-day inoculation-access period on healthy pea plants. Back inoculation from pea to chickpea reproduced the characteristic symptoms in chickpea.

Host range studies

At least six plants of each species were grown in a glasshouse with temperatures ranging from 25 to 30°C. Young adults of *O. orientalis*, given access to infected pea plants for 2 days, were used. Inoculation feeding periods on test plants were 2 days using 25 leafhoppers per pot containing 2-3 test plants. The test plants were observed visually for symptoms and tested by DAS-ELISA and immunosorbent electron microscopy (ISEM) for the presence of the virus.

Virus purification

The procedure described by Van den Heuvel *et al.* (1990) for potato leafroll virus was used with slight modifications. Pea plants showing stunting, leaf rolling and chlorosis were harvested 10-15 days after inoculation with infective leafhoppers and stored at -70°C. Normally 100 g of frozen tissue was processed each time. All steps were carried out at room temperature while the centrifugations were done at 15°C. Tissue was homogenized in a blender with 2 volumes of 100 mM sodium citrate buffer, pH 6.0 (SCB), containing ethanol (5 ml/litre), thioglycollic acid (1 ml/litre) and celluclast[R] (30 ml/litre). The extract was stirred for 3 h, whereafter chloroform (250 ml/litre) and butanol (250 ml/litre) were added. The mixture was stirred for 5 min and clarified by centrifugation (13,680 g, 15 min). Triton X-100 was added (1.0 ml/litre) to the aqueous phase and the mixture stirred for 30 min, then polyethylene glycol (mol. weight 8000) at 80 g/litre and sodium chloride at 23.6 g/litre were added and the mixture was stirred for 20 min. before keeping at room temperature for 2h. The resulting precipitate was collected by centrifugation (13,680 g, 20 min) and resuspended in 30 ml of 100 mM SCB, containing ethanol (50 ml/litre). This mixture was stirred for 30 min, held at room temperature for 16 h and clarified by centrifugation (7100 g, 15 min). The supernatant was loaded on to 15 ml of 30% sucrose, prepared in SCB in Beckman R45 rotor tubes and centrifuged at 185,500 g for 4 h. Each pellet was resuspended in 1 ml SCB, loaded on to a 100-400 g/litre sucrose gradient (in Beckman SW 40 rotor) and centrifuged for 3 h at 110,000 g. The gradients were prepared by layering 2.7 ml of each of 100, 200, 300 and 400 g/litre sucrose and stored overnight at 4°C. Fractions of the gradient were collected with a bent needle attached to a syringe, diluted in SCB, and centrifuged in a Beckman R50 rotor at 150,000 g for 4 h. Pellets were resuspended in 100 µl of 10 mM Tris-HCl buffer, pH 8, containing 1 mM EDTA.

Electron microscopy

Three-hundred mesh copper grids coated with a carbon film were inverted onto 10-ul drops of purified virus for 10 min, stained with 1% uranylacetate and examined with a Philips 201 C electron microscope.

Antiserum production

Purified virus (20-80 µg) was emulsified with an equal volume of Freund's incomplete adjuvant and injected intramuscularly into a New Zealand White inbred rabbit at weekly intervals. After four injections the rabbit was bled at weekly intervals.

Immunosorbent electron microscopy (ISEM)

The method used was essentially that of Roberts and Harrison (1979). Grids were inverted on a 10-µl drop of antiserum (1:1000 dilution in 70 mM phosphate buffer, pH 6.5) for 1 h at 37°C followed by 10 min washing with the same buffer. The grids were inverted onto a 10-µl drop of partially purified virus suspension for 1 h, washed with distilled water, and stained with 2% ammonium molybdate, pH 6.5. Virus particles were counted in fifty viewing fields of a JEOL 100S electron microscope at 30,000 x magnification, and the number of dimers per 1000 um^2 was calculated (Roberts, 1980).

Enzyme-linked immunosorbent assay (ELISA)

Double antibody sandwich (DAS) ELISA, as described by Clark and Adams (1977), and direct antigen coating (DAC) ELISA, as described by Hobbs *et al.* (1987), were used. Gammaglobulins were extracted with sodium sulphate (Hobbs *et al.*, 1987) and conjugated to alkaline phosphatase as described by Clark and Adams (1977), p-nitrophenyl phosphate was used at 1 mg/ml as substrate. In DAC-ELISA penicillinase was used as enzyme. Sodium penicillin (0.5 mg/ml) prepared in a bromothymolblue solution (0.15 g/litre) was then used as substrate (Sudarshana and Reddy, 1989). Various antisera used and their sources are given in Table 2.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was carried out in a discontinuous slab gel for 16 h at 35V. (Laemmli, 1970). Samples were prepared in 62.5 mM Tris pH 6.8, containing SDS (20 g/litre), mercaptoethanol (50 ml/litre) and glycerol (100 ml/litre) and boiled for 5 min prior to loading onto the gels. Protein markers (Biorad) were phosphorylase b (97.4 kD), bovine serum albumin (66.2 kD), ovalbumin (42.6 kD), carbonic anhydrase (31 kD) and soybean trypsin inhibitor (21.5 kD). The gel was stained with silver nitrate as described by Reddy *et al.* (1990).

Nucleic acid extraction and gel analysis

Purified virus was treated with 0.5% SDS and protease K (5 µg/100pg of virus) in

25 mM EDTA for 1 h at 55°C. The nucleic acid was extracted twice with a mixture of chloroform, phenol, isoamyl alcohol (24:24:1) and once with chloroform and isoamyl alcohol (48:1), and precipitated with ethanol and 2 M ammonium acetate at -20°C. Samples were suspended in 10 mM Tris buffer containing 1 mM EDTA, pH 8.0, and were run for 1 h at 100V at room temperature in a 1% agarose gel in 40 mM Tris acetate buffer containing 1 mM EDTA. Gels were stained for 5 min in ethidium bromide (1 µg/ml) and observed on a transilluminator. For treatment with RNase A (at 10 µg/ml) the nucleic acid was suspended in 10mM Tris-HCL, pH 7.8 containing 1mM EDTA. For DNase I (100 µg/ml) treatment samples were suspended in 10 mM Tris-HCL, pH 7.8, 10 mM $MgCl_2$, and for treatment with nuclease S1 (1 unit per 100 ng nucleic acid) in 30 mM sodium acetate, 50 mM NaCl, 100mM $ZnSO_4$ and 5% glycerol, pH 4.6. The reactions were stopped by the addition of EDTA to a final concentration of 10 mM and samples were analyzed in an agarose gel as described above. Circular ssDNA molecular weight markers were derived from pUC 119 clones containing inserts of different sizes. Bacteriophage M13 KO7 was used as helper phage for production of the markers (Vieira and Messing, 1987). Samples for electron microscopy were prepared and spread as described by Murant *et al.* (1981).

Results

Transmission

Mechanical inoculation from chickpea, pea and tobacco (*Nicotiana tabacum*, White Burley) to chickpea, pea and tobacco was not successful, all possible combinations were tested. The virus could not be transmitted by *B. tabaci* but could be transmitted from chickpea to chickpea and to other plant species by *O. orientalis*, a leafhopper known to transmit sesamum phyllody (Vasudeva and Sahambi, 1955) and potato purple top roll (Singh *et al.*, 1983), both mycoplasma diseases.

Host-range studies

The host range of the virus using *O. orientalis* for transmission is presented in Table 1. The following plant species were not infected by the virus: *Cucumis sativus* L., *Cajanus cajan* (L.) Millsp., *Arachis hypogaea* L., *Medicago sativa* L., *Vigna unguiculata* (L) Walp. L., *V. radiata* (L.) Wilczek var *radiata*, *V. mungo* (L.) Hepper, *Glycine max* (L.) Merr, *Nicotiana rustica* L., *N. occidentalis* L. and *Solanum melongena* L. The characteristic symptoms on chickpea, yellowing, reddening, stunting and phloem browning, could be reproduced on chickpea (Fig. 1 shows the stunting). A number of hosts, including *Nicotiana tabacum* L. ('White Burley' and 'Samsum NN') and *Datura stramonium* L., showed leaf rolling, yellowing and stunting. Plants which showed severe symptoms contained larger amounts of virus, as determined in ELISA and ISEM tests, than those which did not show strong symptoms. Two weeks after inoculation feeding nymphs of *O. orientalis* appeared on faba bean, *Vicia faba* L. and lentil, *Lens esculenta*,

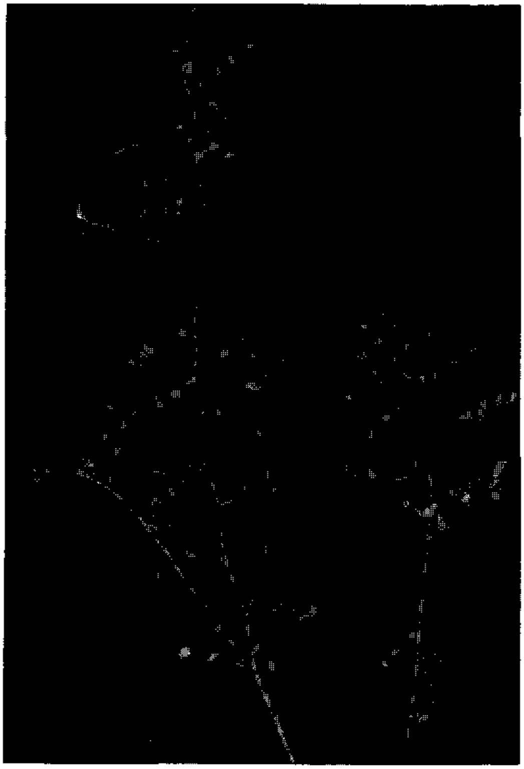


Fig. 1. The symptoms caused by the virus in chickpea after inoculation by *Orosius orientalis* especially the stunting is clear. Left healthy, right inoculated plant, two weeks after inoculation.

Table 1. Host range of the chickpea geminivirus as determined by inoculation using O. orientalis; symptoms observed and virus concentration as estimated by DAS-ELISA and ISEM (H = high, M = medium, L = low).

Family, species and cultivar	Symptoms	Virus concentration
Chenopodiaceae		
Beta vulgaris L. (sugarbeet)	chlorosis	H
Leguminosae		
Cicer arietinum L. (chickpea)	stunting, leaf rolling reddening, yellowing phloem browning small leaves	M
Lens esculenta Moench (lentil)	chlorosis, severe stunting	H
Phaseolus vulgaris		
cv. Top crop	stunting, leaf dropping, rapid death	H
cv. Burpy	none	M
Pisum sativum L. cv. Bonneville (pea)	stunting, leaf rolling chlorosis	H
Vicia faba L. cv. Compakta (faba bean)	none	L
Solanaceae		
Datura stramonium L.	chlorosis, leaf rolling, severe stunting	H
Lycopersicon esculentum Mill (tomato)	none	M
Nicotiana benthamiana L.	stunting, chlorosis	H
Nicotiana glutinosa L.	stunting, vein chlorosis	H
Nicotiana tabacum L.		
cv. White Burley	stunting, leaf rolling	H
cv. Samsun NN	small leaves, chlorosis	H

Moench, indicating that the leafhopper deposited eggs on these plants. On none of the other plants tested nymphs appeared although the leafhopper survived well on all of them, including the plant species that were not infected by the virus.

Purification

The virus did not scatter adequate light to visualize the zones following centrifugation in a sucrose gradient. Therefore 1.5 ml fractions were drawn and pellets from each fraction were observed with an electron microscope. The absorption spectrum (200-300 nm) was recorded for samples containing virus particles. Fraction 2 (drawn at a depth of 30-40 mm from the top of the tube) contained the highest virus concentration (Fig. 2). Fraction 3, drawn at 40-50 mm from the top of the tube, contained largely dimers and some trimers and fraction 4 (drawn 50-60 mm from the top of the tube) contained relatively more tri- and tetramers (Fig. 3). Other fractions contained negligible amounts of virus particles. Virus yields of 0.5-0.6 mg/kg tissue were obtained from pea tissue assuming an extinction coefficient of 7.7 (Goodman and Bird, 1978). The A260/A280 ratio was 1.4 and the dimers were 25x15 nm in size.

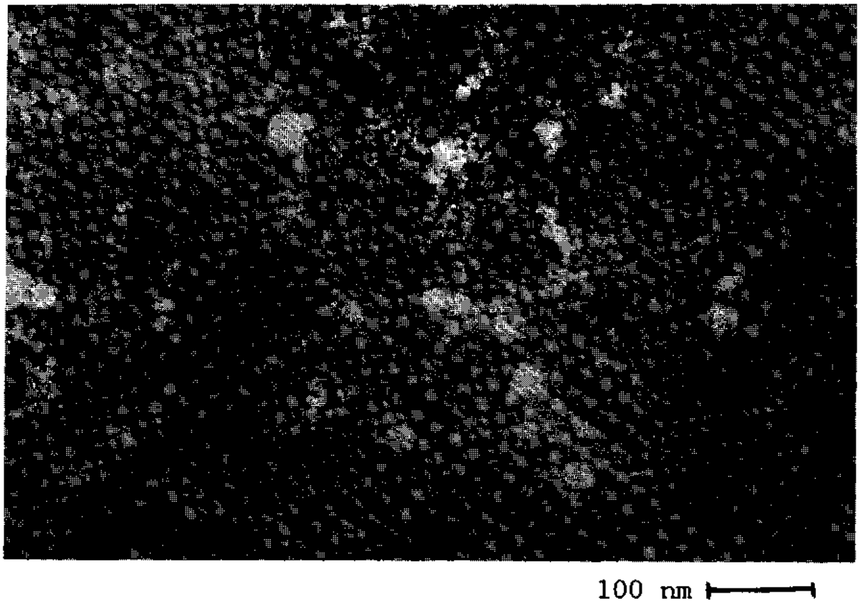
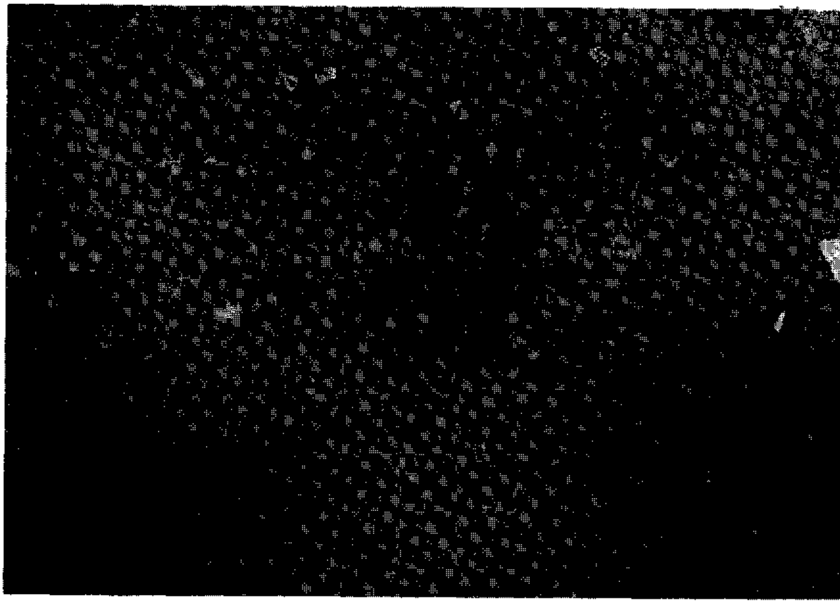


Fig. 2. Electron micrograph of the virus particles present in fraction 2 of the sucrose gradient after purification from pea.



