VIRUSES OF FABA BEAN (*Vicia faba* L.) IN MOROCCO; SURVEYING, IDENTIFICATION, AND ECOLOGICAL ASPECTS

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PROPOSITIONS

1. The demarcation between potyviruses on the basis of coat protein sequence homologies will fade as information on more sequences of potyvirus isolates becomes available.

2. The evaluation of breeding lines in international programmes without testing for resistance to viruses entails the risk of provoking new virus problems in recipient countries.

3. The paucity of ecological information on broad bean mottle virus was due to the non-occurrence of this virus in developed countries.

4. The newly proposed name bean necrotic mosaic virus (Vetten *et al.*, 1992) for the potyvirus distinct from bean common mosaic virus and inducing necrosis on certain bean cultivars is confusing and unacceptable.

   Vetten, H.J., Lesemann, D.-E. & Maiss, E., 1992. Serotype A and B strains of bean common mosaic virus are two distinct potyviruses. Archives of Virology. Suppl. 5 : 415-431.

5. Resistance to impatiens necrotic spot virus of plants transformed with the tomato spotted wilt virus N gene, as claimed by Pang *et al.* (1993), is not documented in their publication.

   Pang, S-Z., Slightom, J.L. & Gonsalves, D., 1993. Different mechanisms protect transgenic tobacco against tomato spotted wilt and impatiens necrotic spot tospoviruses. Biotechnology 11: 819-824.

6. The indication 'definitive member' for the luteoviruses listed by the International Committee on Taxonomy of Viruses is premature.

   Francki, R.I.B., Fauquet, C.M., Knudson, D.L. & Brown, F., 1991. Classification and nomenclature of viruses. Fifth report of the International Committee on Taxonomy of viruses. Archives of Virology. Suppl. 2 : 309-311.

7. Virus strain designation should take host-range properties into consideration.

8. The method employed by Hara *et al.* (1990) to reveal the localization of symbionin in *Acyrthosiphon pisum* does not justify the conclusion that this protein is exclusively confined to aphid's mycetocytes harbouring the primary endosymbionts.

Hara, E., Fukatsu, T. & and Ishikawa, H., 1990. Characterization of symbionin with anti-symbionin antiserum. Insect Biochemistry 20: 429-436.

9. Considering bean leaf roll and pea leaf roll luteoviruses synonymous (Francki *et al.*, 1991) conflicts with the use of the aphid vector specificity to designate strains of barley yellow dwarf luteovirus.

Francki, R.I.B., Fauquet, C.M., Knudson, D.L. & Brown, F., 1991. Classification and nomenclature of viruses. Fifth report of the International Committee on Taxonomy of Viruses. Archives of Virology. Suppl. 2 : 309-311.

10. For full enjoyment of a sunny day in the Netherlands, the availability of an umbrella is required.

PREFACE

During the last four years, the help and support of many people have been essential for the completion of this work. I would like first to express my sincere gratitude to Lute Bos and Rob Goldbach for their encouragement and their continuous guidance with fruitful discussions and criticism.

The kind hospitality of the DLO Research Institute for Plant Protection (IPO-DLO) and the Department of Virology of the Wageningen Agricultural University (LUW) has been greatly appreciated. I want to thank Hans van den Heuvel and Frank van der Wilk for their cooperation and helpful discussions, and many others from IPO-DLO, especially Harm Huttinga, Dick Maat, Emmy van Balen, Rikus Pomp, Chris Cuperus, and Nick Huijberts for their kind help. My thanks are also due to the colleagues and staff members of the Department of Virology (LUW), particularly Jeanne Dijkstra, Dick Lohuis, Jawaid Khan, and Marcel Prins, for interesting discussions. I like to thank also my friend and colleague Mohamed El Yamani (INRA-MIAC, Settat, Morocco) for his cooperation and the use of his research facilities.

Several Dutch people made my stay in the Netherlands pleasant, and I like to thank the families Bos and Zonnenberg, and all my friends in Wageningen, especially the former occupants of Dijkgraaf 2A, for the 'gezellige' moments spent together.

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CONTENTS

CHAPTER 1

Introduction

Faba bean (*Vicia faba* L.), formerly known as broad bean or field bean, is one of the oldest cultivated plants. This leguminous species originated in the Mediterranean region or Southwest Asia, probably during the late neolithic period (Purseglove, 1968). It is grown in home gardens as a vegetable crop for the shell beans, which are eaten when still green, and as a field crop for the dry beans used for human consumption and livestock feeding.

Faba bean is a major crop in many countries such as China, Egypt, and the Sudan. Throughout the Mediterranean region, and in Ethiopia, West Asia, and parts of Latin America, it is widely grown for human food. In these countries, the crop is of special social, nutritional, and economic significance because it provides an important fraction of protein to the diets of millions of people, mainly in the poorer sectors of the population. In the Sudan, for instance, faba bean is a staple food which is consumed in the three daily meals. In recent years, there has been a growing interest in faba-bean production as a source of protein for animal feed in the European Community, North America, and Australia.

As a cool season crop, faba bean is grown in sub-tropical regions with mild winters during winter, under tropical conditions at high elevations (above 1200m), and in temperate regions in spring. The range of its cultivation extends from about 9° to more than 40°N and from near sea level to more than 2000 m (Saxena, 1982).

In Morocco, where only 10% of the surface of the country is cultivated, cereals are dominant with a percentage of 82, and the second place is occupied by food legumes with merely 11% of the cultivated area. Among food legumes, faba bean is by far the most important crop. It occupies ca. 45% of the surface reserved for food legumes, or on average 200 000 ha yearly. It is grown in non-irrigated areas, and extensively in the northcentral part (Meknès, Fès, and Taounate), the central western plain (Settat), and the

northern part of the country (Chaouen and Tetouan) (Anonymous, 1990). Faba bean is a well-established and important component of the cropping system, mainly on small farms. It is grown every year because of its importance for human subsistence and as a cash crop, its nutritive value due to high protein content (ca. 25% of the dry seeds), and its beneficial effect in crop rotation on soil fertility due to its nitrogen-binding capacity. On small farms located in the non-irrigated areas, the annual crop rotation adopted is usually cereal - faba bean. The cultural practices are still traditional. The yields are very low and generally do not exceed 1000 kg/ha. They are estimated to be only half to one fourth of the potential yield in Morocco (Dahan, 1985). The hectarage occupied by faba bean fluctuates yearly because of many problems such as variation in rainfall and pest damage.

Among the pests attacking faba bean in Morocco, the fungi *Botrytis fabae* and *Uromyces fabae*, the insects *Sitona lineatus*, *Aphis fabae*, and *Aphis craccivora*, the stem nematode *Ditylenchus dipsaci*, and the plant-parasitic broomrape *Orobanche crenata* are occurring most frequently (Schlüter *et al.*, 1976). Equally, and sometimes more important are viruses (Schlüter *et al.*, 1976; Fischer, 1979).

Faba bean is widely used as a test plant in virus research because of its susceptibility and sensitivity to a wide range of viruses. As a crop, it is therefore potentially vulnerable to virus infection. Indeed, so far, 44 viruses have been reported capable of infecting this species (Cockbain, 1983). Few years later, when reviewing the viruses naturally occurring on faba bean, Bos *et al.* (1988) listed some 44 viruses belonging to at least 16 taxonomic groups. They are continuously increasing in number, as are the reports on the occurrence of viruses described elsewhere in countries where they had not been detected before. A new ungrouped, persistently aphid-transmitted, single-stranded DNA virus, named faba bean necrotic yellows virus, has been described lately from Syria (Katul, 1992). It was already found to occur on faba bean in a number of countries in West Asia and North Africa (K.M. Makkouk, personal communication), and on chickpea (Cicer arietinum) in Syria and Turkey (N. Horn, personal communication). The broad bean yellow band serotype of pea early-browning tobravirus (PEBV) described from Italy (Russo *et al.*, 1984) has recently been found in Algeria (Mahir *et al.*, 1992), and a

common strain of PEBV in Libya, where the virus had not been reported before (Bos *et al.*, 1993). Pea seed-borne mosaic potyvirus, another seed-transmitted virus infecting faba bean, has lately been reported from Ethiopia and Libya (Makkouk *et al.*, 1993). Especially in the developing countries, with a paucity of information on viruses occurring in their crops, more investigations are needed to monitor the viruses of faba bean.

In Morocco, the research on faba-bean viruses was initiated in 1976, when diseased samples were collected for identification of the viruses occurring on faba bean in the country. Only six viruses were then detected, namely alfalfa mosaic virus (AMV), bean yellow mosaic potyvirus (BYMV), broad bean mottle bromovirus (BBMV), broad bean stain comovirus (BBSV), broad bean wilt fabavirus (BBWV), and PEBV (El Maataoui and Fischer, 1976). BBSV and BYMV were considered economically the most important viruses. BBWV and BBMV caused severe diseases, but their distribution was limited, while AMV and PEBV were rarely found (Fischer, 1979). Further studies, mainly dealing with yield losses, were conducted in the area of Meknès. N'Aït Mbarek (1978) reported that BBMV, with a spread limited to the northern part of the area, can cause an actual yield loss of dry faba beans up to 52%, whereas BBSV, widespread in the region, caused at places a yield loss of 78%. One year later, Fezzaz and Bourbah (1979) reported that infection by BBSV of faba bean from the seeds leads to a decrease in yield of individual plants developing from such seeds by 64%, and that infections resulting from secondary spread reduced yield by 41%, while for BBMV these figures were 76 and 41%, respectively. Much later, a virus survey in a number of Arab countries showed that out of 15 faba-bean samples collected from farmers' fields in Morocco, one sample contained BBMV, two were found infected by BBSV, one by BYMV, while in 11 samples no virus was detected (Makkouk *et al.*, 1988a). This survey revealed that other non-identified viruses do occur on faba bean in Morocco, and that further studies were needed. At that time, a BBSV isolate and a BBMV isolate from Morocco were studied for some of their properties (Makkouk *et al.*, 1987, 1988b).

This thesis resulted from a 'sandwich' research programme performed alternately at Ecole Nationale d'Agriculture, Meknès, Morocco, and at the Research Institute for Plant

Protection (IPO-DLO) and the Department of Virology of the Agricultural University (LUW), both in Wageningen, the Netherlands. At the beginning, a linkage project existed between IPO and the International Centre for Agricultural Research in the dry Areas (ICARDA), Aleppo, Syria. The latter institute aims at improving agricultural development in West Asia and North Africa, and faba bean is one of the crops on which it had a mandate among the International Agricultural Research Centres (IARCs). More recently, responsibility for the faba-bean improvement programme has largely been transferred to national institutions. The present study was undertaken in support of the programme and to prevent future virus problems which might result from developing agriculture (Bos, 1991), and because the information on faba-bean viruses in the country is still limited.