100 nm

Fig. 3. Electron micrograph of the virus particles present in fraction 4 of the sucrose gradient after purification from pea. Note the presence of dimer, trimer and tetramer particles.

Immunosorbent electron microscopy (ISEM)

The homologous antiserum could trap upto 2840 times as many particles as non treated grids (Table 2). Except for the homologous antiserum, none of the antisera tested in ISEM gave a considerable increase in the number of particles trapped. Only bean summer death virus (BSDV) and tobacco yellow dwarf virus (TYDV) antisera gave a slight increase (2-8 times) over the control. This suggests a distant serological relationship with TYDV and BSDV.

ELISA

In DAS-ELISA, using tissue from *Phaseolus vulgaris* 'Top Crop', the virus did not react with African cassava mosaic virus (ACMV) or Indian cassava mosaic virus (ICMV) polyclonal antisera nor with 27 monoclonal antibodies produced against ACMV and ICMV. In DAC-ELISA, using the chickpea virus purified from pea, the virus reacted strongly with antisera to BSDV and weakly with antisera to beet curly top virus (BCTV) and TYDV (Table 3).

Table 2. Trapping of the chickpea geminivirus particles in immunosorbent electron microscopy by homologous and heterologous antisera (a = whitefly-transmitted geminivirus, b = leafhopper-transmitted geminivirus, c = nepovirus), as found in three seperate experiments. The increase factor shows the increase in particle numbers on antiserum-coated grids as compared with the uncoated control. Sources of antisera: African cassava mosaic virus, B.D. Harrison; beet curly top virus, D.L. Mumford; chloris striate mosaic virus, R.I.B. Francki; maize streak virus, K.R. Bock; raspberry ringspot virus, SCRI; squash leaf curl virus, J.E. Duffus; tobacco yellow dwarf virus, J.E. Thomas; bean summer death virus, J.E. Thomas; wheat dwarf virus, K. Lindsten.

	Number of dimers per 1000 μm^2			
Virus antiserum	Expt 1	Expt 2	Expt 3	Increase factor
Homologous	21336	34111	6090	130-2840
African cassava mosaic [a]	13	-	-	0.37
Beet curly top [b]	27	-	-	0.77
Chloris striate mosaic [b]	-	0	-	-
Maize streak [b]	-	13	-	1.08
Raspberry ringspot [c]	0	37	-	-
Squash leaf curl [a]	0	-	-	-
Tobacco yellow dwarf [b]	289	51	92	2-8
Bean summer death [b]	-	-	118	2.5
Wheat dwarf [b]	-	0	-	-
Control (= no serum)	35	12	48	-

SDS-PAGE

A single protein band of 32 kD (average of 4 determinations) was detected from purified virus preparations (Fig. 4).

Nucleic acid characterization

A single nucleic acid band of 2900 nucleotides (average of 4 determinations) was observed from purified virus samples (Fig. 5). It was digested by DNase I and nuclease S1 but not by RNase A, (Fig. 5). Circular nucleic-acid strands was observed by electron microscopy (Fig. 6).

Table 3. The serological relationship of the chickpea geminivirus with BCTV, BSDV and TYDV as tested by DAC-ELISA using chickpea geminivirus purified from pea, homologous antiserum (1/1000), three heterologous antisera (BCTV, BSDV, TYDV, all 1/500) and penicillinase as enzyme. Absorption at 620 nm subtracted from the values for comparable healthy plants. Readings were taken 90 min after addition of substrate. The figures given are means of three replicates.

Amount of virus per well	homologous antiserum	Antisera to BCTV	Antisera to BSDV	Antisera to TYDV
1 µg	1.830	0.658	1.480	0.479
100 ng	0.752	0.010	0.770	0.033
10 ng	0.106	0.021	0.029	0.025

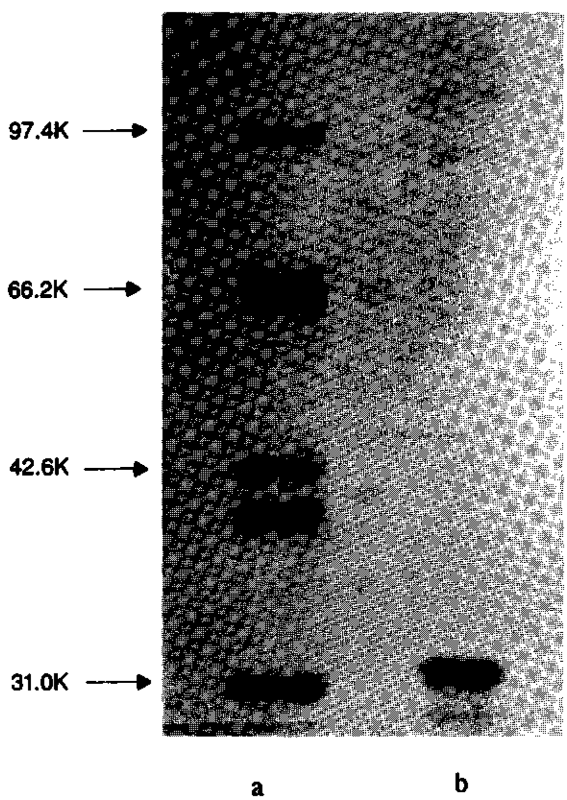


Fig. 4. The results of SDS-PAGE of (a) protein markers (97.4; 66.2; 42.6 and 31 kD) and (b) the virus coat protein. The 42.6 kD protein forms two bands, the lower one being a degradation product. The coat protein is approximately 32 kD.

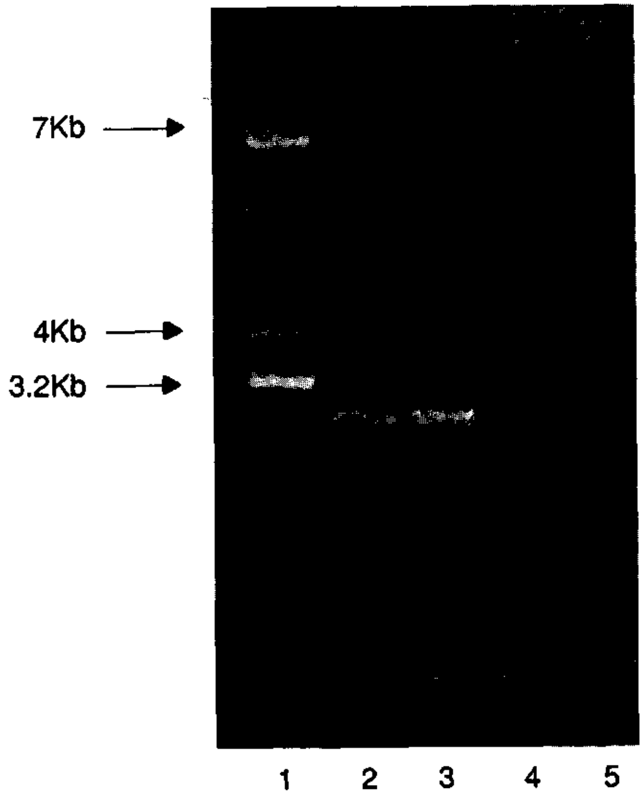


Fig. 5. The results of an agarose gel for the characterization and the determination of the size of the viral nucleic acid. (1) Three single-stranded, circular DNA markers (7,000, 4,000 and 3,200 bases), (2) the virus nucleic acid, untreated, (3) treated with RNase A, (4) treated with DNase I and (5) treated with Nuclease S1.

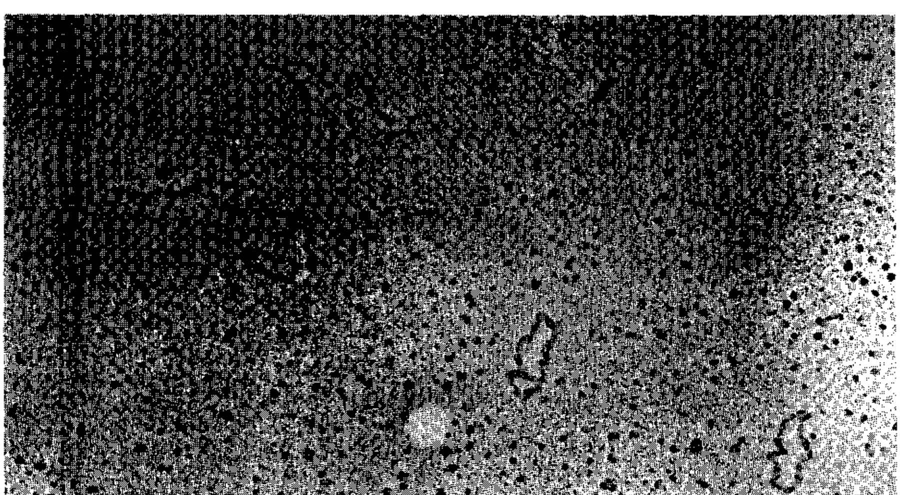


Fig. 6. Electron micrograph of the circular nucleic acid of the chickpea geminivirus.

Discussion

On the basis of the structure of its particles, the virus was recognized as a geminivirus. The presence of circular, single-stranded DNA further reinforced this. Only after many efforts, using different insect species, the vector of the virus was found to be the leafhopper *Orosius orientalis*. The virus possesses a single coat protein subunit with a molecular weight of 32,000 dalton and circular ssDNA with a molecular size of 2900 bases, both within the range of the geminiviruses (Harrison, 1985).

Serological relationships were tested with a range of geminivirus antisera. It is serologically unrelated to ACMV and SLCV. These two viruses were included in the test because the majority of the whitefly-transmitted geminiviruses are serologically related to them (Roberts *et al.*, 1984). The chickpea virus is also unrelated to MSV, CSMV, and WDV, the three leafhopper-transmitted geminiviruses infecting monocotyledonous plant species. In ISEM no relation was found with BCTV but a possibly distant relationship was found with BSDV and TYDV. In DAC-ELISA, however, the chickpea virus reacted strongly with BSDV antiserum and weakly with BCTV and TYDV antisera. The differences found in the serological reactions of the virus in ISEM and DAC-ELISA might be explained by the alkaline conditions (pH 9.8) used for coating the virus in DAC-ELISA. Under those alkaline conditions other epitopes might be exposed than is the case in ISEM. The geminiviruses tested might have some internal epitopes in common while they have no or hardly any external epitopes in common. Moreover, the DNA of the chickpea geminivirus did not hybridize at all with BCTV DNA (J. Stanley, U.K., pers. comm., 1991). These data show that the chickpea geminivirus is a distinct member of the leafhopper-transmitted geminiviruses. The chickpea virus is transmitted by the leafhopper *Orosius orientalis*. Therefore it apparently belongs to the sub-group 2 (Harrison, 1985) including BCTV, BSDV, TYDV. The chickpea virus produces the characteristic symptoms of stunting, yellowing, leaf curling and distortion, as all the members in this subgroup.

As is clear from Table 3 the chickpea geminivirus reacts strongly with BSDV antiserum and weakly with TYDV antiserum despite the fact that the BSDV virus antiserum has a lower titer (1/32) than the TYDV antiserum (1/128). These two viruses are considered to be strains of the same virus (Thomas and Bowyer, 1980). This finding may suggest that BSDV is closer related to the chickpea geminivirus than TYDV and might even imply that BSDV and TYDV are more distantly related than they were originally thought to be (Thomas and Bowyer, 1980).

The chickpea virus could infect species in the Leguminosae, the Solanaceae and the Chenopodiaceae (Table 3). Symptoms produced on different hosts by the chickpea virus differ from those produced by BCTV (Bennet, 1971; Thomas and Mink, 1979), TYDV (Thomas and Bowyer, 1984; Hill, 1950) and BSDV (Bowyer and Atherton, 1971) (Table 4). The chickpea geminivirus causes only mild or no symptoms on sugarbeet and tomato, respectively, while BCTV, BSDV and TYDV cause clear symptoms on these hosts. Moreover, the virus from chickpea does not infect *Nicotiana rustica* and *Cucumis sativus* while the other viruses do (Table 4). On the other hand, the chickpea virus causes more severe symptoms on *Datura stramonium* and *Nicotiana tabacum*. These differences in host range support our conclusion that the chickpea geminivirus is a distinct virus.

Moreover, the coat protein of TYDV (27.5 kD, Thomas and Bowyer, 1980) is considerably smaller than that of the chickpea virus (32 kD). On the basis of host range and serological relationships the chickpea virus is considered to be a distinct leafhopper-transmitted geminivirus. Although it appears to be strongly serologically related to BSDV in DAC-ELISA, no clear relationship was found with this virus in ISEM. Moreover, the symptoms caused by these two viruses are considerably different and BSDV is unstable (Thomas and Bowyer, 1980) while the chickpea virus is very stable. On the basis of the characteristic symptoms produced by this virus in chickpea, the virus was named chickpea chlorotic dwarf virus (CCDV). CCDV seems to be restricted to the cool-season legumes: pea, chickpea, faba bean and lentil (Table 1). None of the tropical legumes were infected. Since *O. orientalis* breeds on faba bean and lentil, the occurrence of CCDV in these two crops could be important and should be further looked into.

Interestingly, the symptoms caused by CCDV are similar to those produced by the

Table 4. Comparison of the host range and other characteristics of the chickpea geminivirus, BCTV (Bennet, 1971; Thomas and Mink, 1979), TYDV (Hill, 1949; Thomas and Bowyer, 1984) and BSDV (Bowyer and Atherton, 1971), na = not available.

	Chickpea virus	BCTV	TYDV	BSDV
Beta vulgaris	chlorosis, mild symptoms severe symptoms	chlorosis, leaf rolling,	stunting	stunting, reddening
Cucumis sativus	not infected dwarfing, leaf rolling	seedlings killed,	na	na
Lycopersicon esculentum	symptomless infection plant dies	leaf twisting, leaf rolling,	infected, symptoms na curling	chlorosis, downward leaflets
Nicotiana tabacum	leaf dwarfing, leaf rolling, plant stunting, no recovery	leaf dwarfing, leaf rolling, recovery	leaf rolling, plant stunting	na
Datura stramonium	chlorosis, leaf rolling, severe plant stunting, plant dies	infected, symptoms na	chlorosis, leaf rolling, slight plant stunting	chlorosis, leaf rolling, slight plant stunting
Nicotiana rustica	not infected	infected, symptoms na	symptomless infection	infected, symptoms na
Coat protein	32 kD	32 kD + 36 kD	27.5 kD	na
Vector	*Orosius orientalis*	*Circulifer tenellus*	*Orosius argentatus*	*Orosius argentatus*

luteovirus causing the chickpea stunt disease (Nene *et al.*, 1991; Nene and Reddy, 1987). In our surveys CCDV was found to be widely distributed in India (Chapter 3). We are currently assessing its economic importance in chickpea.

Acknowledgements

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7. Virus-vector relationships of chickpea chlorotic dwarf geminivirus and the leafhopper *Orosius orientalis* (Hemiptera: Cicadellidae)[1]

N.M. HORN, S.V. REDDY and D.V.R. REDDY

International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, Andhra Pradesh 502 324, India

Summary

Chickpea chlorotic dwarf geminivirus (CCDV) is one of the viruses associated with chickpea stunt disease. It is transmitted by the leafhopper *Orosius orientalis*. The minimum acquisition access period (AAP_{min}) and inoculation access period (IAP_{min}) were found to be less than 2 min, while the minimum latency period (LP_{min}) was less than 2 h. The median AAP, IAP and LP were 8.0 h, 2.3 h and 27.7 h, respectively. No difference in transmission rates (proportion of leafhoppers able to transmit) was observed between male and female leafhoppers. In serial transmission experiments, transmission was shown to be persistent, and after a 2-day AAP about 80% of the leafhoppers transmitted the virus for most of their life. The virus could be detected in individual leafhoppers by DAS-ELISA. It did not multiply in the leafhopper, but, instead, decreased in concentration during leafhopper feeding on a non-host of the virus.

Introduction

Chickpea chlorotic dwarf geminivirus (CCDV) causes symptoms in chickpea plants which include stunting, phloem browning in the collar region, and, in the case of desi types, leaf reddening (Horn *et al.*, 1993; Chapter 6). CCDV is widely distributed in several chickpea-growing areas in India and Pakistan (Chapter 3). The symptoms are

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very similar, if not identical, to those associated with chickpea stunt disease, in India previously thought to be caused by bean leafroll luteovirus (BLRV) (Nene *et al.*, 1991). Elsewhere, similar symptoms in chickpea have been associated with other luteoviruses (Bosque-Perez and Buddenhagen, 1990; Duffus, 1979). Thus, it seemed likely that aphids, as the vectors of luteoviruses, would be the only type of vectors involved in spreading chickpea stunt. The discovery that CCDV is also associated with chickpea stunt, and of its transmissibility by the leafhopper *Orosius orientalis* (Matsumura), is therefore new information on the ecology of the disease (Horn *et al.*, 1993; Chapter 6).

O. orientalis has been found on 12 plant species, including chickpea, in North India throughout the year (Bindra and Singh, 1970) and is considered to be economically important on sesame throughout India (Choudhary *et al.*, 1986). The leafhopper has also been reported from Turkey (Lodos and Kalhadelen, 1985) and Egypt (Habib *et al.*, 1976; El-Nahal *et al.*, 1989). *O. orientalis*, earlier described as *Orosius albicinctus* (Ghauri, 1966), is also known to be the vector of five mycoplasma diseases in India, viz. aster phyllody (Rangaswamy *et al.*, 1988), groundnut witches' broom (Yang and Wu, 1990), potato purple top (Singh *et al.*, 1983), phyllody of *Sesamum* (Vasudeva and Sahambi, 1955), and sweet potato witches' broom (Yang and Chou, 1982). CCDV is the first virus reported to be transmitted by *O. orientalis*, in the Indian subcontinent (Horn *et al.*, 1993; Chapter 6).

Information on the relationships between geminiviruses and their leafhopper vectors is very limited. Storey (1928) investigated the relationships between maize streak geminivirus (MSV) and its vector *Balclutha mbila* (= *Cicadulina mbila*), and Severin (1931), Freitag (1936) and Bennett and Wallace (1938) studied the transmission of beet curly top geminivirus (BCTV) by its vector *Eutettix tenellus* (= *Circulifer tenellus*). MSV and BCTV were shown to be transmitted in a persistent manner, and Freitag (1936) presented indirect evidence that BCTV does not multiply in its vector.

We have now examined the qualitative and quantitative characteristics of CCDV transmission by its leafhopper vector *O. orientalis* to better understand the epidemiology of the virus.

Materials and Methods

Virus isolate, insects, and test plants

The virus isolate used was described by Horn *et al.* (1993; Chapter 6). It was maintained in pea, *Pisum sativum* (cv. Bonneville). Leafhoppers were given an acquisition access period (AAP) of 3 days on infected pea plants (between 10 to 20 days after inoculation), followed by a 3-day inoculation access period (IAP) on healthy pea plants for virus propagation and maintenance. The culture of the leafhopper *O. orientalis* and its maintenance on *Crotalaria juncea* (sunnhemp), and *Sesamum indicum* (sesame) were also described by Horn *et al.* (1993; Chapter 6).

The adult leafhoppers, used for the transmission studies, were from two different cultures. The original culture had a transmission rate (proportion of leafhoppers able to transmit) of 38%. The second culture used was the progeny of a single male and female from the original culture and had a transmission rate of 85%. For the determination of median and minimum transmission values, leafhoppers from the original culture were used. For all other experiments leafhoppers from the culture with the high transmission rate were used. The experiments were carried out in a glasshouse at temperatures between 25 and 32°C. CCDV-infected pea plants (cv. Bonneville) were used for virus acquisition, and healthy pea plants for inoculation in all transmission tests. Inoculated test plants were scored for external symptoms and tested by ELISA, usually 14-20 days after the start of IAP.

Determination of minimum acquisition access period (AAP_{min}), minimum inoculation access period (IAP_{min}), and minimum latency period (LP_{min})

To determine the AAP_{min}, groups of 50 leafhoppers were given varying AAPs on three infected pea plants followed by an IAP of 4 days on three healthy pea plants. To assess the IAP_{min}, leafhoppers were given an AAP of 4 days and then starved for 2 h prior to transferring them in groups of 50 leafhoppers to three healthy pea plants for varying IAPs. The LP is defined as the time between the start of the AAP and the end of the first IAP in which the insects were able to transmit the virus. To determine the

LP_{min}, a group of 100 leafhoppers was given an AAP of 1 h, and transferred serially to pea seedlings at 1-h intervals.

Determination of AAP_{50}, IAP_{50}, and LP_{50}

To determine AAP_{50}, leafhoppers were given an AAP of 1, 3, 8, 24, or 48 h. After each AAP, 50 leafhoppers were transferred individually to healthy pea seedlings for an IAP of 3 days. The leafhoppers were recovered after 3 days and their sex determined. To determine IAP_{50}, leafhoppers were given a 4-day AAP. They were then starved for 1 h, and groups of 50 leafhoppers were given an IAP of 0.5, 1, 3, 8, or 24 h. They were confined individually to healthy pea seedlings for the duration indicated. The insects were recovered after the IAP and their sex was determined.

To determine the LP_{50}, leafhoppers were given a 14-h AAP, followed by five successive IAPs. During the IAPs, individual leafhoppers were kept on pea seedlings, a new seedling for each IAP. The first four IAPs were 8 h and the last one 42 h, to determine the maximum transmission.

For calculation of the median values of AAP, IAP and LP, the method described by Sylvester (1965) was used. The time was converted to $\log_{10}$ (time). The transmission percentages were transformed by putting the transmission percentage for the longest period tested at 100%. This permitted compensation for the exposed leafhoppers which were not able to transmit the virus. The converted percentages were transformed to probits, and then a linear regression of the probit value against $\log_{10}$ (time) was carried out.

Test for non-persistent transmission and transmission by nymphs through moulting

Two groups of 50 leafhoppers were given a short AAP immediately followed by a short IAP on three healthy pea plants per group, to check for possible non-persistent transmission. To test whether nymphs of *O. orientalis* can transmit the virus, and if it persists through moulting, 15 nymphs were given a 2-day AAP, and then transferred serially at daily intervals to healthy pea seedlings. The dates on which the nymphs moulted were recorded.

Serial transmission

Leafhoppers were given a 2-day AAP and then transferred individually to healthy pea seedlings at 1-day intervals, except on Sunday, to study their ability to serially transmit the virus to healthy pea plants until death.

Virus detection by ELISA

DAS-ELISA (Clark and Adams, 1977) was used for the detection of the virus in pea plants. Plates were coated with CCDV IgG (2 µg/ml for detection in plants, and 1 µg/ml for detection in insects) in carbonate buffer (pH 9.8). The antigen was extracted using phosphate-buffered saline (pH 7.2), containing 0.05% Tween 20 and 2% poly-vinylpyrrolidone (= extraction buffer). For antigen extraction from insects, Nonidet P40 (NP40) was added to the buffer at 2ml/l. Extracts from single leafhoppers were prepared in 200 µl buffer, clarified at 8000g for 10 min, and 100 µl of the supernatant was added to ELISA plates and incubated overnight at 4°C. The alkaline phosphatase conjugate was used at 2µg/ml for detection in plants and at 0.5 µg/ml for detection in insects. For virus

Table 1. Number of plants infected with CCDV out of three pea plants inoculated with 50 leafhoppers after varying AAPs and IAPs.

		Number of plants infected			
AAP	IAP	Exp. 1	Exp. 2	Exp. 3	Exp. 4
2 min	4 days	3	3		
5 min	4 days	3	3		
10 min	4 days	3	3		
30 min	4 days	3	3		
24 h	4 days	3	3		
4 days	2 min			1	0
4 days	5 min			2	0
4 days	10 min			1	0
4 days	60 min			3	3
4 days	48 h			3	3

detection in plants, p-nitrophenyl phosphate (0.2 mg/ml) was used as a substrate, and absorbance readings were taken at 405 nm with a Titertek Multiskan. For detection in insects, a more sensitive enzyme-amplification procedure was used (Van den Heuvel and Peters, 1989) and absorption values were recorded at 492 nm.

Quantitative virus assay in leafhoppers

One hundred leafhoppers were given a 3-day AAP on infected pea plants, and then transferred to groundnut, *Arachis hypogaea*, a non-host of CCDV (Horn *et al.*, 1993; Chapter 6) on which the leafhoppers survive. Individual leafhoppers were tested by ELISA immediately after the 3-day AAP, and other individual leafhoppers were tested 9 days after transfer to groundnut. Leafhoppers, which were allowed to feed for 3 days on virus-free pea plants, were treated in a similar manner and served as controls.

Results

AAP_{min}, IAP_{min}, and LP_{min}

The results of the transmission experiments are summarized in Tables 1 and 2. An AAP of 2 min, an IAP of 2 min, and an LP of 2 h still resulted in transmission of the virus. Therefore the AAP_{min}, IAP_{min}, and LP_{min} are likely to be shorter than 2 min, 2 min, and 2 h, respectively. Even an AAP of 2 min resulted in good acquisition of the virus by its vector. For efficient inoculation, more time appears to be required, since the number of plants infected decreased when the IAP was shorter than 1 h (Table 1). The transmission efficiency decreases when the LP is less than 7 h (Table 2).

AAP_{50}, IAP_{50}, and LP_{50}

The values determined in nine independent experiments are summarized in Table 3. As an example, the linear regression of the transmission percentages transformed to probits is given in Fig. 1, for one of the AAP_{50} experiments. The average values for AAP_{50} , IAP_{50}, and LP_{50} were 8.0 h, 2.3 h, and 27.7 h, respectively.

Table 2. Number of plants infected with CCDV out of three pea plants inoculated with 100 leafhoppers after a 1-h AAP and successive IAPs.

	Number of plants infected	
IAP	Exp. 1	Exp. 2
1-2 h[a]	0	2
2-3 h	0	2
3-4 h	2	2
4-5 h	2	3
5-6 h	1	2
6-7 h	0	2
7-24 h	3	3
24-72 h	3	3

[a] Time interval after start of AAP

Test for non-persistent transmission and transmission by nymphs through moulting

None of the two groups of 50 leafhoppers transmitted the virus after a 10-min AAP followed by a 10-min IAP without a LP. Thirteen of the 15 nymphs tested transmitted the virus as nymphs and did so for 2 to 3 days. Then they moulted and continued to transmit the virus as adults. Thus, they retained their transmission ability through moulting.

Transmission rates of male and female leafhoppers

The sex of 795 individual leafhoppers used in the experiments described above was determined: 39% were males and 61% were females. Of the 145 insects that transmitted the virus, 41% were males and 59% were females. Therefore males and females appear to have the same rate of virus transmission.

Table 3. Calculated AAP_{50}, IAP_{50} and LP_{50} (all expressed in hours) for the O. orientalis - CCDV relationship.

	Rep[a]. 1	Rep. 2	Rep. 3	Average (± S.E.)	
AAP_{50}	7.3	8.4	8.2	8.0	(0.4)
IAP_{50}	1.7	0.7	4.7	2.3	(1.2)
LP_{50}	26.3	31.4	25.4	27.7	(1.9)

[a] Replicate

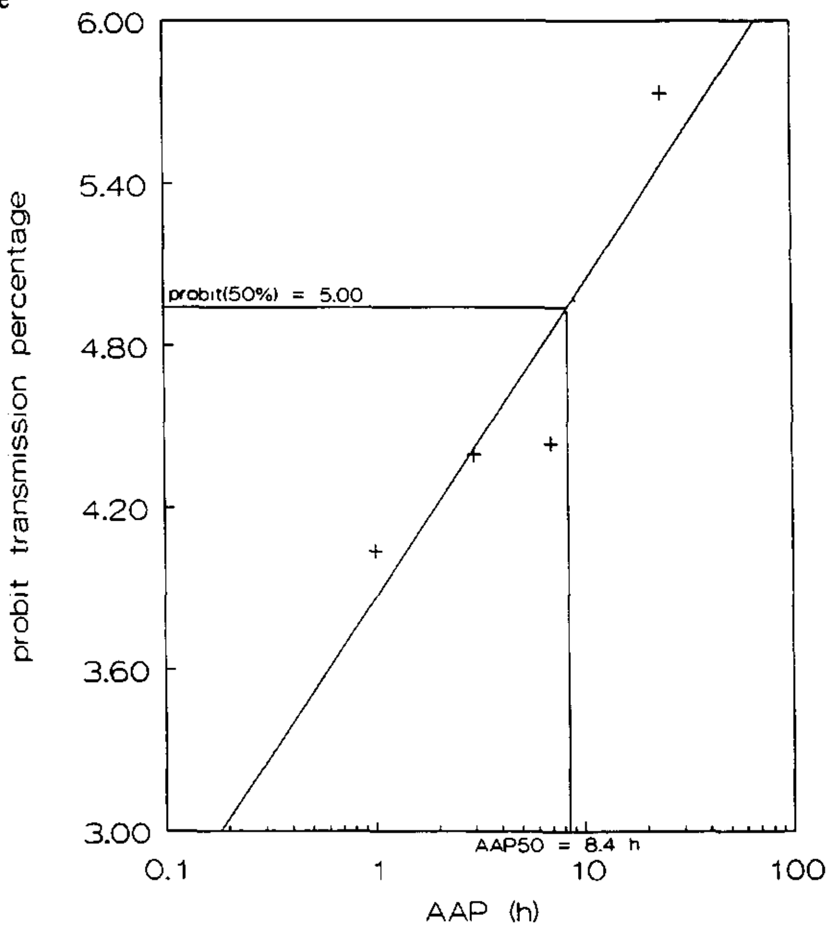

Fig. 1. Example of linear regression of the transmission percentages after probit transformation to determine the median acquisition access period (AAP_{50}) of CCDV transmission by O. orientalis. In this instance the estimated AAP_{50} was 8.4 h (repl. 2).