For this purpose, a survey was first carried out to identify the viruses occurring on faba bean in Morocco and their geographical distribution and incidence were determined (Chapter 2). BBMV and members of the luteovirus group turned out to be prevalent, and BYMV to be of potential importance. Therefore, these viruses were further investigated. Since BBMV isolates were found to differ in symptom severity, and this pathogenic variation is of paramount importance to breeding programmes, investigations were made on the variability of the virus, its interaction with food-legume species, and its seed transmission (Chapter 3). The high incidence of BBMV and the vulnerability of food legumes other than faba bean suggested the involvement of vectors as well as the natural occurrence of the virus in leguminous weeds and cultivated food legumes, and these ecological aspects were also studied (Chapter 4). Proper identification of the luteoviruses infecting faba bean in the country by means of serology only proved insufficient. Therefore, the use of other tools, such as the polymerase chain reaction (PCR) and molecular hybridization was investigated (Chapter 5). Since a number of potyvirus isolates resembling BYMV were detected during the survey, and given the problem of variation within the cluster of legume potyviruses, further studies were aimed at the identification of potyvirus isolates from faba bean and this required more investigations on the differentiation between BYMV and the closely related clover yellow vein virus (Chapter 6). Chapter 7 reviews the recent developments in the identification and the taxonomy of the BYMV-subgroup of potyviruses, and reports on preliminary PCR study

using a set of oligonucleotide primers designed from the known coat-protein-gene sequences of strains of BYMV and CYVV in order to discriminate between these related viruses. Chapter 8 finally discusses the achievements of the investigations, presents some recommendations, and outlines prospects for further research on the viruses of faba bean and other food legumes in Morocco.

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CHAPTER 2

Survey of faba bean (*Vicia faba* L.) for viruses in Morocco

M. FORTASS and L. BOS

SUMMARY

A total of 52 faba-bean fields, located in the main growing areas in Morocco were surveyed for viruses. From the 240 collected samples with symptoms suggestive of virus infection, the following viruses were detected using electron microscopy, serology, and biological indexing: alfalfa mosaic virus (AMV), bean yellow mosaic virus (BYMV), broad bean mottle virus (BBMV), broad bean stain virus (BBSV), broad bean true mosaic virus (BBTMV), pea early-browning virus (PEBV), pea enation mosaic virus (PEMV), pea seed-borne mosaic virus (PSbMV), and a complex of luteoviruses including bean leafroll virus (BLRV). This is the first report of the occurrence of BBTMV, PEMV, PSbMV, and the luteoviruses (including BLRV) on faba bean in Morocco.

The luteoviruses and BBMV were found to be the most prevalent. They were detected in 56 and 50%, respectively, of the surveyed fields; while AMV, BBSV, and PEBV were found in single fields only. The remaining viruses were less prevalent, and were detected in a range of 4 to 15% of the fields surveyed. The incidences per field of the prevalent viruses varied and ranged from 1 to 33% for BBMV and up to 20% in the case of luteoviruses. BBMV was found confined to the central and northern parts of the country, BBTMV and PEMV mainly in the central area, while the luteoviruses and BYMV were spread over the faba-bean growing regions of the country.

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INTRODUCTION

Faba bean (*Vicia faba* L.) is the major food legume grown in Morocco. It occupies around 200.000 ha yearly, and is extensively grown in the central part (Meknès area), the plain of Chaouia (Settat area), and the northwest of the country (Anonymous, 1990). Faba bean is a main component in the cropping system on small farms and in non-irrigated areas, where the recurrent annual crop rotation is cereal - faba bean.

The yields are generally very low, and viruses represent one of the most important constraints(Schluter *et al.*, 1976). On a world basis, 44 viruses have been reported to infect the crop naturally (Bos *et al.*, 1988), but only six viruses have been reported from faba bean in Morocco, without information on their incidence and geographical distribution (El Maataoui and Fischer, 1976). Later, it was claimed that broad bean stain virus (BBSV) and bean yellow mosaic virus (BYMV) are economically the most important viruses infecting faba bean in the country (Fischer, 1979). Recently, a limited survey revealed the occurrence of three viruses only viz. BYMV, broad bean mottle virus (BBMV), and BBSV (Makkouk *et al.* 1988a). However, the number of samples tested so far is extremely low, and no survey covering the different faba-bean growing areas in the country has been conducted.

This chapter describes a systematic survey of the major faba-bean growing regions of Morocco for naturally occurring viruses in faba bean crops and their geographical distribution, incidence and relative importance.

MATERIALS AND METHODS

Survey planning

In March 1988, a preliminary survey was conducted in order to locate the main faba-bean growing areas, to examine the development of the crop with time in different regions and to get a first evaluation of the occurrence of virus infections. The resulting information, and the national crop statistics (Anonymous, 1990), helped to develop the

itinerary of the survey to representatively cover the main faba-bean regions in the country (Fig. 1). The southern area has thereafter been surveyed during the third week of March 1990, and the central and northern areas, one week later. At that time, crops were at the stage of flowering up to early podding.

Choice of fields

The fields to be sampled were chosen systematically by making a stop after each 25 km along the itinerary, and then taking the nearest field. The total number of fields surveyed was 52. Field sizes varied from 0.25 to 2 ha.

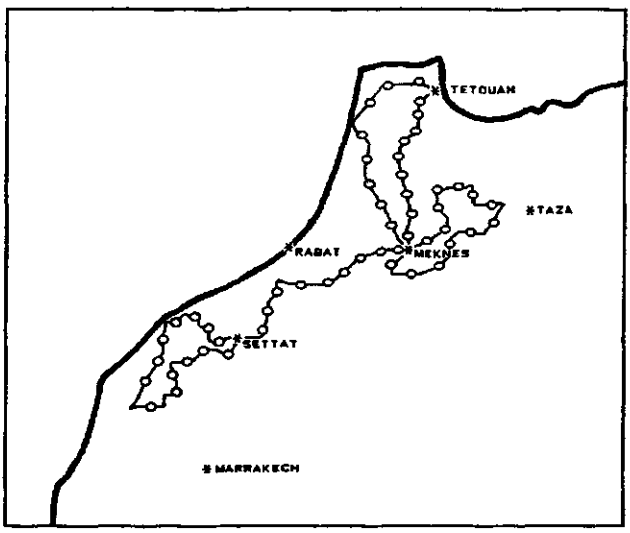

Fig. 1. Itinerary of the survey of faba bean viruses in Morocco (-o-: surveyed field).

Field observations and sample collection

The survey was limited to the viruses causing apparent infections. In each field sampled, the different syndromes suggestive of virus infection in the whole field were described. A sample representing each syndrome was thereafter collected, sealed in a plastic bag, labelled, and put in an ice box for transport. Then, the incidence of plants with each syndrome was assessed by counting the number of plants showing that syndrome in 25%

of the total number of rows regularly distributed over the field. These rows were examined systematically, starting with the first row of the field (Barnett, 1986). In the laboratory, all the collected samples (240 in total) were dried over calcium chloride for later virus identification in Wageningen.

Virus identification

The viruses were identified by electron microscopy, biological indexing to a limited number of test plants, and serological tests.

Electron microscopy. The samples were chopped in 0.2% sodium sulfite, stained in 2% uranyl acetate and viewed in a Philips CM 12 transmission electron microscope.

Biological indexing. The samples were extracted in 0.03 M potassium phosphate buffer, pH 7.7, and inoculated to four plants each of : *Chenopodium amaranticolor*, *C. quinoa*, *Phaseolus vulgaris* 'Bataaf', *Pisum sativum* 'Castro', and *Vicia faba* 'Compacta'. The plants were kept in an insect-free glasshouse for symptom development for at least four weeks.

Serological tests. The samples were extracted in phosphate-buffered saline containing 0.1% Tween 20 and 2% polyvinylpyrrolidone, and tested in DAS-ELISA as described by Clark and Adams (1977). For pea enation mosaic virus (PEMV), the biotin-avidin ELISA was adopted according to Zrein *et al.* (1986).

The antisera to BYMV and clover yellow vein virus (CYVV) were our own (Fortass *et al.*, 1991). Antisera to BBMV, broad bean wilt virus (BBWV), pea early-browning virus (PEBV), alfalfa mosaic virus (AMV), pea seed-borne mosaic virus (PSbMV), and cucumber mosaic virus (CMV) were provided by D.Z. Maat (IPO-DLO, Wageningen, The Netherlands), to PEMV by G. Adam (BBA, Braunschweig, Germany), to BBSV by K.M. Makkouk (ICARDA, Aleppo, Syria), to broad bean true mosaic virus (BBTMV) by H. Rohloff (BBA), and to bean leafroll virus (BLRV) by L. Katul (BBA).

The samples showing symptoms suggestive of luteovirus infection were tested in DAS-ELISA with antisera to potato leafroll virus (PLRV) and BLRV, and in triple antibody sandwich ELISA (TAS-ELISA) with a panel of monoclonal antibodies to PLRV (Van den Heuvel *et al.*, 1990) provided by J.F.J.M. Van den Heuvel (IPO-DLO).

RESULTS

Viruses identified

Among the 52 fields surveyed, 42 (81%) were found to harbour at least one virus. The viruses identified from the collected samples are: AMV, BBMV, BBSV, BBTMV, BYMV, PEBV, PEMV, PSbMV, and a number of luteoviruses including BLRV. The viruses BBTMV, PEMV, PSbMV, and the luteoviruses have not been previously reported from faba bean in Morocco.

BBMV was readily identified by its reactions on test plants. Both *Chenopodium* species reacted with pin-point local lesions two to three days after inoculation, and pea 'Castro' reacted also rapidly with necrotic local lesions, which enlarged and led to withering of inoculated leaves, and stem necrosis. Thus, sample infection by BBMV was recognized as early as three days after inoculation, and identity of the isolates was confirmed serologically. Also in the electron microscope, this virus was readily detected by its high concentration and the dark center of the particles. The variability of the symptoms in 'Compacta' demonstrated the existence of different isolates of BBMV, indistinguishable by ELISA.

Characteristic test-plant reactions also enabled the diagnosis of BYMV, and three different isolates, varying in their symptoms on 'Bataaf' and 'Castro', were identified.

The AMV isolate detected was virulent on bean 'Bataaf', which reacted with vein necrosis and wilting of the inoculated leaves as early as two days after inoculation. Both *Chenopodium* species reacted with systemic chlorotic lesions and severe stunting and leaf deformation. The PEBV isolate was found of low pathogenicity compared to the Dutch isolates. The remaining viruses were not easily identified by biological indexing.

The reactivity in ELISA of the samples with field symptoms suggestive of luteovirus infection was complex. Some samples reacted with BLRV antiserum only, others with PLRV and the PLRV monoclonal antibodies(MAb) WAU-A12 and WAU-A13, whereas twelve samples reacted only with WAU-A12. This MAb was found to react strongly with beet western yellows virus and not with the Dutch isolate of BLRV (Van den Heuvel *et al.*, 1990). These serological patterns suggest that in addition to BLRV, and its possible

deviant strains, other luteovirus(es) naturally occur on faba bean in Morocco. In this report, they are treated as a group.

Field symptoms

The symptoms produced by the different viruses were extremely variable. This may have been due to the genetical variability of the faba-bean landraces grown, and to the time of infection.

The symptoms produced by BBMV varied from green mottling or distinct green veinbanding mosaic in most of the leaves (most likely due to early infection) to vein chlorosis or interveinal mosaic on the upper leaves of the plant (in case of late infection). BYMV induced symptoms varying from severe green blotching to green or yellow mosaic in the upper leaves. The plants infected with PEMV showed either vein clearing or characteristic translucent leaf spotting. Symptoms induced by the luteoviruses consisted of interveinal yellowing, upward rolling and brittleness of the leaves. The early infected plants were generally stunted, necrotic, and produced few or no pods at all (Fig. 2).

Fig. 2. Field symptoms induced by early infection of a luteovirus on faba bean. Healthy plant on the right.

For the remaining viruses, the symptoms consisted of mosaic and reduction in growth and leaf size in case of BBSV infection, leaf narrowing and growth reduction in case of PSbMV, and clear mosaic of the upper leaves in case of BBTMV.

Virus incidence

The number of samples from which the viruses have been identified and the percentages of samples infected with them are shown in Table 1. The luteoviruses and BBMV appear to be the most prevalent viruses in faba bean in Morocco. Next in incidence rank BYMV, PEMV, and BBTMV. The remaining viruses were found incidentally only.

Because of the great variation of the symptoms, especially of BBMV, the number of samples from a given field in which a virus is detected does not reflect the actual incidence of that virus. Table 2 records the incidence of the fields infested with the respective viruses. It shows that the viruses found can be grouped into three categories based on their incidence. The first category includes the luteoviruses and BBMV which were detected in 56 and 50%, respectively, of the surveyed fields. They are thus the most prevalent viruses in faba-bean crops in Morocco. The second category comprises the viruses PEMV, BBTMV, BYMV, and PSbMV which were less frequent (detected in a range decreasing from 15 to 4% of the fields). The third category of viruses includes AMV, BBSV, and PEBV, each of these was detected in one field only.

Mixed infections

Most viruses were found to occur in single infections. The mixed infections and their relative incidences are listed in Table 3. The mixed infections by BBMV and BBTMV and by PEMV and BBMV were the most prevalent. They were found each in 8% of the surveyed fields.

Number of viruses detected per field

The number of viruses detected per field varied from one to four (Table 4). Among the surveyed fields, 48% harboured two viruses, generally BBMV and a luteovirus, and 36% of the fields harboured only one virus. Four viruses were detected in a single field. Thus,

the number of fields harbouring more than one virus is high.

Table 1. Viruses identified from the faba-bean samples collected during the survey arranged according to incidence.

Virus(es)[1]	Number of infected samples	% of total number of samples
BBMV	73	30.4
Luteoviruses	28	11.6
BYMV	9	3.7
PEMV	8	3.3
BBTMV	6	2.5
PSbMV	2	0.8
AMV	1	0.4
BBSV	1	0.4
PEBV	1	0.4

[1] AMV: alfalfa mosaic virus, BBMV: broad bean mottle virus, BBSV: broad bean stain virus, BBTMV: broad bean true mosaic virus, BYMV: bean yellow mosaic virus, PEBV: pea early-browning virus, PEMV: pea enation mosaic virus, PSbMV: pea seed-borne mosaic virus.

Table 2. Prevalence of the viruses identified from the survey.

Virus(es)[1]	Number of fields where found	% of total number of fields
Luteoviruses	28	55.7
BBMV	26	50.0
PEMV	8	15.4
BBTMV	5	9.6
BYMV	5	9.6
PSbMV	2	3.8
AMV	1	1.9
BBSV	1	1.9
PEBV	1	1.9

[1] For explanation of acronyms, see Table 1.

Geographical distribution

The geographical locations at which the viruses were detected in faba bean in Morocco

are shown in Fig. 3. BBMV (Fig. 3A) appears to be more or less confined to the central and northern parts of the country, although also found in the area of Settat. The luteoviruses (Fig. 3B) were spread nearly all along the route of the survey. BYMV (Fig. 3E) occurred incidentally only and in different regions. BBTMV (Fig. 3C) and PEMV (Fig. 3D) were found in the central part, and the remaining viruses were detected in one or two fields in the southern part of the surveyed area.

Table 3. Mixed infections detected in faba-bean samples collected during the virus survey.

Virus mixture[1]	Number of positive samples	Number of fields where found	% of total number of fields
AMV - BYMV	1	1	1.9
BBMV - BBTMV	4	4	7.7
BBMV - PEMV	4	4	7.7

[1] For explanation of acronyms, see Table 1.

Table 4. Number of viruses detected per field, and incidences of multiple infestation.

Number of viruses[1]	Number of fields	% of infested fields
1	15	35.7
2	20	47.6
3	6	14.3
4	1	2.4

[1] For explanation of acronyms, see Table 1.

Field incidences of the prevalent viruses

The number of virus-infected plants per field is generally very low, except for the prevalent viruses (BBMV and the luteoviruses). The variability of their incidences is represented in Fig. 4. The incidence of BBMV per field is generally less than 10%, with

a maximum of 33% recorded in one field. The incidence of the luteoviruses recorded in the majority of the fields ranged between 1 and 10%, the highest incidence recorded is 20%.

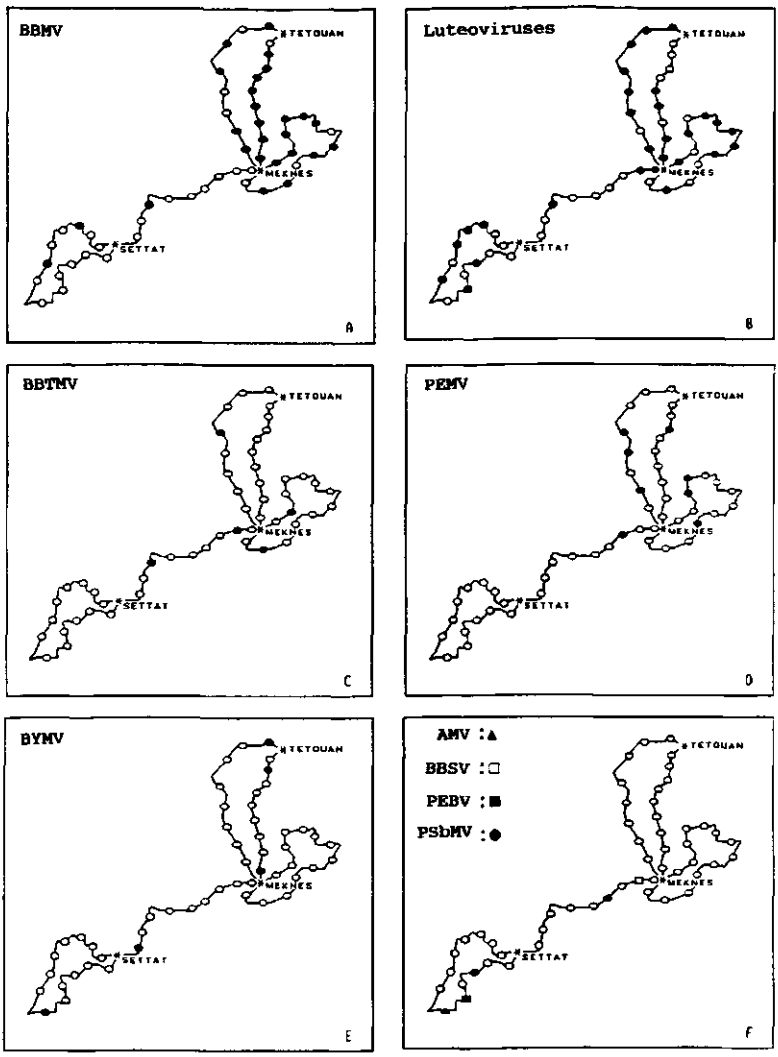

Fig. 3. Geographical distribution of the faba-bean viruses in Morocco.

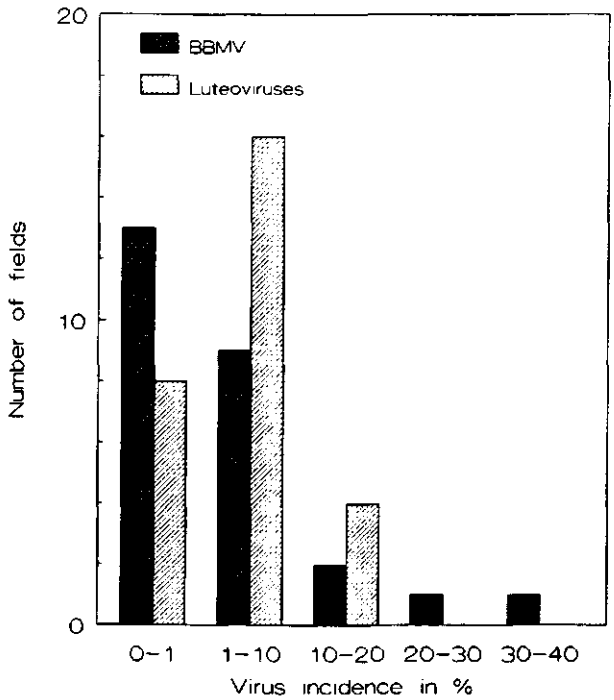

Fig. 4. Incidence of BBMV and the luteoviruses in surveyed faba-bean fields.

DISCUSSION

Of the viruses identified from the samples collected during the survey, PEMV, PSbMV, BBTMV, and the luteovirus complex (including BLRV) have not been previously reported from Morocco. The first three have been reported from most of the countries in West Asia and North Africa (Makkouk *et al.*, 1988a), and this paper now reports their natural occurrence in faba bean in Morocco. Our results show that the luteoviruses and BBMV are the most prevalent viruses in faba bean crops in Morocco.

Although earlier reports considered BBMV of limited distribution and of no economical importance (Fischer, 1979), it now appears to be prevalent in the country. On a world basis, BBMV seemed to be of restricted geographical distribution. It has been reported

for the first time from England (Bawden *et al.*, 1951) and later incidentally from Portugal (Borges and Louro, 1974), Sudan (Murant *et al.*, 1974), Morocco (El Maataoui and Fischer, 1976), China (Ford *et al.*, 1981), and Algeria (Ouffroukh, 1985). It was recently reported from Tunisia, Egypt, Lebanon, and Syria (Makkouk *et al.*, 1988b). Although so far considered of mere academic interest only, it is now known to be widespread throughout the West Asia and North Africa, and to have high incidence in Morocco. The transmission of BBMV is not yet known, although seed transmission is suspected (Bawden *et al.*, 1951; N'Ait Mbarek, 1978), and has been reported but at low rate when the virus occurs in mixed infection with BYMV (Murant *et al.*, 1974; Makkouk *et al.*, 1988b). Information on the role of vectors is also limited. Walters and Surin (1973) reported experimental inefficient transmission by the beetles *Acalymma trivittata*, *Diabrotica undecimpunctata* and *Colaspis flavida*, while Borges and Louro (1974) reported a similarly poor transmission by the weevil *Sitona lineatus*.

The prevalence of the virus in Morocco and the high incidence recorded in some fields may be due to a high rate of seed transmission and/or the existence of efficient vector(s). Its reported wide host range, mainly among legume species (Makkouk *et al.*, 1988b), suggests the existence of natural sources of infection contributing to its potentiel importance. Further investigations are needed to determine the ways of transmission of this virus in Morocco, survey other legumes for natural infection, study the variability of the virus, and evaluate the genetic vulnerability of the promising breeding lines of food legume species to the variants of the virus.

Our serological data revealed that faba bean in Morocco is infected by a complex of luteoviruses including BLRV, or deviant strains of the latter. The recorded incidences of this group of viruses did not exceed 20%, but fields in the area of Meknés visited late in the season showed very high incidences. Thus a field survey around May would give a different assessment of the incidence (number of fields infested and number of plants infected per field) of the luteoviruses. In addition, the transmission by aphids in a persistent manner, and the dramatic damage to faba bean make the luteoviruses of prime importance. Further studies are required for complete identification through specificity of aphid transmission and host range (Johnstone *et al.*, 1984) and serology.

BBSV which was considered earlier to be widespread in Morocco (Fischer and Lockhart, 1976) has been found in our survey in one single field only, and is now considered of no economical importance. This decrease in the incidence of this virus may be due to a reduction in the population density of its vector, and therefore to a situation of self-elimination as reported in Scotland by Jones (1978).