Serial transmission

The leafhoppers used in this experiment lived for 2 to 23 days after the AAP, although most survived 17 - 20 days. Most of the 60 leafhoppers tested, transmitted the virus until their death or till a few days before they died (e.g. LH54, Table 4). Some ofthem had a few interspersed failures in transmission (e.g. LH37, LH48, LH51, Table 4). A few leafhoppers stopped transmitting the virus long before they died (e.g. LH11, Table 4). Very few leafhoppers failed to transmit because a colony with a high transmission rate (85%) was used.

Quantitative virus assay in leafhoppers

The virus titre of individual insects, determined by ELISA, varied widely. The average absorbance values ($\pm$ standard errors) of the leafhoppers fed on infected plants (exposed leafhoppers) was 0.252 ($\pm$ 0.018) immediately after the 3-day AAP and 0.056 ($\pm$ 0.040) 9 days later (12 days after start of the AAP). For the leafhoppers fed on virus-free plants (unexposed leafhoppers) these values were 0.047 ($\pm$ 0.010) and 0.022

Table 4. Range of variation in serial transmission of CCDV by seven leafhoppers selected from 60 tested after daily transfers to pea test plants.

	1[a]	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
LH25[b]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-[d]							
LH11	+[c]	+	+	-	-	-	-	-	-	-	-	-[d]											
LH18	+	+	+	+	+	+	+	+	+	+	+	-	-	-[d]									
LH37	+	+	+	+	+	+	+	+	+	+	-	+	+	-	-	+	+	+	-	-	-	-	-[d]
LH48	+	+	+	+	+	+	+	+	+	+	-	+	+	-	-	-	-[d]						
LH51	+	+	+	-	+	+	+	+	+	+	+	+	+	+	-	+	+[d]						
LH54	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+[d]			

[a] Serial number of daily transfer to pea test plants after initial 2-day AAP
[b] Code number of leafhopper
[c] + = pea plant infected
- = pea plant not infected
[d] Death of leafhopper

(± 0.018), for 3 and 12 days, respectively. These results are shown in Fig. 2. There were substantial differences in OD values of exposed and unexposed leafhoppers immediately after the 3-day AAP. However, 9 days later many of the exposed leafhoppers gave ELISA readings similar to those of the unexposed leafhoppers and only a few of the exposed leafhoppers still gave values that were substantially higher than comparable controls.

Discussion

The minimum values for AAP, IAP and LP found in this study represent extremes, whereas median values can be quantified better, and are ecologically more important, than minimum values. Although *O. orientalis* can acquire CCDV and inoculate the virus

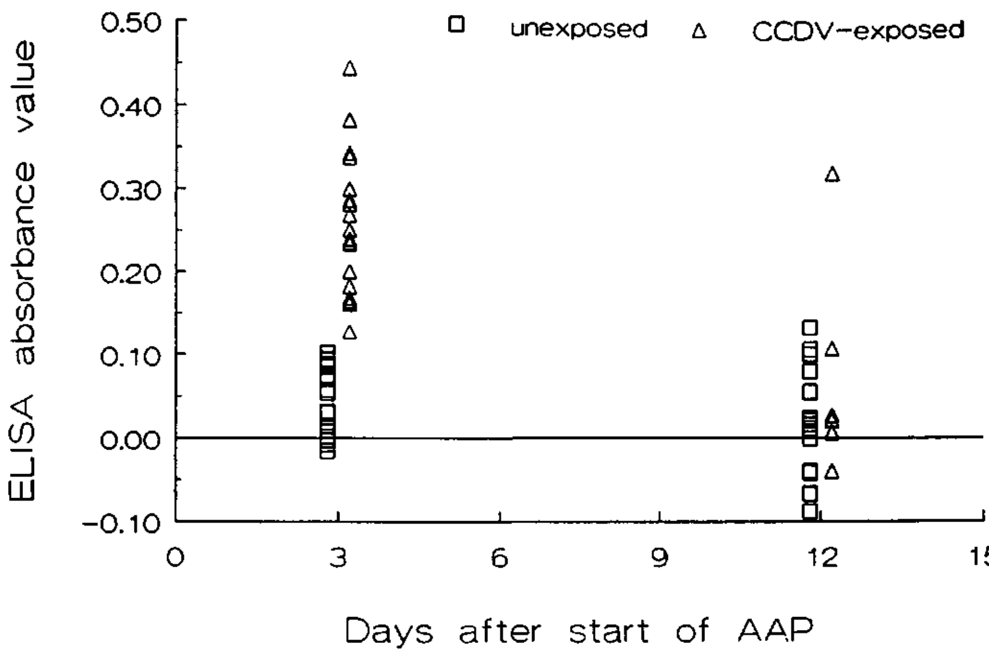

Fig. 2. ELISA absorbance values of unexposed and CCDV-exposed leafhoppers immediately after a 3-day AAP and after another 9 days on a non-host of CCDV. Every square or triangle represents the absorbance value for one leafhopper. Absorbance values measured at 492 nm, 30 min after addition of the final substrate; the average value of six buffer controls was subtracted.

into a plant in short access periods, longer periods are needed for efficient transmission. Nevertheless, *O. orientalis* can be considered an efficient transmitter of CCDV, as especially shown in serial transmission tests by the persistence of the virus in the vector. In similar experiments with MSV there were many more interspersed failures during a period of transmission (Storey, 1928).

In studies on transmission of other geminiviruses, minimum values are generally given (Table 5). The minimum values now reported for CCDV agree with those given for beet curly top geminivirus (BCTV) (Severin, 1921, 1931; Bennett and Wallace, 1938). BCTV and CCDV can both be acquired and inoculated very quickly. This would indicate that either the leafhoppers can reach the phloem rapidly, or CCDV and BCTV are not phloem limited.

Secondary spread within a crop can occur readily when the vector requires a relatively short time for acquisition and inoculation and the virus has a short latency period. Secondary spread has not been widely studied for leafhopper-transmitted pathogens (Chiykowski, 1981), but it is now known that both BCTV and CCDV can be acquired and inoculated in very short periods of feeding. This indicates the potential of CCDV to reach high infection levels in crops.

CCDV is retained by *O. orientalis* for up to 21 days (Table 4). Moreover, *O. orientalis* does not lose the virus through moulting. These results and the non-transmission of CCDV in a 10-min AAP immediately followed by a 10-min IAP suggests that CCDV is transmitted by *O. orientalis* persistently rather than non-persistently.

Loss of ability to transmit CCDV 10 or more days after acquisition (Table 4) provides indirect evidence that CCDV does not multiply in its vector. Furthermore, the reduced virus concentration on the 9th day after the AAP as compared to immediately after a 3-day AAP, suggests that the virus does not multiply in *O. orientalis* (Fig. 2). Indeed, the majority of leafhoppers from CCDV-infected plants gave absorbance values similar to those of leafhoppers from virus-free plants 9 days after the AAP. The serial-transmission experiment (Table 4) showed that the majority of leafhoppers still transmitted the virus 9 days after AAP. The amount of virus present in leafhoppers 9 days

Table 5. Minimum values of AAP, IAP and LP for the leafhopper transmission of geminiviruses as reported in this and other studies.

Virus[a]	Vector	AAP_{min}	IAP_{min}	LP_{min}	Reference
MSV	*C. mbila*	1 h	nd[b]	12 h	Storey, 1928
		15 sec	5 min	nd	Goodman, 1981
BCTV	*C. tenellus*	2 min	10 min	4 h	Severin 1921, 1931
		1 min	1 min	5 h	Bennett, 1938
BSDV	*O. argentatus*	nd	nd	48 h	Bowyer and Atherton, 1971
CCDV	*O. orientalis*	2 min	2 min	2 h	This publication

[a] MSV = maize streak virus, BCTV = beet curly top virus, BSDV = bean summer death virus, CCDV = chickpea chlorotic dwarf virus.
[b] not determined.

after the AAP must therefore have been below the detection level. Loss of virus during feeding on a non-host of the virus is compatible with non-propagative, merely circulative transmission. BCTV was shown indirectly to be transmitted non-propagatively by *E. tenellus* and the proportion of insects transmitting the virus gradually decreased when the vector was confined to a non-host of the virus (Freitag, 1936). Bennet and Wallace (1938), when indirectly assaying *E. tenellus* for BCTV, also found that the virus content in the insects decreased with increasing time on maize, when transferred to maize after feeding on infected beet. The non-propagative character of MSV transmission was shown in infectivity tests and ELISA by Reynaud and Peterschmitt (1992), who also reported that the concentration of the virus decreased in most of the insects, although a few did retain high virus concentrations.

This is the first report of median values for a leafhopper-transmitted virus. The median values found for CCDV transmission are in the same range as those of the persistent transmission of potato leafroll virus (PLRV) by *Myzus persicae* (AAP_{50} 12 h, IAP_{50} 45-105 min, LP_{50} 24-124 h; Peters, 1986; Van den Heuvel *et al.*, 1991). The median IAP values for transmission of both PLRV and CCDV varied widely.

Female *O. orientalis* are not more efficient transmitters of CCDV than males in contrast to the early results concerning MSV and its vector *Cicadulina mbila* (Storey 1928, 1932). Our results with CCDV are in agreement with those of Freitag (1936) for BCTV, who found that males and females of *E. tenellus* were equally efficient transmitters.

The above facts lead to the conclusion that CCDV is transmitted by the leafhopper *O. orientalis* in a persistent, non-propagative and circulative manner. This leafhopper is an efficient vector of CCDV, it can transmit the virus even after short feeding periods and the virus persists even up to 21 days after acquisition. The transmission characteristics of CCDV by its vector resemble more closely those of BCTV, another member of the same sub-group of geminiviruses, than those of MSV, a member of another sub-group of geminiviruses.

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8. Assessment of yield losses caused by chickpea chlorotic dwarf geminivirus in chickpea (*Cicer arietinum*) in India[1]

N.M. HORN, S.V. REDDY and D.V.R. REDDY

International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, Andhra Pradesh 502 324, India

Summary

Yield losses caused by chickpea chlorotic dwarf virus in chickpea were estimated by comparing uninfected and infected plants in the field at two locations in India. When infected before flowering, yield losses of individual plants amounted to nearly 100% in the three cultivars studied. Plants that became infected during flowering had yield losses of 75-100%. Percentage of crop loss is likely to equal percentage of disease incidence, since plant densities in farmers' fields are probably too low to allow uninfected plants around infected ones to compensate the yield losses of infected plants.

Chickpea chlorotic dwarf virus (CCDV) is a newly-described, leafhopper-transmitted geminivirus infecting chickpea, *Cicer arietinum* (Horn *et al.*, 1993; Chapter 6). Surveys conducted during the 1991/1992 season revealed that CCDV is widely distributed in India, and that it was the most important chickpea virus in Rajasthan (India) and in Pakistan (Chapter 3). It is one of the viruses causing symptoms similar to those described for chickpea stunt by Nene *et al.* (1991). They include plant stunting, internode shortening, phloem browning in the collar region, and leaf reddening in desi-type, and yellowing in kabuli-type chickpeas (Horn *et al.*, 1993; Chapter 6). Other viruses associated with chickpea stunt symptoms in the literature are bean leafroll luteovirus (synonym for pea leafroll virus) in Iran and India (BLRV; Kaiser, 1972;

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Reddy *et al.*, 1979), beet western yellows luteovirus, legume yellows luteovirus (probably a strain of BLRV), and subterranean clover red leaf luteovirus in California, USA (Bosque-Perez and Buddenhagen, 1990). An additional luteovirus, tentatively called chickpea luteovirus (CpLV), found in India and Pakistan (Chapter 3), and in Syria and Turkey (Chapter 4), is yet to be characterized. Since these viruses cannot be distinguished by the symptoms they cause on chickpea, serological techniques, such as ELISA, are necessary to identify them.

Chickpea plants that become infected with CCDV at an early stage of development normally do not produce any pods. The above-described symptoms are followed by rapid plant decline, and very few early infected plants survive. Kaiser and Danesh (1971) reported that in Iran BLRV caused 90-100% yield loss in chickpea when plants were aphid-inoculated. In chickpea naturally infected with chickpea stunt in India, Kotasthane and Gupta (1978) reported 80-95% yield reduction.

This paper reports on the yield losses caused by CCDV in chickpea under natural conditions as determined by comparing the yield of infected plants with those of uninfected neighbouring plants during the 1991/1992 season.