BYMV, also previously reported as an economically important faba-bean virus in Morocco (Fischer, 1979), appears to be less prevalent. However, it occurs in different areas of the country, is seed and aphid transmitted, and therefore remains be of potential importance.

The newly reported viruses PEMV and BBTMV occur in widely separate regions, mainly in the central-northern part of the country, while PSbMV was encountered in two fields only. Further surveys would evaluate the dynamic of their occurrences, and surveying of peas for viruses would most probably reveal the occurrence of PEMV and PSbMV in this crop.

In our survey we did not come across BBWV, although the virus has been reported from Morocco in faba bean (El Maataoui and Fischer, 1976) and in peppers (Lockhart and Fischer, 1976). CMV, which has been reported from faba bean in most of the countries in West Asia and North Africa, is still not encountered in faba bean in Morocco, neither does CYVV. More and repeated surveying is undoubtedly needed to monitor the dynamic occurrence of these viruses. Surveys at different times of the year may give an assessment of their dynamic build-up during the season. Meanwhile, more studies on the prevalent viruses, i.e. BBMV and the luteoviruses, including yield loss assessement are required for faba-bean improvement in Morocco.

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CHAPTER 3

Broad bean mottle virus in Morocco; variability, interaction with food legume species, and seed transmission in faba bean, pea, and chickpea

M. FORTASS and L. BOS

SUMMARY

Biological indexing of faba-bean samples collected during an earlier virus survey in Morocco revealed variation in symptom severity among isolates of BBMV. When further comparing seven selected isolates from Morocco and three from Algeria, Sudan, and Tunisia, they could be divided into mild, severe, and intermediate isolates, according to their pathogenicity on a number of food-legume genotypes tested. The Moroccan isolate SN1 and the Sudanese SuV256 were very mild, and deviant also in their effect on *Gomphrena globosa*, whereas the Tunisian TV75-85 and the Moroccan VN5 were virulent. Representative isolates were indistinguishable, however, in coat-protein molecular weights, and they reacted similarly to the antisera to a Moroccan and a Syrian isolate in electro-blot immunoassay.

Promising ICARDA breeding lines and accessions - ten each of pea and lentil, nine of chickpea, and twelve of faba bean - were all found vulnerable (susceptible and sensitive) to all isolates. Within each food-legume species, vulnerability varied from high to moderate, and no immunity was detected. Virus concentrations in faba-bean lines suggest that isolates differ in virulence rather than in aggressiveness, and that the differences in

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vulnerability among the lines are due to differences in sensitivity rather than in susceptibility.

When pooled seed samples were germinated and seedlings were tested for BBMV in DAS-ELISA, the virus was found seed-transmitted in faba bean, chickpea, and pea at transmission rates of ca. 1.2, 0.9, and 0.1 %, respectively. This is the first report on seed transmission of BBMV in faba bean, when occurring on its own, and the first record of such seed transmission in chickpea and pea.

INTRODUCTION

Broad bean mottle virus (BBMV) was first described from faba bean (*Vicia faba* L.) in England by Bawden *et al.* (1951). Much later, it was reported from the same crop in Portugal (Borges and Louro, 1974), and then occasionally from Sudan and North Africa (Murant *et al.*, 1974; Fischer, 1979; Ouffroukh, 1985; Makkouk *et al.*, 1988a), and from China (Ford *et al.*, 1981) and West Asia (Makkouk *et al.*, 1988a). The virus is now known to be able to infect a wide range of legume species and to be widespread in faba bean in West Asia and North Africa, the outreach region of the International Center for Agricultural Research in the Dry Areas (ICARDA), Aleppo, Syria (Makkouk *et al.*, 1988b).

During systematic surveys of faba bean for viruses in Morocco, BBMV was found to be widely distributed there and to occur in high incidences in farmers' fields (Fortass and Bos, 1991). The biological indexing of the survey samples revealed BBMV isolates which differed in symptom severity. Information on such pathogenic variation is essential for breeding programmes. The variability of the virus in Morocco, the interaction of Moroccan isolates with agronomically promising ICARDA breeding lines of major food legume species, and seed transmission in faba bean, pea, and chickpea were therefore studied in detail.

MATERIALS AND METHODS

Virus isolates

Seven Moroccan isolates of BBMV, collected from different faba-bean growing areas and found to differ in symptom severity on some host plants, were used in this study. They were given the codes BN1, F2, FN1, ON4, SN1, UN2, and VN5; referring to the areas of collection during the survey (Fortass and Bos, 1991). The isolates were screened by ELISA, electron microscopy, and biological indexing for absence of other viruses infecting faba bean. Three other isolates from Algeria (AlgB1), Sudan (SuV256), and Tunisia (TV75-85) were included for comparison. TV75-85 and SuV256 were from the IPO-DLO collection and earlier described by Makkouk *et al.* (1988b) and Bos *et al.* (1992). AlgB1 was provided by A. Ouffroukh (INPV, El Harrach, Algiers, Algeria). All virus isolates were obtained from faba-bean field samples, and were stored dessicated over calcium chloride. They were revived and maintained in the glasshouse on *Vicia faba* 'Compacta'.

Host-range studies

The virus isolates were extracted in 0.03 M potassium phosphate buffer, pH 7.7, and inoculated to four plants of selected test species. Carborundum, 400 mesh, was used as abrasive. The plants were kept in a glasshouse and observed for symptom development for at least four weeks. The plants, or parts of them showing no visible symptoms, were tested for latent infection in double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) as described by Clark and Adams (1977). The antiserum used was the one to the Moroccan isolate MV90-85 (Makkouk *et al.*, 1988b) since it was found to react with all the isolates under investigation when tested in preliminary assays.

Virus purification

Two selected Moroccan isolates (SN1 and VN5) and the isolates AlgB1, SuV256, TV75-85, and MV90-85 were propagated in *Nicotiana clevelandii*, and purified by two cycles of differential centrifugation according to Hollings and Horváth (1981).

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE of purified virus preparations was carried out according to Laemmli and Favre (1973). The separating gel contained 12% acrylamide, and about 2ug of virus preparation was loaded per slot.

Electro-blot immunoassay (EBIA)

EBIA's of the purified virus preparations were carried out as described earlier (Fortass *et al.*, 1991), using the antiserum to the Moroccan isolate MV90-85 and an antiserum to the Syrian isolate SV48-86 provided by K. M. Makkouk (ICARDA, Aleppo, Syria).

Interaction with food-legume breeding lines

A number of agronomically promising breeding lines and accessions of faba bean, chickpea (*Cicer arietinum*), lentil (*Lens culinaris*), and pea (*Pisum sativum*) were received from the Genetic Resources Unit of ICARDA. Ten lines each of pea and lentil, nine of chickpea, and twelve of faba bean were used to investigate their behaviour to the BBMV isolates, and to evaluate possible differences between isolates in pathogenicity and between host genotypes in vulnerability (susceptibility and sensitivity). Five plants per entry were mechanically inoculated with each virus isolate as described before.

Virus concentration in faba-bean lines

In order to detect possible differences in aggressiveness among virus isolates, and susceptibility among faba-bean lines, the concentrations of all isolates (except SuV256) in inoculated faba-bean breeding lines were evaluated weekly, until five weeks after inoculation, using DAS-ELISA. At a sampling date, tip leaves were selected and ground. The dilutions of the crude extract were the same for all lines, isolates, and sampling times.

Seed-transmission tests

All faba-bean, chickpea and pea plants, used for studying the interaction between food-legume breeding lines and BBMV isolates, were kept in the glasshouse until seed

maturity. All seeds were harvested and sown in steam-sterilized soil. The developing seedlings were collected and tested for BBMV in groups of three, five, or ten in DAS-ELISA. The rates of seed transmission were calculated using the formula of Maury *et al.* (1985): $p=[1-(\frac{Y}{N})^{1/n}]\times 100$, where p is the percentage of infection, Y the number of seedling groups free of virus, N the number of groups tested, and n the number of seedlings per group.

RESULTS

Host range and symptomatology

The test-plant reactions to the virus isolates are summarized in Table 1. All test plants inoculated became infected except *Nicotiana tabacum* 'Samsun'. Infection remained restricted to the inoculated leaves in *Chenopodium amaranticolor*, *Chenopodium quinoa*, both cucumber cultivars, and *Phaseolus vulgaris* 'Bataaf'. These species and cultivars were found good local-lesion hosts, reacting as early as two to three days after inoculation. No differences between isolates were recorded on the basis of the reactions of non-legume species, except that SN1 and SuV256 appeared deviant in their effect on *Gomphrena globosa*.

On the basis of the reactions of the food legume lines and cultivars tested, the BBMV isolates could be divided into three categories, viz. mild (poorly pathogenic) isolates (FN1, SN1, and SuV256), severe (highly pathogenic) isolates (ON4, TV75-85, and VN5), and intermediate isolates (AlgB1, BN1, F2, and UN2). A selected set of genotypes, including *Lens culinaris* ILC 6437 and ILC 5876, *Pisum sativum* Acc. 21 and Acc. 167, and *Vicia faba* FLIP 84-230 and FLIP 86-122, differentiated between the mild and severe isolates.

The severe isolates could be distinguished by the systemic necrosis induced on both pea-breeding accessions 21 and 167, and by the stem necrosis on faba-bean FLIP 84-2037. In addition, the isolate ON4 was the only one causing a systemic necrosis followed by wilting on pea 'Castro' (not listed in Table 1), which reacted typically with necrosis and withering of the inoculated leaves and stem necrosis (Fig. 1) to the remaining

isolates. The mild isolates, on the other hand, did not induce any systemic necrosis on any of the food legume lines and cultivars tested. They did not infect the pea accessions systemically, and both lentil lines did not react locally. Moreover, the Sudanese isolate SuV256 appeared still milder as it failed to induce a local reaction on the chickpea lines tested. The remaining isolates behaved differently from the severe and mild ones, depending on the line or cultivar inoculated. All the isolates behaved similarly on *Phaseolus vulgaris* 'Bataaf', and also on *Trifolium incarnatum* except for SuV256, which did not induce a local reaction on the latter.

Table 1. Reactions[a] of selected host plants to different BBMV isolates.

Host plants	BBMV isolates									
	AlgB1	BN1	F2	FN1	ON4	SN1	SuV256	TV75-85	UN2	VN5
Non-legume species										
Chenopodium amaranticolor	L,–	L,–	L,–	L,–	L,–	L,–	L,–	L,–	L,–	L,–
Chenopodium quinoa	L,–	L,–	L,–	L,–	L,–	L,–	L,–	L,–	L,–	L,–
Cucumis sativus										
'Chinese Slangen'	L,–	L,–	L,–	L,–	L,–	L,–	L,–	–,–	L,–	L,–
'Gele Tros'	L,–	L,–	L,–	L,–	L,–	L,–	*,*	L,–	L,–	L,–
Gomphrena globosa	L,S	L,S	L,S	L,S	L,S	l,–	–,s	L,S	L,S	L,S
Nicotiana clevelandii	L,S	L,S	L,S	L,S	L,S	L,S	L,S	L,S	L,S	L,S
Nicotiana tabacum										
'Sumsun'	–,–	–,–	–,–	–,–	–,–	–,–	–,–	–,–	–,–	–,–
Legume species										
Cicer arietinum										
FLIP 85-15	L^n,S	L^n,S	L^n,S	–,S	L^n,S	–,S	–,S	L^n,S	L^n,S	L^n,S
Lens culinaris										
ILC 5876	–,S	–,S	–,S	–,S	–,S^n	–,S	–,S	–,S^n	–,S	L^n,S^n
ILC 6437	L^n,S^n	–,S	L^n,S	L^n,S	L^n,S^n	–,S	–,S	L^n,S^n	L^n,S^n	L^n,S^n
Paseolus vulgaris										
Bataaf	L,–	L,–	L,–	L,–	L,–	L,–	L,–	L,–	L,–	L,–
Pisum sativum										
Acc. 21	L^n,S^n	L^n,S	–,S	–,S	L^n,S^n	L^n,S	–,S	L^n,S^n	L^n,S^n	L^n,S^n
Acc. 167	L^n,–	L^n,–	L^n,–	L^n,S	L^n,S^n	L^n,–	L^n,–	L^n,–	L^n,S^n	L^n,S^n
Trifolium incarnatum	L,S	L,S	L,S	L,S	L,S	L,S	l,S	L,S	L,S	L,S
Vicia faba										
FLIP 84-230	L,S	–,S	L,S	–,S	L^n,S	–,S	–,S	L^n,S	L,S	L^n,S^n
FLIP 86-122	L,S	L,S	–,S	–,S	L^n,S	–,S	–,S	L^n,S	–,S	L^n,S

[a] L, local symptoms; S, systemic symptoms; l, latent local infection; s, latent systemic infection; n, necrotic; –, no infection; *, not tested.