Two chickpea genotypes, viz. 'ICCV 10' (desi) and 'ICCV 2' (kabuli), were tested at ICRISAT Center (Patancheru, Andhra Pradesh, South India, 18° N), and one, viz. 'WR 315' (desi), at Hisar (Haryana, North India, 29° N). In each experiment, plants with stunt-like symptoms were selected, numbered and tagged on two different dates. From each tagged plant three leaves were collected and tested in DAS-ELISA with CCDV antiserum, as described by Clark and Adams (1977). ELISA plates were incubated with CCDV-IgG (2 µg/ml) for 2h at 37°C and washed. Triturates from the samples in buffer (10 ml/ g tissue) were added to the wells and incubated for 2 h at 37°C. After washing, the plates were incubated with CCDV-IgG alkaline phosphatase conjugate (1 µg/ml) for 1 h at 37°C. After another washing, the substrate p-nitrophenyl phosphate was added. Plants that were found infected with CCDV were used for the yield-loss assessment. When harvesting the tagged, CCDV-infected plants, three healthy-looking neighbouring plants were also harvested (Fig. 1). The yield of individual infected plants was com-

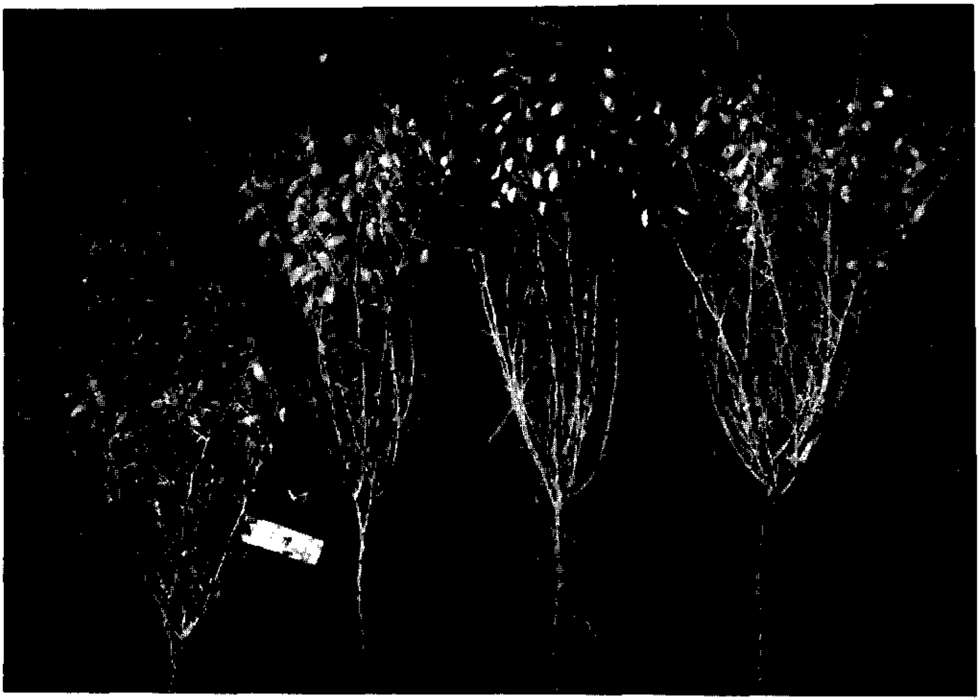

Fig. 1. CCDV-infected chickpea plant (left) and three healthy neighbouring plants, immediately after harvest.

pared with the averageyield of its three apparently healthy neighbouring plants. These differences were then statistically analysed, using a t-test. In each field 50 randomly selected, healthy-looking plants were also harvested individually for measuring their yield.

At Patancheru, 32 plants of 'ICCV 10' were found to be infected during flowering and only 9 plants of 'ICCV 10' in the same field were found to have become infected since the first observation date. In the case of 'ICCV 2', 80 and 39 plants were found to have become infected when the crop was at the flowering and pod-setting stages, respectively. At Hisar, 42 plants of 'WR 315' were found to be infected before flowering and an additional 24 during flowering.

The average yields of diseased and apparently healthy plants, estimated yield losses, and results of the statistical analysis are presented in Table 1. The results show that

CCDV could cause considerable yield losses in chickpea plants, i.e. 75-100%, at both locations, in all three chickpea genotypes, and at both dates of observation. These losses were far beyond the standard error. When symptoms were already present at the flowering stage, yield losses were close to 100%. When they were only present at the pod-filling stage, indicating that the plants had become infected during flowering or later, yield losses were slightly lower, but still considerable (75-90%). The most vulnerable cultivar, WR 315, which suffered 100% yield loss when infected before flowering, is currently widely grown by farmers in North India.

Table 1. Estimation of yield losses to chickpea plants due to CCDV at ICRISAT Center and Hisar.

Genotype	Location	Stage of crop	Number of infected plants	Average yield (grammes/plant)		Yield loss %-age	Standard error %-age
				healthy plants	infected plants		
ICCV 10	ICRISAT	flowering	32	11.9	0.01	99.8	0.2
		pod setting	9	14.6	1.5	90.2	3.4
ICCV 2	ICRISAT	flowering	80	8.6	0.13	98.5	0.3
		pod setting	39	10.9	2.5	75.4	2.8
WR 315	Hisar	preflowering	42	na*	0	100	na
		flowering	24	na	0	100	na

* not applicable

If incidence of diseased plants is low and they are scattered throughout the field, neighbouring plants in dense crops (300,000 plants/ha for chickpea) and at high soil fertility may compensate for declining or dead plants (Bos, 1982). The yields of healthy plants, which were randomly selected in each experiment, were all in the same range as those of healthy plants located near infected plants. This indicates that no significant compensation occurred in these experiments. On this basis we assume that in farmers' fields in North India and Pakistan, where the crops are often raised at medium densities (100,000 - 200,000 plants/ha; Chapter 3), no compensation of losses by diseased plants by enhanced development of healthy neighbouring plants occurs. Since infection often leads to complete loss of yield by the infected plant, under such conditions, percentage of yield loss per field is likely to be equal to percentage of disease incidence. This further emphasizes the potential threat of CCDV to chickpea cultivation.

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9. Screening of chickpea (*Cicer arietinum* L.) and other *Cicer* spp. for resistance to chickpea chlorotic dwarf geminivirus in India

N.M. HORN, M.P. HAWARE, S.V. REDDY and D.V.R. REDDY

International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, Andhra Pradesh 502 324, India

Summary

Chickpea chlorotic dwarf virus (CCDV) was the predominant virus in field trials where chickpea genotypes were screened for resistance to chickpea stunt (Hisar, Haryana, India), indicating that earlier field screening there had mainly been for resistance to the disease caused by CCDV. Thirteen chickpea genotypes, found to be disease resistant in the field, were further tested in the greenhouse along with a vulnerable genotype, 'WR 315', by releasing CCDV-carrying leafhoppers. Although it was possible to obtain infection in all genotypes tested, symptom development was considerably delayed in disease-resistant genotypes as compared to the vulnerable genotype. The virus concentration, as found in ELISA, was initially lower in disease-resistant genotypes than in the vulnerable one, but it reached the same level three weeks after inoculation. Resistance to the disease (disease resistance) thus seems to be a matter of resistance to the virus (virus resistance), i.e. delayed virus multiplication and/or systemic movement. Four wild *Cicer* species, *C. reticulatum*, *C. judaicum*, *C. echinospermum* and *C. cuneatum*, though susceptible to CCDV, expressed symptoms later than *Cicer arietinum* 'WR 315', and symptoms were weaker. Thus, a greenhouse screening approach for resistance to CCDV has been developed and sources of virus resistance were identified.

Introduction

Chickpea chlorotic dwarf virus (CCDV) is a leafhopper-transmitted geminivirus that infects chickpea in India and Pakistan (Horn *et al.*, 1993; Chapter 3 and 6). Symptoms caused by this virus in chickpea are indistinguishable from the symptoms described for chickpea stunt disease (Nene *et al.*, 1991), earlier ascribed to bean leafroll luteovirus (BLRV, Reddy *et al.*, 1979). CCDV and BLRV independently cause plant stunting, internode shortening, leaf chlorosis, phloem discoloration at the collar region, and, in the case of desi-type chickpeas, leaf reddening.

Surveys conducted in North India and in Pakistan showed that CCDV was widely distributed and the predominant virus at Hisar (Chapter 3). In Pakistan, relatively high incidence of CCDV was noticed in exotic germplasm. This virus may rapidly become important in chickpea if such germplasm would be used for plant introduction and breeding, also because plants infected with this virus hardly yield any seed (Chapter 8). Since at the moment the use of genetic resistance seems to be the only promising measure of control, selection of chickpea genotypes resistant to CCDV, or further breeding for resistance to the virus is having high priority.

Screening for resistance to chickpea stunt in India started as early as 1979 at Hisar, Haryana, a "hot spot" for stunt, and over 10,000 chickpea genotypes have since been tested. They were screened in so called 'disease nurseries' in the field, where infection pressure was enhanced by interplanting with alfalfa, a known host of BLRV, and by wide spacing of the rows. Scoring was by visual observation for symptoms. The chickpea genotype 'WR 315' was found in the early years to have a high stunt incidence, and was therefore used as the vulnerable check. Over 100 germplasm entries, showing some resistance to chickpea stunt during these early trials, were further evaluated in the field at Hisar during the 1990/91 and 1991/92 seasons. Diseased plants were then extensively tested by ELISA for CCDV and some luteoviruses to verify for resistance to which viruses the genotypes had actually been tested.

This paper discusses the results of the field trials in the light of the ELISA results on the viruses present. A selected number of genotypes, showing some disease resistance were further screened by inoculation and observation in the greenhouse to better evaluate the degree and type of resistance.

Materials and Methods

Terminology

Virus resistance is used here for resistance to virus multiplication and spread of the virus within the plant, and can only be identified if the virus concentration and multiplication are monitored in plant genotypes. The opposite of virus resistance is susceptibility, where susceptibility is the capacity to contract infection, irrespective symptom development. Disease resistance is the overall result of either resistance to the virus, resistance to the vector, tolerance to virus infection, or a combination of these factors. The antonym of disease resistance is vulnerability. The higher disease resistance, the lower vulnerability, and the reverse. The same holds for virus resistance and susceptibility.

Virus identification and incidence

To check the incidence and relative importance of CCDV in trial fields, plants were visually selected for symptoms, and tested by ELISA with antiserum to CCDV, as described by Horn *et al.* (1993; Chapter 6). Since luteoviruses can produce symptoms similar to those produced by CCDV, all collected plants were also tested with one or more luteovirus antisera (bean leafroll virus antiserum from Dr Bos, The Netherlands; potato leafroll virus antiserum from Mr Maat, The Netherlands; subterranean clover red leaf virus antiserum from Dr Johnstone, New Zealand; and chickpea luteovirus antiserum produced at ICRISAT; Chapter 3).

CCDV isolate and maintenance

The isolate of CCDV used for greenhouse screening, the procedure employed for maintenance in pea, *Pisum sativum* 'Bonneville', and the maintenance of the leafhopper vector *Orosius orientalis* were according to Horn *et al.* (1993; Chapter 6).

Cicer genotypes

Thirteen chickpea genotypes, that had shown less than 10% stunt incidence during the 1989/1990 and 1990/1991 seasons in the trial fields at Hisar while the vulnerable check 'WR 315' had over 30% incidence, were chosen for greenhouse screening. These 13 genotypes and four wild *Cicer* spp. were obtained from the ICRISAT germplasm collection. The chickpea genotype 'WR 315' was used as a vulnerable check in all experiments.

Greenhouse screening

For resistance screening in the greenhouse, plants were inoculated just after emerging in groups of three plants per cage by adding 30 leafhoppers that had completed a 3-day acquisition-access period on infected pea plants (between the 10^{th} and the 20^{th} day after inoculation). Per genotype 12 plants were inoculated and the leafhoppers were removed after a 3-day inoculation-access period. Inoculated plants were observed visually for symptoms, which included reddening of leaf edges, reduction in size of tip leaves, leaf epinasty and leaf chlorosis. All exposed plants, irrespective symptom production, were tested by ELISA four weeks after inoculation. Symptom development was recorded at regular intervals beginning one week after the start of the IAP.

Virus titration

ELISA was used to estimate the relative CCDV concentration in genotypes 'ICCC 10', 'GG 669' and 'WR 315' at 7, 10, 12, 14, 17 and 20 days after the start of the IAP. Four plants of each genotype were tested individually and four young leaves from each plant were ground in extraction buffer. From each sample, four dilutions were tested in duplicate. Sap from uninfected chickpea plants treated similarly served as control. The absorbance was measured at 405 nm, one hour after addition of the substrate. The absorbance values for control plants were subtracted from the values for inoculated plants.

All plants were kept till the end of the experiment. They were then all observed visually and retested by ELISA to detect actual infection. The absorbance values of plants that did not become infected were not included in the calculation of averages and standard errors.

Results

Virus identification and incidence

In December 1990 and January 1991, a total of 97 plants with stunt-like symptoms were collected in the trial fields at Hisar. The majority of these plants reacted with CCDV antiserum (71 plants = 73%). Of the 308 stunted plants collected in December 1991 and January 1992, 37% reacted with CCDV antiserum (Chapter 3). Only a small number (9 plants) reacted with one or more of the luteovirus antisera, and only one plant was infected with a luteovirus and CCDV. Good virus detection methods were not yet available for the stunt viruses during the earlier 1989/1990 season. At that time, plants were collected and subjected to several purification methods, and geminivirus particles, as observed by electron microscopy, were recovered. These are now known to be the particles of CCDV (Horn *et al.*, 1993; Chapter 6). In January 1992, an additional 200 samples were collected from chickpea plants without symptoms, to check for symptomless infections. None of them reacted with CCDV antiserum.

Greenhouse screening

The 13 chickpea genotypes selected in the field for some degree of disease resistance, were further tested in the greenhouse. Only the results obtained with five representative genotypes and the vulnerable check 'WR 315' are presented in Fig. 1. The symptom development was slower, and thereby the incubation period longer, in the disease-resistant genotypes as compared to 'WR 315'. The results obtained in the greenhouse varied considerably among the disease-resistant genotypes (Fig. 1), but they were clearly more resistant than 'WR 315'.

Two extremes ('WR 315' and 'ICCC 10') and one intermediate genotype ('GG 669') were selected from those listed in Fig. 1 for measurement of relative CCDV concentrations in another experiment. The absorbance values for the 10^{-1} dilution of plant sap and their increase with time after inoculation are shown in Fig. 2A. Symptom development in the ELISA-tested plants is shown in Fig. 2B. Virus multiplication was slower in 'GG 669' and 'ICCC 10' than in 'WR 315'. At 7 DAI the virus concentration

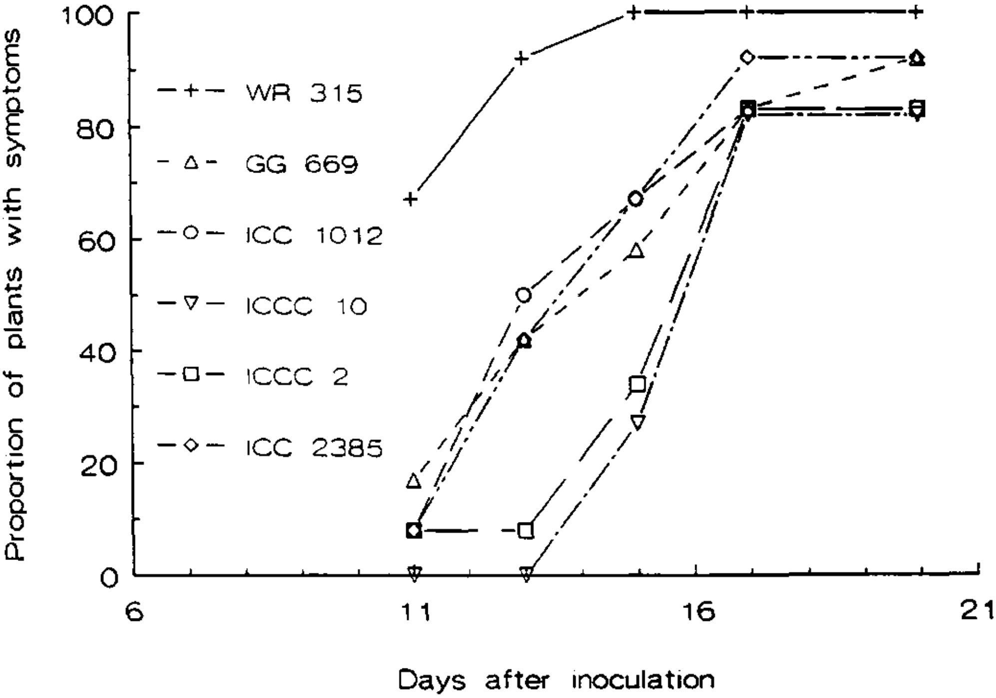

Fig. 1. Increase in proportion of plants with symptoms for the vulnerable ('WR 315') and five disease-resistant genotypes of chickpea after inoculation with leafhoppers in the greenhouse. Of each genotype 12 plants were inoculated.

in 'WR 315' was approximately 13 times that in 'GG 669'. At 14 DAI in 'WR 315' it was approximately 1.3 times that in 'GG 669'. Interestingly there was practically no difference in virus concentration between the three genotypes at 20 DAI (Fig. 2A). Symptom development in ELISA-tested plants (Fig. 2B) largely conforms to the virus concentrations.

The results of the screening of wild *Cicer* species are summarized in Table 1. Although the majority of the plants of these species became infected, symptom development in them was slower than in the vulnerable 'WR 315' genotype of *C. arietinum*. Additionally, symptoms were much less severe than in 'WR 315', especially in 'ICCW 6', which showed very mild symptoms, viz. only leaf chlorosis and slight leaf reddening but no clear stunting. None of the plants that remained symptomless showed virus presence in ELISA.

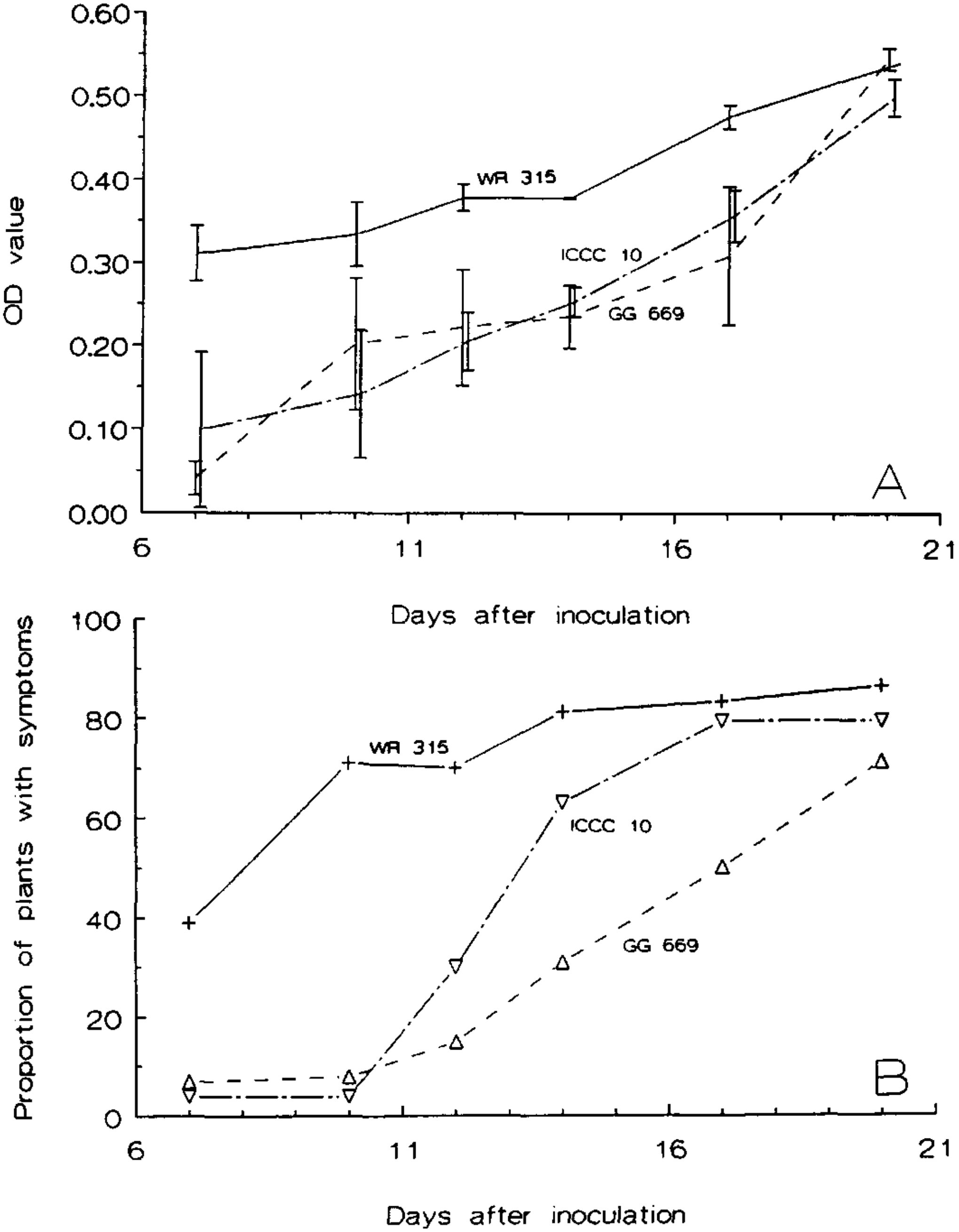

Fig. 2. Increase in virus concentration in three chickpea genotypes, as measured by ELISA (A) and increase in proportion of plants with symptoms (B) after inoculation with leafhoppers in the greenhouse. Of each genotype 24 plants were inoculated. OD values are for plant sap at 10^{-1} dilution and the range presented for each OD value is the standard error. At 14 DAI, for 'WR 315' the standard error was 0.

Table 1. Proportion of plants of *Cicer* spp. infected by CCDV after inoculation with leafhoppers in the greenhouse, and the type of symptoms observed 25 days after inoculation.

Genotype	Species	Proportion of plants infected	Symptoms
ICCW 6	*C. reticulatum*	7/10 (= 70%)	chlorosis, slight leaf reddening no clear stunting
ICCW 36	*C. judaicum*	4/6 (= 67%)	chlorosis, stunting
ICCW 44	*C. echinospermum*	4/4 (= 100%)	chlorosis, leaf reddening, stunting
ICCW 47	*C. cuneatum*	4/6 (= 67%)	chlorosis, reduction in size of tip leaves, stunting
WR 315	*C. arietinum*	10/10 (= 100%)	chlorosis, leaf reddening, leaf epinasty, reduction in size of tip leaves, stunting

Discussion

Serological tests of a large number of stunted plants (over 400) in field screening trials at Hisar during 1990/91 and 1991/1992 have shown that CCDV was the predominant virus there. Consequently, genotypes with low stunt incidence at Hisar during these years have some degree of resistance to stunt disease caused by CCDV.

Genotypes with lower stunt incidence in the field could all become infected in the greenhouse. They developed typical stunt symptoms, and the proportion of plants infected was nearly as high as for 'WR 315'. However, in these genotypes the appearance of symptoms was delayed as compared to 'WR 315', and this indicates some degree of resistance. The infection percentages at 10 DAI corresponded with stunt incidence observed in the field.

The mechanism of resistance was further studied in three genotypes. The resistance in 'GG 669' and 'ICCC 10' as compared to 'WR 315', is probably due to the slower virus multiplication or movement, and thus to true resistance to infection by the virus, hence to the virus itself.

Comparison of the proportion of plants with symptoms for 'GG 669' and 'ICCC 10' in two experiments (Fig. 1 and 2B) shows that also factors other than the chickpea genotype influence the appearance of symptoms, e.g. the infection pressure, which was only partly standardized. Nevertheless, 'WR 315' always was the chickpea genotype in which incidence of disease increased first and most rapidly.

The field and greenhouse screening of chickpea genotypes has clearly shown that 'WR 315' is a highly susceptible and vulnerable genotype, and that 'ICCC 10' and 'GG 669' are disease and, more specifically, virus resistant. The severity of symptoms in the chickpea genotypes did not differ and none of the symptomless plants contained CCDV. Thus, no tolerance seems to be involved.

In the field, chickpea genotypes can be selected for disease resistance and such testing may suffice for preliminary screening of large numbers of germplasm entries or breeding lines, provided infection pressure is sufficiently high. To specify for which viruses the genotypes so selected may be resistant, serological monitoring of the trials for the viruses occurring there is essential. The 13 chickpea genotypes that had shown less than 10% incidence at Hisar, and were considered to be less vulnerable for the disease caused by CCDV, were also planted in Junagadh (Gujarat, India) during the 1991/92 season. There all 13 genotypes showed high levels of infection (40 - 70%) and this is explained by the fact that a luteovirus was predominant in that field screening (Chapter 3). Greenhouse screening, which is more laborious, can help in further evaluating chickpea genotypes that have shown lower stunt incidence under field conditions. In the greenhouse, testing for resistance can be done with specific viruses and virus strains of known pathogenicity, and also the mechanism of disease resistance (e.g. tolerance, vector resistance, virus resistance) can be identified and studied separately. Detailed information on the mechanism of resistance will make selection and breeding more precise. For example, the selection of tolerant genotypes is undesirable from an ecological point of view. Further surveys, throughout chickpea-growing areas, and yield-loss studies are meanwhile essential to show which virus(es) are of actual and potential economic importance in chickpea production to set priorities for further selection and breeding. Virus identification, which is still incomplete and often difficult, as for the luteoviruses (Waterhouse *et al.*, 1988), is prerequisite for obtaining meaningful data from field screening trials and to enable comparison of results obtained at different locations and in different years.

The research reported here has led to a practicable screening approach for chickpea resistance to the non-mechanically transmissible CCDV and has tentatively identified sources of some degree of CCDV resistance in chickpea and in some wild *Cicer* spp. No immunity was found, but it is worth testing more wild species to verify whether immunity is present in some of them.

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CONCLUSION

10. General discussion

Chickpea stunt is a disease characterized by plant stunting, internode shortening, reduction in size of tip leaves, leaf reddening in desi-type and leaf yellowing in kabuli-type chickpeas, and phloem browning in the collar region of stems (Nene and Reddy, 1976). This study has shown that bean leafroll virus (BLRV) is not the only incitant of stunt in chickpea. It can also be caused by at least four other viruses. Surveying of chickpea crops for the viruses involved in chickpea stunt turned out to be far from simple, as is the etiology of the disease. At different places different viruses, causing identical symptoms, were found to be involved or to prevail. So, the surveying was not merely a matter of routine detection of viruses with a standard range of antisera to known viruses. It gradually led to the detection of a number of new viruses and to their full or partial characterization.

BLRV is only one of a series of luteoviruses involved. BLRV had been alleged to be associated with the typical stunt symptoms in Iran by Kaiser (1972) and in India by Reddy *et al.* (1979), but at that time BLRV had not yet been characterized physico-chemically, so that the identity of the virus originally reported from Iran and India remains uncertain. A large number of aphid species were shown to transmit BLRV in Iran (Kaiser, 1972). This led to the assumption that not only BLRV, which is transmitted by a few aphid species only, but also BWYV may have been associated with the disease there (Ashby, 1984). BWYV has recently indeed been shown to be involved in chickpea stunt in California (Bosque-Perez and Buddenhagen, 1990) and in Spain (Carazo *et al.*, 1993).

During surveys in India, Pakistan, Syria, Turkey and Lebanon, BLRV-like, BWYV-like, and CpLV-like isolates were found to be associated with the disease (Chapters 3 and 4). The different patterns of reaction with the Mabs of the BLRV-like isolates (Chapters 3 and 4) indicate variation within this group of luteoviruses. CpLV has not yet been characterized because of difficult aphid transmission and problems in transferring the isolate to plants in the greenhouse.

From a single field-infected chickpea plant three luteovirus isolates were obtained (Chapter 5). One of them was characterized using host-range studies, ELISA, SDS-PAGE and Western blot analysis, and was identified as a distinct strain of BWYV. This isolate and another isolate, identified as PLRV-like by its reactions with poly- and monoclonal antibodies, could not cause chickpea stunt on their own. A third isolate from the same field-infected chickpea plant could cause all symptoms characteristic of chickpea stunt. This isolate was BWYV-like in its serological affinities, and could have been the same as the BWYV-like isolate which was characterized in Chapter 5. In that case, it would need coinfection with the PLRV-like isolate to cause the characteristic symptoms. A Dutch isolate of BWYV (from lettuce) and another one of BLRV (Ashby and Huttinga, 1979) were now both shown to cause chickpea stunt on their own. Thus, luteoviruses can cause the disease, but it remains unclear which components of luteovirus mixtures from naturally infected chickpea play a role in the etiology of chickpea stunt. Transfer with aphids from a field-infected plant to a plant in the greenhouse may favour certain variants and may thereby make the study of their role in chickpea stunt more difficult.

The newly described geminivirus CCDV was unequivocally shown to cause plant stunting, internode shortening, leaf chlorosis and reddening, and phloem discoloration in chickpea (Chapter 6). This virus was found to be widely distributed in India and Pakistan (Chapter 3). It occurred up to 12 % in vulnerable chickpea genotypes already early during the season, while in experiments at Hisar in India natural incidences of up to 90 % were found. The virus was efficiently transmitted by the leafhopper *Orosius orientalis* (Chapter 7), which has been reported to occur at many places in India (Bindra and Singh, 1970) and is known there as a pest of sesame (Choudhary *et al.*, 1986). Experiments demonstrated that this virus can cause considerable reduction in yield (Chapter 8). In resistance-screening experiments immunity to CCDV was not found in chickpea and in a small number of wild *Cicer* genotypes tested. However, genotypes with resistance to the virus were detected in greenhouse tests (Chapter 9). They might be useful for resistance breeding.

Faba bean necrotic yellows virus (FBNYV), a still unclassified virus, taxonomically different from luteoviruses, appeared to cause similar, if not identical, symptoms (Chapter 4). Earlier laboratory tests had demonstrated that this virus could cause plant stunting and leaf chlorosis in kabuli-type chickpeas (Katul *et al.*, 1993), but then the plants were not examined for phloem browning (L. Katul, pers. comm., 1992). During the surveys in Syria, Turkey and Lebanon (Chapter 4), FBNYV was repeatedly detected in field-infected chickpea plants with characteristic stunt symptoms, and it was the virus with the highest incidences in these regions. FBNYV was often the only virus that could be detected in these plants.

In Madhya Pradesh, India, stunt incidence in the field was high during the 1991/1992 season (Chapter 3). The causal agent could be transmitted by grafting from chickpea to chickpea. It did not react with CCDV antiserum, nor with any of the luteovirus antisera used. This suggests that another, yet unidentified, agent was involved there.