Electrophoretic analysis

All purified isolates revealed, on SDS-polyacrylamide gels, a single polypeptide with a molecular weight of ca. 22 Kd. They behaved similarly and were indistinguishable in coat-protein molecular weights.

EBIA

The reactivity of the purified isolates to the antiserum to the Moroccan isolate MV90-85 in EBIA is shown in Fig. 2. All isolates reacted similarly and with the same intensity. The reactions were also found similar when an antiserum to the Syrian isolate SV48-86 was used (data not shown).

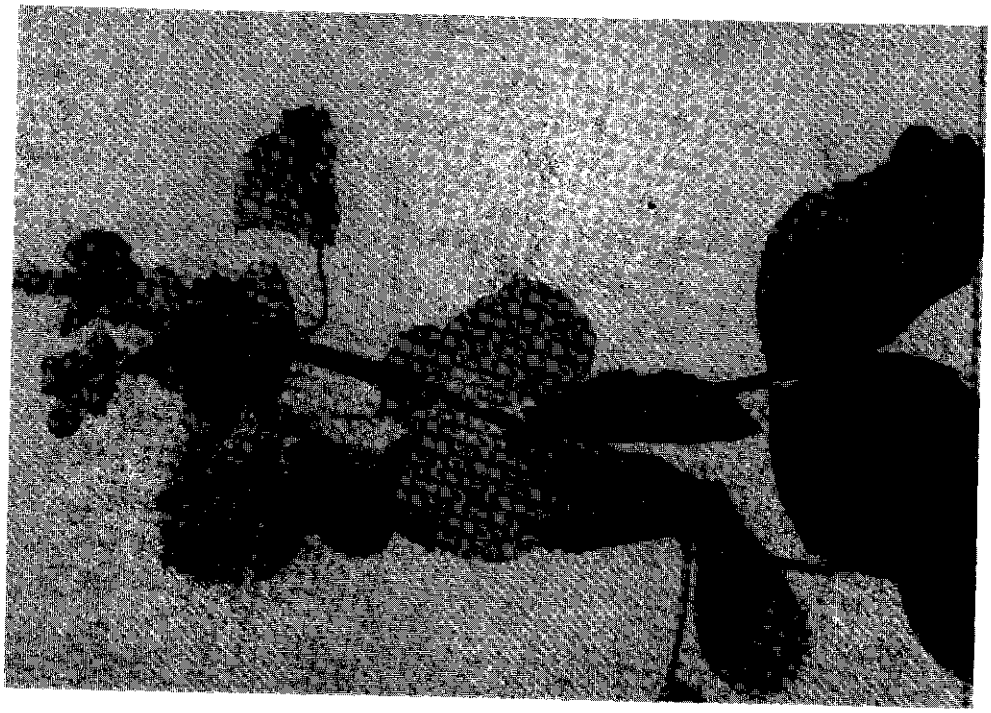

Fig. 1. Withering of inoculated leaves and stem necrosis induced by BBMV-FN1 on *Pisum sativum* 'Castro', 17 days after mechanical inoculation.

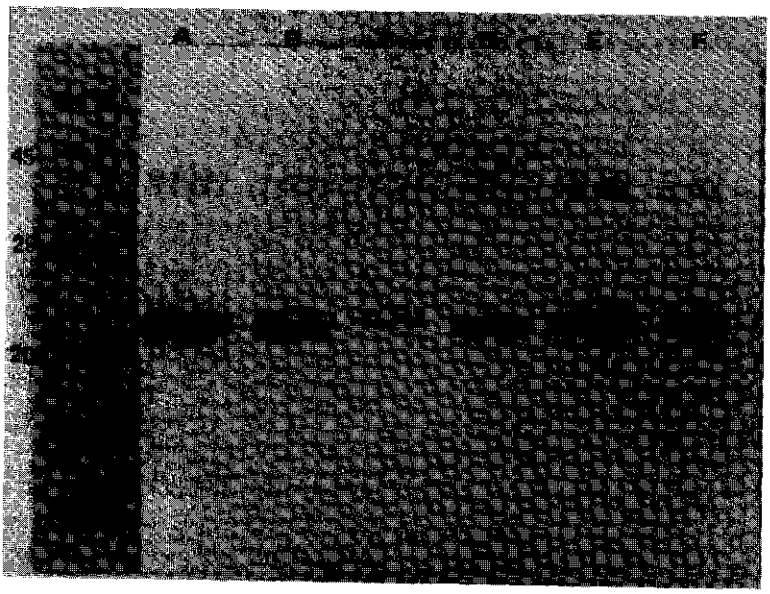

Fig. 2. Reactivity in EBIA of BBMV isolates to the antiserum to the Moroccan isolate MV90-85. A: SN1, B: VN5, C: AlgB1, D: SuV256, E: TV75-85, and F: MV90-85.

Food-legume genotype reactions

Faba bean. The reactions of the different ICARDA breeding lines of faba bean to the virus isolates under investigation are summarized in Table 2. All lines were found vulnerable to all the isolates. They all reacted with the mottling characteristic of BBMV infection (Fig. 3), but differences could be observed on the basis of presence or absence of local symptom and the severity of stunting. On faba bean, the isolates VN5, ON4, TV75-85, and AlgB1 were more pathogenic than the remaining isolates. They induced a local reaction (necrotic in some combinations) and varying degrees of stunting on most lines. The isolate VN5 appeared to be the most pathogenic one as judged by the necrotic local reaction and stunting it induced. The Sudanese isolate SuV256 and the Moroccan SN1 induced neither a local reaction (except on the most sensitive lines) nor a stunting on any of the lines tested. The ICARDA breeding lines FLIP86-114, 86-117, and 86-119 appeared to be the most vulnerable, and the lines FLIP 87-26, 86-146, and 86-122 the least.

Pea. All tested accessions of pea were found vulnerable (Table 3). The isolates VN5, TV75-85, and UN2 were highly pathogenic on this host species. In addition to the typical reaction consisting of wilting of the inoculated leaves and stem necrosis, some highly vulnerable accessions reacted with stunting, systemic necrosis, or wilting (Fig. 4). The pea lines tested can be grouped into highly vulnerable (ICARDA accessions 21, 22, 62, and 125) and less vulnerable (accessions 101, 154, 167, 169, and 30). No seeds were produced by the plants of accession 22 inoculated with the different BBMV isolates. The accessions 8, 21, and 22 did not react with symptoms typical of BBMV, but with an unusual yellow mosaic (Fig.4, middle).

Chickpea. The reactions of the chickpea lines tested are summarized in Table 4. These data show that the isolates VN5, TV75-85, and to a lesser extent UN2 , are the most pathogenic on chickpea, and that the isolates SuV256, FN1, and SN1 are mild (Fig. 5). All lines tested were found vulnerable to all isolates. They can be grouped into the highly vulnerable (ILC 482, FLIP 82-150, and FLIP 83-47), the less vulnerable (FLIP 87-69, and 85-15), and intermediate lines with reactions varying according to isolate.

Lentil. All lentil lines tested were found vulnerable, except ILC 5845, 5999, 6442, and 6773 which did not react to the mild isolate SuV256. Isolates VN5, TV75-85, ON4, and UN2 were the most pathogenic. They often induced a local necrotic reaction and systemic yellow mosaic and necrosis (depending on the line). Isolates SuV256, SN1, BN1, and FN1 did not induce any local reaction on most lines tested, while the systemic reaction to them was limited to a mosaic or mild stunting. The breeding lines ILC 5845 and 6437 were found highly vulnerable, whereas ILC 6442, 6216, 6763, and 6773 were less vulnerable. The remaining lines behaved differently, depending on the isolate.

Virus concentration in faba-bean lines

The evolution of the isolate concentrations in faba-bean lines shows that they reach the maximum around the second week after inoculation (Fig. 6). No clear differences could be seen between isolates nor between breeding lines. The virus concentration appears

independant of the vulnerability of the line and the pathogenicity of the isolate.

Fig. 3. Systemic mottling on Vicia faba 'Compacta' induced by BBMV-FN1, three weeks after mechanical inoculation. Healthy control on the left.

Fig. 4. Reaction of the pea Acc. 8 to BBMV-UN2 (right) and BBMV-SN1 (middle), 17 days after mechanical inoculation. Uninoculated control on the left.

Fig. 5. Reaction of the chickpea line FLIP 84-92 to two isolates of BBMV, TV75-85 (right) and SN1 (middle), four weeks after inoculation. Uninoculated control on the left.

Table 2. Reactions[a] of the faba-bean breeding lines, arranged from top to bottom according to decrease in vulnerability, to the BBMV isolates arranged from left to right according to increase in severity.

	SN1	F2	SuV256	FN1	BN1	UN2	AlgB1	ON4	TV75-85	VN5
FLIP 86-117	C/Mo	C/Mo	N/Mo	N/Mo	N/Mo	C/Mo	C/Mo ST	N/Mo SN	C/Mo ST	N/Mo sST
FLIP 86-119	C/Mo	C/Mo	N/Mo	N/Mo	N/Mo	C/Mo	C/Mo ST	N/Mo	C/Mo ST	N/Mo sST
FLIP 86-114	–/Mo	C/Mo	N/Mo	N/Mo	–/Mo Nc	C/Mo	N/Mo	N/Mo	N/Mo sST	N/Mo sST
FLIP 86-107	–/Mo	C/Mo	–/Mo	N/Mo	–/Mo	C/Mo	N/Mo ST	N/Mo SN,sST	C/Mo sST	N/Mo SN,sST
FLIP 86-115	–/Mo	C/Mo	–/Mo	–/Mo	N/Mo	C/Mo	N/Mo ST	N/Mo	C/Mo sST	N/Mo SN,sST
FLIP 86-116	–/Mo	–/Mo	–/Mo	N/Mo	–/Mo	C/Mo	N/Mo ST	N/Mo SN	C/Mo sST	N/Mo sST
FLIP 84-230	–/Mo	C/Mo	–/Mo	–/Mo	–/Mo	C/Mo	C/Mo	N/Mo	N/Mo ST	N/Mo VN, ST
FLIP 84-237	–/Mo	–/Mo	–/Mo	–/Mo	N/Mo	C/Mo	C/Mo ST	N/Mo	C/Mo ST	N/Mo sST
FLIP 85-172	–/Mo	–/Mo	–/Mo	–/Mo	–/Mo	N/Mo	C/Mo ST	N/Mo	–/Mo ST	N/Mo SN, ST
FLIP 87-26	–/Mo	–/Mo	–/Mo	–/Mo	C/Mo	N/Mo	C/Mo	N/Mo	C/Mo ST	N/Mo ST
FLIP 86-146	–/Mo	C/Mo	–/Mo	–/Mo	C/Mo	C/Mo	C/Mo	–/Mo	N/Mo ST	N/Mo ST
FLIP 86-122	–/Mo	–/Mo	–/Mo	–/Mo	C/Mo	–/Mo	C/Mo	N/Mo	N/Mo ST	N/Mo ST

[a] Local reaction/systemic reaction: C, local chlorosis; N, local necrosis; –, no reaction; Mo, mottle; SN, stem necrosis; ST, stunting; sST, severe stunting; Nc, necrosis; VN, vein necrosis.

Table 3. Reactions[a] of the pea accessions, arranged from top to bottom according to decrease in vulnerability, to the BBMV isolates arranged from left to right according to increase in severity.

	FN1	SuV256	F2	BN1	SN1	AlgB1	TV75-85	ON4	VN5	UN2
Acc. 22	W/Mo ST	–/Mo	W/YMo sST	W/Mo ST	W/Mo SN,ST	W/Mo SN	W/YMo ST	W/Mo SN,ST	W/YMo ST	W/Mo SN,ST
Acc. 125	W/SN	W/Mo SN	W/SN	W/SN	W/Nc W	W/SN Nc,W	W/SN Nc,W	W/Mo SN	W/Nc W	W/SN Nc,W
Acc. 62	W/SN	W/SN	W/SN	W/SN	W/SN	W/SN W	W/SN W	W/SN W	W/SN W	W/SN W
Acc. 21	–/YMo	–/Mo	–/YMo	W/YMo	W/YMo	W/Nc W	W/Nc W	W/YMo Nc	W/Mo Nc	W/Nc W
Acc. 8	–/YMo	W/Mo	–/YMo ST	–/YMo	W/YMo	W/Nc W	W/Nc W	W/YMo	W/Mo	W/Nc W
Acc. 101	W/SN	W/SN	W/SN	W/SN	W/SN	W/SN	W/SN	W/SN	W/SN	W/SN
Acc. 154	W/SN	W/SN	W/SN	W/SN	W/SN	W/SN	W/SN	W/SN	W/SN	W/SN
Acc. 30	W/SN	W/SN	W/SN	W/SN	W/SN	W/SN	W/SN	W/SN	W/SN	W/SN
Acc. 169	W/SN	W/SN	W/–	W/–	W/SN	W/SN	W/–	W/SN	W/SN	W/SN
Acc. 167	W/–	W/–	W/–	W/–	W/–	W/–	W/–	W/SN	W/SN	W/SN

[a] Local reaction/systemic reaction: W, wilting; –, no reaction; Mo, mosaic; YMo, yellow mosaic; ST, stunting; sST, severe stunting; SN, stem necrosis; Nc, necrosis.

Table 4. Reactions[a] of the chickpea breeding lines, arranged from top to bottom according to decrease in vulnerability, to the BBMV isolates arranged from left to right according to increase in severity.

	SuV256	SN1	FN1	BN1	F2	AlgB1	ON4	UN2	VN5	TV75-85
FLIP 82-150	–/mMo ST	N/VY ST	N/Mo ST	N/Mo ST	N/Mo ST	N/Mo ST	N/Mo ST,FL	N/Mo ST,FL	N/Mo ST,FL	N/sMo sST,FL
FLIP 83-47	–/mMo	N/VY ST	N/Mo ST	N/Mo ST,FL	N/Mo ST	N/Mo ST	N/Mo ST	N/Mo ST,FL	N/Mo ST,FL	N/sMo sST,FL
ILC 482	–/mMo	N/VY ST	N/Mo ST,FL	N/Mo ST	N/Mo ST,FL	N/Mo ST	N/Mo ST	N/Mo ST	N/Nc W	N/sMo sST,FL
FLIP 84-15	–/mMo	N/VY ST	N/mMo ST	N/Mo ST	N/Mo ST	N/Mo ST	N/Mo ST	N/Mo ST	N/Mo ST,FL	N/sMo sST
FLIP 84-92	–/mMo	–/VY	N/Mo ST	N/Mo ST	N/Mo ST	–/Mo ST	N/Mo ST	N/Mo ST	N/Mo ST	N/sMo sST
ILC 263	–/Mo	–/VY ST	–/Mo ST	N/Mo ST	N/Mo ST	N/Mo ST	–/Mo ST	N/Mo ST	N/Mo ST	N/sMo sST
FLIP 81-293	–/mMo	–/Mo ST	–/Mo ST	N/Mo ST	N/Mo ST	–/Mo ST	–/Mo ST	N/Mo ST	N/Mo ST	N/sMo sST
FLIP 87-69	–/mMo	–/VY ST	–/VY ST	N/Mo ST	–/Mo ST	N/Mo ST	–/Mo ST	N/Mo ST	N/Mo ST	N/Mo ST
FLIP 85-15	–/mMo	–/VY	–/VY	N/Mo	N/Mo	N/Mo ST	N/Mo ST	N/Mo ST	N/Mo ST	N/Mo ST

[a] Local reaction/systemic reaction: N, local necrosis; –, no reaction; Mo, mosaic; mMo, mild mosaic; sMo, severe mosaic; VY, vein yellowing; ST, stunting; sST, severe stunting; FL, filiform leaves; W, wilting; Nc, necrosis.

Table 5. Reactions[a] of the lentil breeding lines, arranged from top to bottom according to decrease in vulnerability, to the BBMV isolates arranged according to increase in severity.

	SuV256	BN1	SN1	FN1	F2	AlgB1	ON4	TV75-85	UN2	VN5
ILC 6437	–/Mo	–/Mo	–/Mo	N/Mo	N/Mo	N/Mo Nc	N/Mo Nc	N/Mo Nc	N/Nc	N/Mo Nc
ILC 5845	–/–	–/Mo	N/Mo	N/Mo	N/Mo	–/Mo	N/Nc	N/Mo Nc	N/Nc	N/Nc
ILC 5876	–/ST	–/Mo	–/Mo	–/Mo	–/Mo	–/Mo	–/Mo Nc	–/Mo Nc	–/Mo	N/Mo Nc
ILC 5722	–/ST	–/Mo	–/Mo ST	–/Mo	–/Mo ST	N/Mo	N/Mo Nc	–/Mo Nc	N/Mo Nc	N/Mo Nc
ILC 5999	–/–	–/Mo	–/Mo	–/Mo	–/Mo	–/Nc	–/Mo	–/Mo	N/Mo Nc	W/Mo Nc
ILC 6246	–/Mo	N/–	–/Mo	–/Mo	N/Mo	N/–	–/Mo	–/Mo Nc	–/Mo	N/Mo Nc
ILC 6442	–/–	–/Mo	–/Mo	–/Mo	–/Mo	–/Mo	N/Mo	–/Mo Nc	–/Mo	–/Mo Nc
ILC 6763	–/ST	–/Mo	–/Mo	–/Mo	–/Mo	–/Mo	N/Mo	–/Mo	N/Mo	–/Mo Nc
ILC 6216	–/Mo	–/Mo	–/Mo	–/Mo	–/Mo	–/Mo	–/Mo	–/Mo	–/Mo	N/Mo Nc
ILC 6773	–/–	–/VY	–/Mo	–/Mo	–/Mo	–/Mo	–/Mo	–/Mo	–/Mo	–/Mo

[a] Local reaction/systemic reaction: N, local necrosis, –, no reaction; W, wilting; –, no reaction; Mo, mosaic; ST, stunting; VY, vein yellowing; Nc, necrosis.

Seed transmission

The results of seed-transmission tests are shown in Table 6. From the 330, 315, and 1300 seedlings grown from pooled seeds produced by inoculated faba bean, chickpea, and pea plants, respectively, groups were found infected after ELISA testing. The transmission rates were calculated and found to be 1.21, 0.95, and 0.07 % in faba bean, chickpea, and pea respectively.

Table 6. Seed-transmission rates of BBMV in faba bean, chickpea, and pea.

Food-legume species	Number of seedlings tested	Number of seedlings per group	Number of groups positive in ELISA	Rate of transmission in %
Faba bean	330	3	4	1.21
Chickpea	315	3	3	0.95
Pea	1300	10	1	0.07

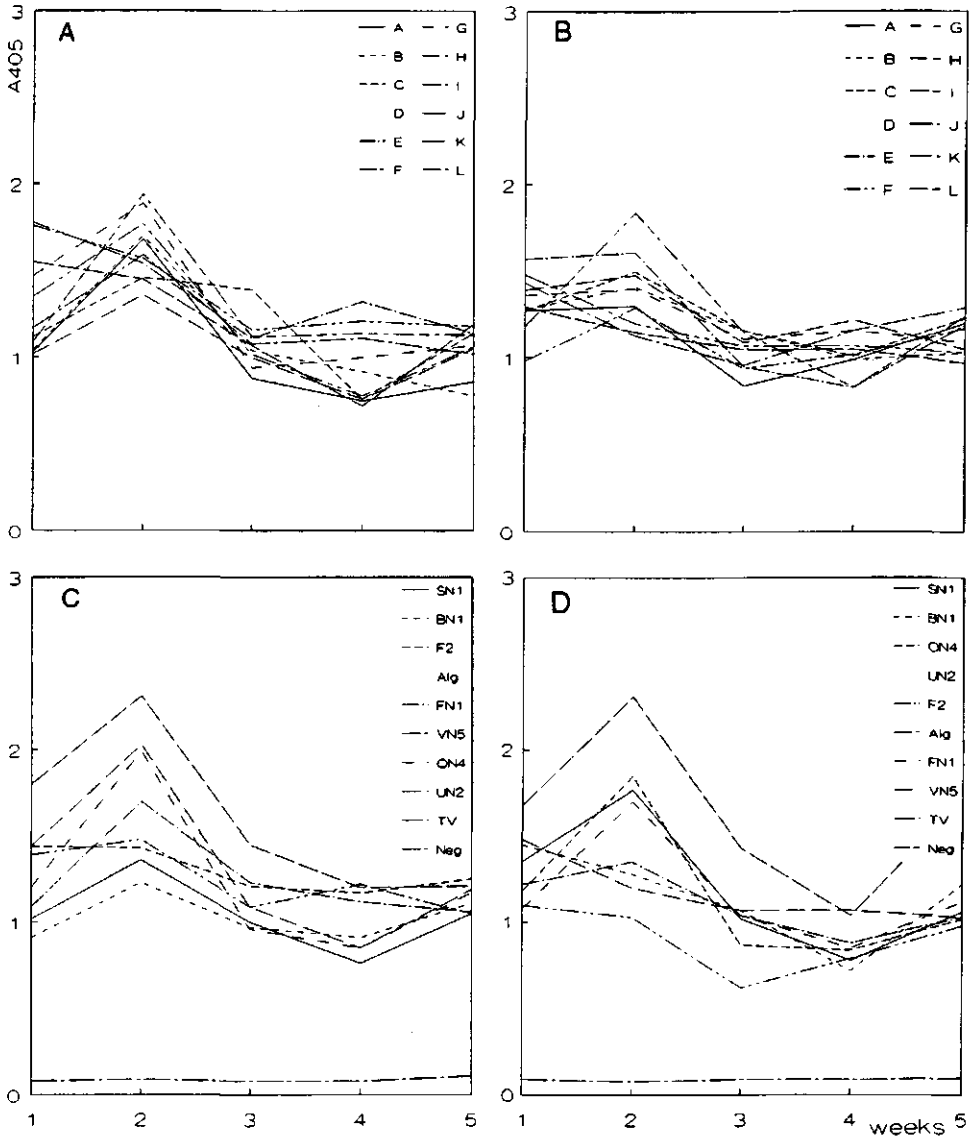

Fig. 6. Virus concentration measured by optical density (405 nm) at different weeks following mechanical inoculation. **A:** concentration of the mild isoalte SN1 in different faba-bean lines (A to L). **B:** concentration of the severe isolate VN5 in the same faba-bean lines. A to L are the FLIP lines 84-230, 84-237, 85-172, 86-107, 86-114, 86-115, 86-116, 86-117, 86-119, 86-122, 86-146, and 87-26, respectively. **C:** concentration of different isolates in the highly vulnerable faba-bean line FLIP 86-117. **D:** concentration of different isolates in the less vulnerable line FLIP 86-146. Neg is the uninoculated control.