Thus, a number of luteoviruses (BLRV-like, BWYV-like and CpLV-like), a leafhopper-transmitted geminivirus (CCDV), FBNYV, and in Madhya Pradesh, India, possibly yet another agent, were involved in causing chickpea stunt. These viruses occur in the field either alone, or in combinations of two or more in single plants. Currently no data on the effects of mixed infections are available.

During the surveys (Chapters 3 and 4), 200 - 300 field-collected plants with stunt symptoms from India, Pakistan, Syria, Turkey and Lebanon were especially examined for the presence and intensity of leaf chlorosis, leaf reddening and phloem discoloration. No correlation was found between the presence or severity of these symptoms, and the type of virus(es) detected by ELISA (unpublished data). Under field conditions, age of the crop at the time of infection, the period of time elapsed since infection started, chickpea genotype, and environmental conditions vary, and they may contribute to variation in symptoms. Such variation in symptom expression has already been shown to occur for other luteoviruses. In the Netherlands, BLRV infection was symptomless in alfalfa in summer, whereas during spring and autumn infection of this crop by BLRV led to prominent symptoms (vein yellowing; Van der

Want and Bos, 1959). Irrespective of their taxonomy, all viruses currently known to be associated with chickpea stunt are phloem limited. In all instances, the external symptoms are likely to identically result from vascular blocking due to phloem degeneration primarily produced by the viruses. The non-phloem-limited viruses known from chickpea cause mosaic, narrow leaves, bushy growth, and other symptoms, but no stunting or phloem discoloration (Nene and Reddy, 1987).

Extensive greenhouse studies might reveal slightly different symptoms for different viruses on certain chickpea genotypes. Such a comparison was not possible within the scope of this study. FBNYV was not known to occur in India, and for experimentation with the luteoviruses efficient aphid vectors were not available. However, such symptoms will certainly vary greatly, even for individual viruses, in extent and severity according to genotype, plant age at time of infection, and conditions. Therefore, differences in symptoms or symptom development will undoubtedly be of limited practical value, if at all, for distinguishing the viruses in the field, and thus for disease diagnosis. No basic differences in symptoms could be found in chickpea plants, although different viruses were found to be associated with the stunt symptoms.

Therefore, it is now proposed that the use of the name 'chickpea stunt', introduced by Nene and Reddy (1976) for the virus disease they described symptomatologically (Nene and Reddy, 1976; 1987), be continued, irrespective of the causal virus. Kaiser (1972) and Reddy *et al.* (1979) were the only authors ascribing the symptoms to a, then not reliably characterized, virus. All other, even recent, reports on chickpea stunt are on symptomatological evidence only (Kothastane and Gupta, 1978; Nene and Reddy, 1987; Kannaiyan and Hariwa, 1989; Abdalla and Van Rheenen, 1991; Reddy *et al.*, 1991; Mutshiya *et al*, 1991; Woldeamlak Araya *et al.*, 1991; Ayub *et al.*, 1992). and BLRV (earlier described as pea leafroll virus) was assumed to be the incitant. Publications on a similar disease of chickpea in the USA (Bosque-Perez and Buddenhagen, 1990) and Spain (Carazo *et al.*, 1993) did not mention the name chickpea stunt, but associated the disease with the luteoviruses BLRV, BWYV, subterranean clover red leaf virus (SCRLV) and legume yellows virus (LYV, probably a strain of BLRV), as detected in ELISA and by aphid transmission. It is now shown that other viruses can cause identical symptoms and thereby the stunt disease. Retention of the 17-year-old name 'chickpea stunt' is likely to create less confusion than now inventing a range of different names for similar, if not identical, syndromes caused by different viruses.

The disease can have a dramatic effect on yield (Chapter 8; Kaiser and Danesh, 1971; Kotasthane and Gupta, 1978; Ayub *et al.*, 1992). High incidences now found in certain regions and at certain research stations (Chapters 3 and 4), indicate its potential threat to the cultivation of chickpea. Therefore it is essential to precisely characterize the causal viruses, and study them for their differences in host ranges, vector specificity and efficiency, and other ecological characters, to facilitate specific strategies of control.

The only method available at the moment to distinguish the viruses involved in chickpea stunt is serology, particularly ELISA. This technique should, therefore, be more extensively used. Since it may be difficult for scientists in developing countries to use ELISA, the International Agricultural Research Centers (ICARDA and ICRISAT) can play an active role in reliably diagnosing the disease including its causative viruses.

When disease incidence is high, as in experimental fields at Hisar and in Junagadh (Chapter 3), orientational distinction of the viruses involved might be possible with a series of differentiating host genotypes. This might be especially useful when facilities for ELISA are not available. In plant pathology, such differentiating sets are mostly based on the presence or absence of symptoms in these genotypes, as caused by susceptibility or immunity, respectively. Since no immunity has been found yet in the case of chickpea stunt, genotypes with differences in vulnerability should be used and incidence of disease be assessed. 'WR 315' should be included in the set of differentials since this genotype appeared to be highly vulnerable to CCDV and to the luteoviruses studied. 'ICCC 10' could be included as a genotype less vulnerable to CCDV and highly vulnerable to the luteovirus prevalent in Gujarat (Chapter 3). This genotype had always less than 10% stunt incidence at Hisar where CCDV was prevalent (Chapter 9). Another genotype, which is less vulnerable to luteovirus(es) and highly vulnerable to CCDV, is still needed. Such a set of at least three genotypes would allow discrimination in the field between CCDV and luteoviruses. 'WR 315' would be a good indicator of disease incidence, and the other two genotypes would give an indication of the type of virus present. It should, however, be kept in mind that other unknown or undescribed (luteo)viruses, if also present, will still escape detection with this set, and may even confuse the identification of known viruses. The testing of at least part of the samples on wider host ranges in the greenhouse is, therefore, wise to detect the possible involvement of other hitherto undetected viruses.

The method employed to screen for resistance to CCDV (Chapter 9), demonstrates that it is effective in selecting genotypes resistant to the viruses causing chickpea stunt. Earlier, screening in the field had been performed by selecting genotypes with low stunt incidence, as judged by typical symptoms (ICRISAT, 1989). In this way, genotypes were selected for resistance to the disease. It was assumed that this meant resistance to BLRV, since this was, at that time, the only virus known to cause such symptoms. Such screening in the field by visual observation for symptoms, however, is for disease resistance and not for resistance to virus infection (for definitions see Chapter 9). Disease resistance could be a matter of virus resistance or of tolerance, that is of resistance to virus multiplication or merely of the absence of symptoms despite virus multiplication. Tolerance is ecologically undesirable since the virus concentration in tolerant genotypes may become high and lead to an increased inoculum pressure in the environment, which may threaten vulnerable genotypes of chickpea or even of other crops. Field screening should, therefore, be accompanied by the testing of symptomless plants of promising genotypes by ELISA for the presence and concentration of the virus(es) to clearly identify the type of resistance.

Furthermore, field screening for resistance by mere visual scoring for symptoms does not allow specification of the virus(es) to which resistance is found. Resistance found at one location does not guarantee resistance at other locations, where other viruses or other virus strains may occur. This was first observed for chickpea stunt in 1980, when chickpea genotypes found to be disease resistant at Hisar, India, were shown to be extremely vulnerable to BLRV in the Netherlands (Dr L. Bos, IPO Wageningen, correspondence with Dr Y.L. Nene, 1980; Plate 22 in: Bos, 1983). This was now also found when chickpea genotypes, selected for disease resistance at Hisar, were planted in Junagadh. These genotypes performed well at Hisar, where CCDV was the predominant virus, whereas they contracted high incidences of stunt in Junagadh, where a luteovirus prevailed. By identifying the virus(es) present in each field trial, the resistance found there can be specified. Thus, field screening must be accompanied by monitoring the virus(es) present. Additional testing of the promising genotypes under insect-proof conditions in the greenhouse with well identified viruses, will help further specifying to which viruses resistance has actually been found, and enable specific testing under controlled conditions excluding natural contaminations (Chapter 9).

A highly important aspect of future research should be the further characterization of the luteoviruses now found to be associated with chickpea stunt and the characterization of the pathogen (possibly a virus) that caused the stunt symptoms in chickpea in Madhya Pradesh (Chapter 3). Since luteoviruses appeared to occur in mixtures in chickpea and not all isolates were able to cause the disease (Chapter 5), the different components of the mixtures, and their role in the etiology of chickpea stunt should also be studied.

Many attempts have been made to distinguish between luteoviruses and to classify them (Waterhouse *et al.*, 1988; Martin *et al.*, 1990; Duffus *et al.*, 1990; Ward, 1993), but the taxonomy of this group is still evolving. Serologically, the viruses of this group form a continuum with some degree of clustering (Waterhouse *et al.*, 1988). On the basis of their host ranges, the luteoviruses can be divided into subgroups (Martin *et al.*, 1990), but considerable variation in host range within the subgroups, especially the BWYV subgroup, still exists (Duffus *et al.*, 1990). More serological and genome sequence data have become available recently, and these usually match (Martin *et al.*, 1990). However, there are luteovirus isolates which are BWYV-like in their serological reactions, but BLRV-like in DNA hybridization tests (Fortass, 1993). In this study, the BLRV-like isolates showed quite some variation in their reactions with the Mabs (Chapters 3 and 4). Luteoviruses were also found in mixtures by others (Duffus, 1981; Chapter 5), and selection of variants has been possible from a single luteovirus species (Van den Heuvel *et al.*, 1993). The variation within a luteovirus species has till now been judged mainly by host-range and serological studies. The increasing amount of molecular data may soon allow investigation of variation within single luteoviruses at the molecular level. Although the genomes of PLRV isolates showed more than 93% homology (Martin *et al.*, 1990), this may not hold for BWYV, which is more variable. Thus, the so-called 'definite' members of the luteoviruses (Francki *et al.*, 1991) appear to be not yet that clearly defined. This explains the difficulties dealt with in identifying the luteoviruses during the surveys described here (Chapters 3, 4 and 5).

Since the viruses may annually shift in relative importance, as was observed in Gujarat (Chapter 3), the occurrence of the viruses in the different chickpea-growing areas should be monitored from time to time by surveying as done during the 1991-1992 season (Chapters 3 and 4). A change in reaction of cultivars to the disease in farmers' fields may already hint at a change in virus population. Such monitoring will help to concentrate research efforts on economically important viruses, and to adapt research to the actual situation in the field.

The use of resistant genotypes seems now to be one of the main practicable measures of control. Resistance breeding should, therefore, be given high priority. In order to be effective, resistance sources should be identified to those viruses which are currently considered to be economically important as revealed by surveys. For example, the luteovirus that occurred in Junagadh (Chapter 3) should receive high priority, because of its high incidence and damage incurred. CCDV is widely distributed and occurs at high incidences at some locations. Therefore it will be desirable to develop CCDV-resistant cultivars. Genotypes that are introduced into areas where CCDV is endemic, such as the Thal area in Pakistan and Rajasthan in India, should be screened for resistance to CCDV prior to their introduction into these areas to avoid introduction of highly vulnerable genotypes, leading to epidemic development of the disease there. For the West Asia/North Africa (WANA) region, breeding for resistance to FBNYV should receive high priority, with a view to the increasing importance of the virus there (Katul *et al.*, 1993).

There still is a paucity up to complete lack of information on the incidence of stunt, on the virus(es) that are involved in chickpea stunt, and on their incidence in Bangladesh, Myanmar, Ethiopia, and in WANA countries like Iran and Morocco. Surveys of chickpea for chickpea stunt and associated viruses in these countries by scientists of National Agricultural Research Systems (NARS) in collaboration with ICRISAT and ICARDA would be a good way to gain more information on this disease and on the importance of the causal viruses.

Since the viruses causing stunt primarily infect and affect the phloem, detailed anatomical studies of chickpea plants infected by CCDV or the luteoviruses are of interest. Controlled introductions of individual viruses, virus strains, and combinations of them into chickpea, to study their effect on chickpea, may reveal possible interactions. Such investigations, impossible within the limited amount of time available for this PhD study, may help to further comprehend the pathogenesis and ecology of the disease and provide more decisive information on possible differences between the viruses in their effects on plant hosts.

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SUMMARY

Chickpea stunt is the most important virus disease of chickpea (*Cicer arietinum* L.). This disease is characterized by leaf chlorosis or leaf reddening (depending on the chickpea cultivar), plant stunting, internode shortening, reduction in size of tip leaves, and phloem discoloration. Infected plants decline and premature death mostly follows. Till recently, the disease was ascribed in India to bean leafroll luteovirus (BLRV) only. Also in other countries, luteoviruses were reported to be associated with similar stunt symptoms. In India, however, indications emerged that more viruses were involved in the disease. Therefore, the viruses associated with chickpea stunt, and their role in the etiology of the disease were now studied.

Surveys were undertaken in India, Pakistan, Syria, Turkey and Lebanon. No sap-transmissible viruses could be isolated from plants with chickpea stunt. In India and Pakistan, 1804 samples from plants with stunt-like symptoms were collected and tested with poly- and monoclonal antibodies (Chapter 3). In plants with chickpea stunt from farmers' fields, a geminivirus (newly described in this thesis as chickpea chlorotic dwarf virus; CCDV), isolates reacting with an antiserum to a luteovirus isolate from chickpea (tentatively named chickpea luteovirus; CpLV), and BLRV-like isolates were detected. CCDV and CpLV-like isolates occurred widely, whereas the BLRV-like isolates were found at very low incidences in two regions in India only. In Syria, Turkey and Lebanon, the recently characterized, unclassified faba bean necrotic yellows virus (FBNYV) was predominantly associated with chickpea stunt (Chapter 4). More than 50% of the 313 plant samples collected reacted with FBNYV antiserum. Luteoviruses (beet western yellows virus-like, CpLV-like and BLRV-like) were also detected in ELISA using poly- and monoclonal antibodies, but their incidences were lower than those of FBNYV. The geminivirus was not detected in these countries.

The reaction patterns with the Mabs of the luteovirus isolates from these five countries differed from those of a number of known luteoviruses (Chapters 3 and 4). New luteoviruses may well have been traced here. The surveys showed that the etiology of chickpea stunt is much more complex than originally thought. More viruses seem to

be involved, but their role in the etiology is not yet clear.

To further identify the luteoviruses obtained from chickpea, three isolates from a single field-infected chickpea plant with stunt symptoms in India were studied (Chapter 5). One of them was characterized by host range, ELISA and Western blot analysis, and was identified as a distinct strain of beet western yellows luteovirus (BWYV). When chickpea plants were inoculated with this isolate, infection was obtained very rarely, and in case of infection no symptoms were observed. The second isolate was serologically similar to potato leafroll luteovirus (PLRV), and the third to BWYV. Only the latter was associated with stunt symptoms in greenhouse-grown chickpea plants inoculated with *Myzus persicae*. These orientational experiments suggest that luteoviruses occur in mixtures in chickpea. Not all components may be able to cause chickpea stunt on their own, and the behaviour of luteoviruses, and their involvement in the etiology of chickpea stunt appears to be complex.