DISCUSSION

The ten BBMV isolates investigated can be divided into three groups based on their pathogenicity on selected test species (Table 1). These results are largely corroborated by the reactions of the groups of genotypes of four food-legume species when tested separately (Tables 2 to 5), indicating overall differences in virulence between the BBMV isolates. The severe isolates (VN5, ON4, including the Tunisian TV75-85, and to a lesser extent UN2) are characterized by the systemic necrosis induced on pea Acc. 21 and on the lentil lines ILC 5876 and ILC 6437, and by the local necrosis induced on the faba-bean line FLIP 86-122. On the other hand, the mild isolates (FN1, SN1, including the Sudanese SuV256, and to a lesser extent F2) did not cause local necrosis on chickpea line FLIP 85-15 and on lentil line ILC 6437, and did not produce local symptoms on faba-bean line FLIP 86-122. The remaining isolates assumed an intermediate position, but varied largely in the reaction they induced depending on the food-legume species and lines tested. BN1, for instance, appeared less pathogenic on lentil than on chickpea. This indicates that clear differences in host genetic vulnerability further add to the variation in symptoms produced. The severe isolates appeared more pathogenic on some food-legume species than on the others; VN5 is more pathogenic on lentil and faba bean, whereas TV75-85 is so on chickpea, and UN2 on peas. Although all isolates were obtained initially from faba-bean fields, four other food legumes are potential natural hosts, and some isolates are even more pathogenic on food legumes other than faba bean. Within the cluster of mild isolates, SuV256 was the most deviant in its extremely low pathogenicity. It did not induce a local reaction on any of the nine chickpea lines tested, but produced a systemic mild mosaic (Table 4). It is the only isolate which did not induce any symptom on four lentil lines (including ILC 5845, highly vulnerable to all other isolates). It also differs from the remaining mild isolates by the mild reactions it induced on *Gomphrena globosa* and *Trifolium incarnatum*, and by absence of systemic symptoms on the former and of local symptoms on the latter (Table 1).

The investigated virus isolates behaved identically in EBIA when using antisera to a Moroccan and a Syrian isolate, and their coat proteins exhibited identical molecular

weights. They appeared to differ in pathogenicity only. Virus concentrations determined in infected faba-bean lines show that mild isolates occur also in high concentration, and that less vulnerable lines contain virus concentrations comparable with those in highly vulnerable ones. This suggests that the differences in pathogenicity between isolates are due to differences in virulence rather than aggressiveness, and that differences in vulnerability between genotypes are due to differences in sensitivity rather than susceptibility. The terminology used to describe the relationships between virus isolate and host genotype are according to Bos (1983). The vulnerability of a host is its inability to defend itself and overcome the effects of a virus. It depends on its susceptibility (readiness to accept the virus and assist its multiplication) and its sensitivity (severity of the reaction to the attack). The pathogenicity of a virus is its ability to cause disease, and it is determined by its aggressiveness (readiness to infect and multiply in the host) and by its virulence (ability to incite disease symptoms).

Consequently, the isolates VN5, TV75-85, ON4, and UN2 should be regarded as virulent isolates of BBMV, and SuV256, SN1, FN1, and F2 as mild isolates of this virus. More differenciating parameters, and the reproducibility of the severity of symptoms in relation to the temperature are needed in order to consider the extremes as virulent and mild strains. When considering the Moroccan isolates, VN5 and SN1 are considered the extremes. For screening of food-legume germplasm for resistance to BBMV in Morocco, VN5 should be used for faba bean, chickpea, and lentil, while the isolate UN2 for pea. Further surveying of food legumes, including biological testing of samples, in Algeria, Tunisia, and Sudan is likely to reveal the existence of mild and severe isolates in these countries also. This indicates, as reported earlier (Fortass and Bos, 1991), the importance of supplementing ELISA testing with biological indexing as it allows the detection of variation in pathogenicity between virus isolates.

The food-legume breeding lines tested were all found susceptible to all the isolates used, except that ILC-lentil lines 5845, 5999, 6442, and 6773 did not react to the mild strain SuV256. Within each food legume, highly and less vulnerable, or more specifically, highly and less sensitive lines can be distinguished. A cluster of intermediate genotypes sensitive to the virulent isolates and less sensitive to the mild ones, with

varying degrees of interactions, is found within each food legume species. This necessitates the use of virulent isolates when screening germplasm for resistance to the virus. Moreover, local germplasm should be tested and compared with the genetic material to be introduced from international programmes in order to avoid the risks of introduction of genetic vulnerability, as was, for example, the case with Asian genotypes of rice introduced into Africa and found vulnerable to rice yellow mottle virus previously endemic in the continent (John *et al.*, 1986). The food-legume breeding lines and accessions we have tested were all found vulnerable to highly vulnerable, and no immunity could be detected. This implies that within international programmes, before introduction into new regions, germplasm should be screened for resistance to the viruses occurring there.

In this study, BBMV was found to be seed-transmitted in faba bean, pea, and chickpea. The seed transmission in lentil could not be tested since no seed was produced by the infected plants. This is the first conclusive report on seed transmission of BBMV in faba bean, and the first record of such transmission in pea, and chickpea. The seed transmission of this virus in faba bean was already suspected (Bawden *et al.*, 1951), and has been later reported for mixed infections of the virus with bean yellow mosaic virus (Murant *et al.*, 1974; Makkouk *et al.*, 1988b). The rates of transmission now found, differed according to species, and the highest rate was recorded in faba bean (1.2%). The number of seedlings tested was relatively low, and more testing is needed to better quantify the rates of seed transmission of the virus, especially in faba bean and chickpea. The numbers of seeds available were too low to study the effect of genotype and virus isolate on seed transmission. The use of different genotypes of food legumes, and of different isolates of the virus has undoubtedly enhanced the chance of detecting seed transmission of BBMV in our pooled seed samples.

High vulnerability of legumes other than faba bean and high incidence of this virus already found in faba bean, suggest widespread occurrence of BBMV also in other legumes in Morocco and other countries in West Asia and North Africa. Symptoms in these species, including necrosis as described here and before (Makkouk *et al.*, 1988b), are likely to be overlooked as caused by BBMV or by virus at all. Seed transmission in

faba bean and other legume crops may well explain the widespread occurrence of the virus throughout West Asia and North Africa (Makkouk *et al.*, 1988b), and its prevalence in Morocco (Fortass and Bos, 1991). On-going studies have shown already that *Sitona lineatus* is a vector of BBMV in Morocco, and the natural occurrence of the virus in other food-legume species and wild hosts is under investigation.

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CHAPTER 4

Broad bean mottle virus in Morocco; curculionid vectors, and natural occurrence in food legumes other than faba bean

M. FORTASS and S. DIALLO

SUMMARY

Broad bean mottle virus (BBMV) was transmitted from infected to healthy faba-bean plants by the curculionid weevils *Apion radiolus* Kirby, *Hypera variabilis* Herbst, *Pachytychius strumarius* Gyll, *Smicronyx cyaneus* Gyll, and *Sitona lineatus* L. The latter appeared to be an efficient vector: acquisition and inoculation occurred at the first bite, the rate of transmission was ca. 41%, and virus retention lasted for at least seven days. *S. lineatus* transmitted the virus from faba bean to lentil and pea, but not to the three genotypes of chickpea tested. This is the first report on the genera *Hypera*, *Pachytychius*, and *Smicronyx* as virus vectors, and on *Apion radiolus*, *Hypera variabilis*, *Pachytychius strumarius*, and *Smicronyx cyaneus* as vectors of BBMV.

Out of 351 samples of food legumes with symptoms suggestive of virus infection, 16, 11, 19, and 17% of the samples of chickpea, lentil, pea, and common bean, respectively, were found infected when tested for BBMV in DAS-ELISA. This is the first report on the natural occurrence of BBMV in chickpea, lentil, pea, and common bean. The virus should be regarded as a food-legume virus rather than a faba-bean virus solely, and is considered an actual threat to food legume improvement programmes.

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INTRODUCTION

Earlier surveys of faba bean (*Vicia faba* L) for viruses in Morocco have shown broad bean mottle virus (BBMV) to be prevalent. Its incidence per field ranged from 1 to 33%, and the virus was found widespread in the faba-bean growing areas, especially in the central and northern parts of the country (Fortass and Bos, 1991). It was later reported to be seed transmitted in faba bean, chickpea, and pea at transmission rates of ca. 1.2, 0.9, and 0.1%, respectively (Fortass and Bos, 1992).

The prevalence of the virus in Morocco and its high incidence in some fields suggest the existence of efficient vectors, but information on vector transmission of BBMV is still limited. Walters and Surin (1973), in the USA where the virus has not been reported to occur naturally, were the first to report experimental transmission of BBMV by the striped cucumber beetle (*Acalymma trivittata*), the spotted cucumber beetle (*Diabrotica undecimpunctata*), and the grape colaspis (*Colaspis flavida*) at transmission rates of 9.67, 7.76, and 6.66%, respectively. Later, a similarly poor transmission (5 to 6%) by the leaf weevil *Sitona lineatus* var. *viridifrons* Motsch was reported in Portugal (Borges and Louro, 1974). Cockbain (1983) showed that BBMV is transmitted by *Apion* spp., and a transmission rate of 20% was obtained with *A. vorax*. Recently, Makkouk and Koumari (1989) reported that *A. arrogans* is a vector of BBMV in Syria.

Another ecological feature suggesting the potential importance of the virus is its wide host range among legume species (Makkouk *et al.*, 1988). Recently, some promising breeding lines of faba bean, chickpea, pea, and lentil were tested and found susceptible and sensitive to the virus (Fortass and Bos, 1992). Symptoms, such as necrosis, produced in these species are likely to be overlooked as caused by BBMV. This suggests a widespread, yet unknown, occurrence of the virus in food legumes other than faba bean, and the possible existence of vectors spreading the virus between food-legume crops.

This chapter reports on two important aspects of the ecology of BBMV in Morocco i.e. its vectors and its natural occurrence in food legumes of importance in the country.