The Indian geminivirus from chickpea was first detected by electron microscopy after partial purification from extracts of chickpea plants from the field (Chapter 6). This virus was not sap transmissible and could not be transferred with aphids or whiteflies. The leafhopper *Orosius orientalis* was identified as a vector of the virus. The virus particles were geminate, and contained a coat protein of 32 kD and a circular, ssDNA of 2900 nucleotides. The virus was shown to be serologically distinct from the known leafhopper-transmitted geminiviruses, and was considered to be a new, hitherto undescribed, geminivirus. It was named chickpea chlorotic dwarf geminivirus (CCDV) and was shown to cause symptoms in chickpea similar, if not identical, to those described for chickpea stunt.

Plants that became infected with CCDV in the field during or before flowering, suffered from yield losses of 75 - 100% (Chapter 8). Plant densities in farmers' fields are likely to be too low to allow uninfected plants around infected ones to compensate the yield losses of infected plants. Therefore, crop loss is likely to equal percentage of disease incidence.

To better understand the ecology of CCDV, its relationships with the leafhopper *O. orientalis* were studied (Chapter 7). The leafhopper can acquire and introduce the virus in very short feeding periods. The minimum latency period was also very short (2 h). This indicates that the vector can easily transfer the virus. Single leafhoppers could transfer the virus for 10 - 15 consecutive days when they were transferred daily to healthy plants. Application of ELISA to single leafhoppers showed that the amount of virus in the vector decreased when the insects were kept on a non-host of the virus. Thus, the virus is transmitted by *O. orientalis* in a persistent, non-propagative manner.

When a number of chickpea genotypes, identified as disease resistant in field experiments, were inoculated in the greenhouse with CCDV, these genotypes showed slower symptom development than the vulnerable control 'WR 315' (Chapter 9). In two disease-resistant genotypes the virus concentration was initially lower than in the vulnerable control, but it reached the same level three weeks after inoculation. This disease resistance thus seems to be a matter of true virus resistance. Four wild *Cicer* spp. were also leafhopper-inoculated with CCDV in the greenhouse. They all expressed symptoms later than 'WR 315', and their symptoms were weaker than those in 'WR 315'. No immunity was found in the *Cicer* spp. tested. Greenhouse screening and ELISA testing of field samples, both described in this thesis, are a good way to specify the type and degree of resistance found in field screening, and the virus to which resistance is involved.

Several viruses appear to be associated with chickpea stunt, viz., a number of luteoviruses, CCDV and FBNYV. None of the luteovirus isolates obtained from chickpea in this study was shown to cause the disease. However, the luteoviruses BWYV and BLRV (Dutch isolates from lettuce and alfalfa, respectively) were demonstrated to cause all symptoms characteristic of chickpea stunt. Thus, luteoviruses can cause the disease, but it remains unclear which components of luteovirus mixtures from chickpea actually play a role in the etiology of the disease. CCDV was shown to cause the symptoms characteristic of chickpea stunt, and FBNYV most likely causes the same symptoms. The viruses found to be etiologically associated with chickpea stunt are all phloem limited, and probably cause the external symptoms of chickpea stunt indirectly by primarily blocking the phloem.

It is now proposed to retain the name 'chickpea stunt' for the disease irrespective of the causal virus, since these viruses cause similar, if not identical, symptoms, and they cannot be distinguished by the symptoms they cause in chickpea. An important aspect of future research is the further characterization of the luteoviruses occurring in chickpea, and their possible role in the etiology of chickpea stunt. Full characterization of the luteoviruses detected during this study remains difficult awaiting further improvement of the luteovirus taxonomy. This difficulty also explains the problems dealt with in identifying luteoviruses during the surveys.

SAMENVATTING

De kekererwt (*Cicer arietinum* L.) is een belangrijke eiwitbron in Zuid Azië. Dit gewas is ook bekend onder de wellicht meer gebruikte, verbasterde naam kikkererwt en de Engelse naam is 'chickpea'. De belangrijkste virusziekte van kekererwt is 'chickpea stunt'. Het duidelijkste kenmerk van de ziekte is dwerggroei (Engels: stunt) van de plant. Daarnaast vertonen zieke planten geel- of roodverkleuring van de bladeren afhankelijk van het kekererwtras, verkorte internodiën, verkleinde topblaadjes en floëemverbruining. Zieke planten sterven meestal voortijdig af.

Tot voor kort werd gedacht dat in India het erwtetopvergelingsluteovirus (Engels: bean leafroll luteovirus; BLRV) de enige veroorzaker van deze ziekte was. In andere landen werden andere virussen, uit dezelfde luteovirusgroep, gevonden in kekererwtplanten met de symptomen die karakteristiek zijn voor 'chickpea stunt'. In India kwamen er ook steeds meer aanwijzingen dat een aantal virussen bij deze ziekte betrokken was. Daarom werden de desbetreffende virussen bestudeerd, evenals hun aandeel in het veroorzaken van de symptomen die bij deze ziekte horen.

Veldinspekties werden gedaan in India, Pakistan, Syrië, Turkije en Libanon. Sap-overdraagbare virussen konden niet geïsoleerd worden uit planten met 'chickpea stunt'. In India en Pakistan werden 1804 monsters verzameld van planten met dwerggroei en deze werden getoetst met poly- en monoklonale antilichamen in ELISA. In deze planten van boerenvelden werden isolaten aangetoond die reageerden met een antiserum tegen een geminivirus (voor het eerst beschreven in dit proefschrift en genoemd 'chickpea chlorotic dwarf virus'; CCDV), een antiserum tegen een luteovirus (voorlopig 'chickpea' luteovirus genoemd; CpLV) en/of een antiserum tegen BLRV. CCDV en CpLV-achtige isolaten kwamen wijdverspreid voor terwijl de BLRV-achtige isolaten weinig voorkwamen en alleen in twee gebieden in India.

In Syrië, Turkije en Libanon kwam het recentelijk gekarakteriseerde 'faba bean necrotic yellows virus' (FBNYV) het meest voor. Meer dan 50% van de 313 verzamelde plantemonsters reageerde met FBNYV-antiserum. Luteovirussen (slavergelingsvirus, Engels: 'beet western yellows virus', BWYV; CpLV-achtige en BLRV-achtige isolaten) werden ook aangetoond met ELISA, maar zij kwamen veel minder voor dan FBNYV. Het geminivirus werd in deze drie landen niet gevonden.

De reactiepatronen van de luteovirussen uit deze vijf landen met de monoklonale antilichamen verschilden van die van bekende luteovirussen. We zouden hier nieuwe luteovirussen op het spoor kunnen zijn. De veldinspekties toonden aan dat de etiologie van 'chickpea stunt' veel ingewikkelder is dan oorspronkelijk gedacht werd. Er zijn meer virussen bij betrokken, maar de rol van elk ervan in het veroorzaken van de ziekte is nog niet duidelijk.

Om de uit kekererwt geïsoleerde luteovirussen beter te identificeren, werden drie isolaten uit één enkele kekererwtplant uit India beter bestudeerd. Eén van de drie werd gekarakteriseerd met behulp van zijn waardplantenreeks en zijn serologische reacties. Dit isolaat bleek een stam van het slavergelingsluteovirus te zijn. Als kekererwtplanten werden geïnoculeerd met dit isolaat, werden de planten vrijwel nooit ziek, en als ze ziek werden vertoonden ze geen symptomen. Het tweede isolaat was serologisch gelijk aan het aardappelbladrolluteovirus (Engels: potato leafroll virus; PLRV) en het derde aan het slavergelingsvirus (BWYV). Wanneer kekererwtplanten met deze drie isolaten geïnoculeerd werden, bleek alleen het derde isolaat voor te komen in planten met de symptomen die karakteristiek zijn voor 'chickpea stunt'. Deze oriënterende proeven suggereren dat luteovirussen in kekererwt in mengsels voorkomen. Waarschijnlijk zijn niet alle componenten in staat zelfstandig de ziekte te veroorzaken. Het gedrag van de luteovirussen en hun rol in de ziekte zijn gecompliceerd.

Het Indiase geminivirus uit kekererwt werd voor het eerst ontdekt bij elektronenmicroscopisch onderzoek van gedeeltelijk gezuiverd sap van kekererwtplanten uit het veld. Het aangetroffen virus was niet sap-overdraagbaar en kon niet overgebracht worden met bladluizen of wittevliegen. De cicadellide *Orosius orientalis* bleek een vector van het virus te zijn. De virusdeeltjes zijn tweedelig en bevatten een eiwit van 32 kDalton en cirkelvormig, enkelstrengig DNA van 2900 nucleotiden. Het bleek serologisch niet verwant te zijn met de andere geminivirussen die door cicadelliden worden overgedragen. Het bleek een nieuw, tot nu toe niet beschreven, geminivirus te zijn en werd de naam 'chickpea chlorotic dwarf virus' (CCDV) gegeven. Dit virus veroorzaakt in kekererwt symptomen die gelijk zijn aan de symptomen die beschreven zijn voor 'chickpea stunt'.

Kekererwtplanten die voor of tijdens de bloei met CCDV geïnfecteerd raakten, leden opbrengstverliezen van 75 tot 100%. De plantdichtheden op boerenvelden zijn in India waarschijnlijk te laag om gezonde planten in de buurt van zieke tijdens de ontwikkeling van het gewas te laten compenseren voor de opbrengstverliezen van de zieke planten. Daarom is het uiteindelijke oogstverlies in een gewas waarschijnlijk gelijk aan het percentage zieke planten dat in dat gewas voorkomt.

Om de ecologie van CCDV beter te begrijpen, werd de relatie met de cicadellide *O. orientalis* bestudeerd. De cicadellide kan het virus makkelijk opnemen en afgeven. De minimum latentieperiode was heel kort. Dit geeft aan dat het insekt het virus makkelijk kan overdragen. Bovendien konden individuele insekten het virus gedurende 10 tot 15 dagen overdragen wanneer ze dagelijks op een gezonde plant werden overgezet. Toepassing van ELISA op individuele insekten toonde aan dat de hoeveelheid virus in de vector afnam wanneer de insekten zich voedden op een plant die geen waard voor het virus is. Het virus wordt dus op een persistente manier overgedragen door de vector en vermeerdert zich niet in de vector.

Wanneer een aantal kekererwtgenotypen, die in het veld als ziekteresistent geïdentificeerd waren, in de kas met behulp van cicadelliden werden geïnoculeerd met CCDV, vertoonden ze een langzamere symptoomontwikkeling dan de kwetsbare controle 'WR 315'. In twee ziekteresistente genotypen was de virusconcentratie aanvankelijk lager dan in de kwetsbare controle, maar drie weken na inoculatie bereikte de virusconcentratie hetzelfde niveau. Deze ziekteresistentie lijkt dus een kwestie van echte virusresistentie te zijn. Vier wilde *Cicer*-soorten werden ook in de kas met CCDV geïnoculeerd. De symptomen verschenen in deze genotypen later dan in 'WR 315' en ze waren zwakker dan in 'WR 315'. Immuniteit is niet gevonden in de getoetste *Cicer*-soorten. Kas- en ELISA-toetsing, zoals beschreven in dit proefschrift, zijn een goede manier om de aard en mate van resistentie gevonden in het veld te specificeren en om te toetsen welk virus betrokken was bij de veldtoetsing.

Verschillende virussen zijn in het veld betrokken bij 'chickpea stunt', namelijk een aantal luteovirussen, CCDV en FBNYV. Van geen van de luteovirussen, die geïsoleerd zijn uit kekererwt in dit onderzoek, kon worden aangetoond dat het deze ziekte veroorzaakte. Met Nederlandse isolaten van het slavergelings- en het erwtetopvergelingsvirus konden echter wel alle symptomen die karakteristiek zijn voor 'chickpea stunt' teweeggebracht worden in kekererwt. Luteovirussen kunnen dus de ziekte veroorzaken, maar het is nog niet duidelijk welke componenten van de luteovirus-mengsels in kekererwt hiervoor werkelijk verantwoordelijk zijn. CCDV veroorzaakt de karakteristieke symptomen, en waarschijnlijk doet FBNYV hetzelfde. De virussen die als veroorzakers van de ziekte in kekererwt werden gevonden zijn allemaal floëem-gebonden en veroorzaken de externe symptomen waarschijnlijk indirect door primair het floëemtransport te blokkeren.

Het voorstel is nu om de naam 'chickpea stunt' te blijven gebruiken voor de ziekte onafhankelijk van het virus dat de veroorzaker is, omdat deze virussen gelijke, of zelfs identieke, symptomen veroorzaken in kekererwt. De virussen kunnen niet onderscheiden worden op basis van de symptomen in kekererwt. Een belangrijk aspect van toekomstig onderzoek is de verdere karakterisering van de luteovirussen die in kekererwt voorkomen, en de bestudering van hun rol in de etiologie van 'chickpea stunt'. Volledige karakterisering blijft moeilijk zolang de luteovirustaxonomie nog niet verder uitgewerkt is. Dit verklaart ook de problemen ondervonden bij het identificeren van de luteovirussen die geïsoleerd werden tijdens de veldinspekties.

CURRICULUM VITAE

Nicolaas Maria Horn was born in Amsterdam on December 23, 1956. In 1975 he started his studies at the Wageningen Agricultural University (LUW). This scientific education was completed in 1982 with Phytopathology and Virology as major subjects, and Genetics as a minor subject. Thereafter he conducted contract research on development of fungicide resistance of powdery mildew of wheat, at the Department of Phytopathology of LUW. A literature review on the effect of air pollution on fungal diseases of trees was done at the Dorschkamp Research Institute for Forestry and Landscape Planning (IBN-DLO) in Wageningen in 1984 and published in 1985.

Since 1985 he was employed as an associate expert Virology/Mycology by the Directorate General for International Cooperation (DGIS) of the Netherlands Ministry of Foreign Affairs. After a short training in tropical legume virology at IPO-DLO (DLO Research Institute for Plant Protection) Wageningen, and a language course in Indonesian, he was stationed from March 1986 till February 1989 at the Malang Research Institute for Food Crops (MARIF) in Malang (East Java) within the bilateral ATA-272 project. There, he assisted in setting up the Virology Unit and initiated a research programme on viruses of soybean and groundnut.

In July 1989, he continued as associate expert Virology employed by DGIS at the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), near Hyderabad, India, by invitation of this institute. The third year he was employed by ICRISAT. The research was on the viruses possibly involved in the stunt disease of chickpea, and led to this thesis.

In July 1992 he returned to IPO-DLO, Wageningen, to finalize his studies on chickpea stunt by further investigating the luteoviruses of chickpea and writing the thesis. The first 3½ months of his stay at IPO-DLO were spent on DGIS-study leave. During his stay at IPO-DLO, the institute provided hospitality, and laboratory and office facilities. Nico Horn is currently looking for a suitable position to continue research on plant viruses.