MATERIALS AND METHODS

Insect species

The curculionid weevils *Apion radiolus* Kirby, *Sitona lineatus* L., *Hypera variabilis* Herbst, *Smicronyx cyaneus* Gyll, and *Prachytychius strumarius* Gyll (Fig. 1) were collected from faba-bean fields in the areas of Meknès, Fès and Taounate in Morocco. They were maintained in the greenhouse on *V. faba* 'Aguadulce'. All the insects were collected from apparently healthy faba bean plants, and were transferred at 2 to 3-day intervals to three successive healthy 'Aguadulce' plants in order to eliminate viruses acquired by the beetles in the field. Field-collected insects were used since the larvae are soil feeders and the rearing procedure is time consuming. The weevil species were identified by A. Boughdad, Département de Zoologie Agricole, ENA, Meknès, Morocco.

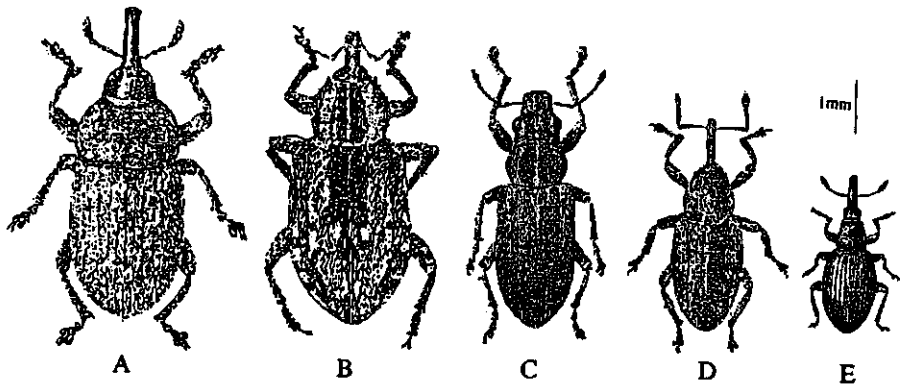

Fig. 1. Drawings and comparative average sizes of the weevil vectors of BBMV. A: *Pachytychius strumarius*, B: *Hypera variabilis*, C: *Sitona lineatus*, D: *Smicronyx cyaneus*, and E: *Apion radiolus*. (Rearranged after Hoffmann, 1959).

Plant species

Plants of *V. faba* 'Aguadulce', mechanically inoculated with the Moroccan isolate VN5 (Fortass and Bos, 1992), were used as virus source. The same cultivar was used for assays. In case of transmission of BBMV by *Sitona lineatus*, other food legumes were

used as assay plants and are listed in Table 1. These genotypes were provided by M. El Yamani, INRA-MIAC, Settat, Morocco.

Transmission tests

The procedure of transmission was according to Bakker (1974) as used for rice yellow mottle sobemovirus. Depending on the acquisition access period (AAP), two approaches were used. For AAPs of 12, 24, and 48 h, the complete plants were used as a source of virus, while for AAPs of 1/4, 1/2, 1, and 10 h, detached leaves placed in petri dishes were used as a virus source. All insects were submitted to pre-acquisition starving for 4 to 24 h. The assay plants were used at the 2- to 3-leaf stage, and the number of insects per plant varied from 1 to 3. All assay plants used in the transmission studies were kept in the greenhouse for 2 to 3 weeks, and then tip leaves were stored at -20°C for later testing in ELISA.

Quantification of transmission by *S. lineatus*

In order to assess the acquisition and inoculation access periods, 412 individuals of *S. lineatus* were given AAPs of 1/4, 1/2, 1, 10, 12, and 24 h and 1, 2, and 4 days of inoculation access periods (IAPs) on 190 'Aguadulce' plants, with 1 to 3 insects per plant. The retention period of BBMV by *S. lineatus* was determined by daily transfers of viruliferous insects to healthy plants, using 1 and 2 individuals per plant. Serial transfers to healthy plants following the first bite were also done with 4 insects (1 per plant). The transmission rate of BBMV by the insect was determined as the ratio of the number of infected plants to the total number of tested plants, using single insects and 24 h as AAP and IAP.

Transmission to other food legumes by *S. lineatus*

A total of 656 individuals of *S. lineatus*, given an AAP of 24 h on infected 'Aguadulce', were transferred to 286 plants of lentil (*Lens culinaris*), 20 plants of chickpea (*Cicer arietinum*), and 22 plants of pea (*Pisum sativum*). The IAP was 24 h and the number of insects per plant varied from 1 to 3.

ELISA testing

The assay plants used in the transmission experiments were tested for BBMV in double-antibody sandwich ELISA as by Clark and Adams (1977). The antiserum used had been raised against the Moroccan isolate MV90-85 (Makkouk *et al.*, 1988). The γ-globulins were used at 1 µg/ml and the alkaline phosphatase conjugated antibodies were diluted to 1:1000 in the extraction buffer. Healthy plants not fed upon by insects were used as negative controls. The leaf material was ground in the extraction buffer 1:4 (w/v). The insects used in the serial transfers were tested for virus retention by ELISA. They were ground with pestle and mortar at a ratio 1 insect per 0.5 ml extraction buffer. The extracts were then centrifuged for 10 mn at low speed (1000 g), and the supernatants were used in ELISA (200 µl per well). Non-viruliferous insects kept on virus-free plants were used as negative controls. The sample was considered positive when the absorbance value exceeded that of the negative control plus three standard deviations. The 'Aguadulce' plants infected by BBMV-VN5, and used in the transmission tests as source plants, were used as a positive control.

Survey of other food legumes and weeds for BBMV

A series of surveys were conducted at different times of the year in the central and northern parts of the country in fields of chickpea, pea, lentil, and common bean (*Phaseolus vulgaris*). Per species, 24 fields were surveyed, and a total of 351 samples of food legumes and 102 samples of legume weeds, showing symptoms suggestive of virus infection, were collected. They were tested for BBMV by ELISA as described above. The weed species were identified by A. Tangi (INRA-MIAC, Settat, Morocco).

RESULTS

Transmission of BBMV by vectors

Sitona lineatus. The results of the experiments are shown in Table 2. The insect species transmitted BBMV from infected to healthy faba bean at all AAPs and IAPs tested. A higher transmission rate was obtained with three individuals than with single

insects per plant, and an AAP of 15 min was enough for *S. lineatus* to transmit the virus.

Smicronyx cyaneus is also able to transmit BBMV from faba bean to faba bean. Transmission occurred at AAPs of 24 h, and a high rate (6/8) was obtained after a 48-h AAP and a 24-h IAP. No transmission was obtained after short AAPs of 15 or 30 min, but the number of plants used for assaying was not high. In daily transfers of two individuals per plant given an AAP of 24 h and an IAP of 48 h, it was found that the virus persisted in *S. cyaneus* for 48 h only (data not shown).

Other Curculionidae. All three species tested transmitted the virus to faba bean. Given an IAP and an AAP of 24h each, *Apion radiolus* transmitted BBMV to 2 plants out of 12, *Hypera variabilis* to 4 out of 6, and *Pachytychius strumarius* to 2 out of 10. The number of insects collected was very low, thus limiting further studies with these species.

Table 2. Transmission of BBMV to faba bean by *Sitona lineatus* at different acquisition and inoculation access periods.

Number of insects per plant	Acquisition access period (h)		Inoculation access period (days)	Results
1	a	1/4	1	33* (1/3**)
		1/2	1	14 (2/14)
		48	1	12 (2/16)
	b	1	2	5 (1/17)
		12	2	12 (2/16)
		24	2	45 (9/20)
3	a	1/4	1	75 (6/8)
		10	1	72 (8/11)
		24	1	33 (4/12)
		48	1	85 (17/20)
	b	1	2	50 (5/10)
		24	2	33 (4/12)
	c	1/2	4	45 (8/17)
		12	4	47 (9/19)

* Percentage of transmission, ** Number of plants infected out of number of plants inoculated

Quantification of the transmission by *S. lineatus*

The shortest AAPs and IAPs tested were 15 minutes and 24h respectively, they still led to virus transmission. Of a total of 100 plants of 'Aguadulce' tested for transmission of BBMV by single *S. lineatus*, given an AAP and an IAP of 24 h each, 41 plants were found to be infected. The rate of transmission was thus estimated to be 41%. The results of the daily transfers show that all the plants used in the experiment were infected, implying that *S. lineatus* retains and transmits the virus until after the seventh transfer. Moreover, in serial transfers following the first insect feeding damage, it was found that the first insect bite already led to virus transmission (data not shown).

Transmission by *S. lineatus* to other food legumes

The results in Table 1 show that *S. lineatus* transmits BBMV from *V. faba* to some but not all genotypes of *L. culinaris* and *P. sativum*, but not to the three genotypes of *C. arietinum* tested.

Table 1. Transmission of BBMV from faba bean to chickpea, lentil, and pea by *Sitona lineatus*, given acquisition and inoculation access periods of 24 h (two insects per plant).

Species and genotype	Results
Chickpea	
F 84-82 C	0* (0/11**)
F 88-75 C	0 (0/13)
F 84-182 C	0 (0/6)
Lentil	
ILL 6212	0 (0/26)
L 121	7 (2/26)
L 56	19 (36/189)
ILL 6001	62 (28/45)
Pea	
SM 790031	0 (0/9)
PH 135 B	66 (4/6)
P 343	71 (5/7)

* Percentage of transmission, ** Number of plants infected out of number of plants inoculated

Natural occurrence of BBMV in other food legumes and legume weeds

Among the 102 symptom-bearing samples of the legume weeds *Trifolium alexandrinum*, *Medicago scutellata*, *Medicago hispida*, and *Vicia sativa*, none was found infected with BBMV when tested in ELISA. In contrast, the legume crops chickpea, pea, lentil, and common bean, were found infected with the virus. Table 3 shows that ca. 16, 11, 19, and 17% of the symptom-bearing samples of chickpea, lentil, pea, and common bean, respectively, were found infected by BBMV. The virus was found in all four food legume crops in the areas surveyed, except in lentil in the area of Meknès.

Table 3. ELISA testing of symptom-bearing samples of food legumes collected from fields.

Legume species	Area of collection	Results*
Cicer arietinum		**15/93** (16%)
	Meknès	4/35
	Taounate	11/58
Lens culinaris		**13/114** (11%)
	Meknès	0/14
	Fès	5/42
	Taounate	8/58
Phaseolus vulgaris		**5/30** (17%)
	Meknès	5/30
Pisum sativum		**22/114** (19%)
	Meknès	19/81
	Fès	3/33

* Number of infected samples over total number of samples tested (percentage of infected samples).

The field symptoms recorded on these species were difficult to define, most likely because of mixed infections. The symptoms consisted of a necrosis of the lower leaves and a mild mosaic on the tip leaves in the case of pea, and vein clearing and mottling in the case of lentil. Infected samples of chickpea revealed a striking wilting of the lower

leaves and a yellow mosaic on the upper foliage, while chlorotic spots were observed on common bean leaves. There is no indication that these symptoms are caused by BBMV infection solely.

DISCUSSION

BBMV was transmitted from infected to healthy faba bean by the leaf weevils *Sitona lineatus*, *Smicronyx cyaneus*, *Hypera variabilis*, *Apion radiolus*, and *Pachytychius strumarius*. Among these, *S. lineatus* appeared to be an efficient vector, since an AAP of 15 minutes or the first bite were sufficient for transmission, the rate of transmission was relatively high (ca. 41%), and the virus was retained by the insect for at least 7 days. Bawden *et al.*, (1951) already suspected *S. lineatus* to be a vector of BBMV in England, but their transmission tests failed. Much later, Borges and Louro (1974) reported transmission of BBMV by *S. lineatus* var. *viridifrons* Motsch in Portugal at a rate of 5 to 6%. The transmission rate obtained by us appears high, but this discrepancy in data may be due to a difference in weevil biotypes, virus strains, number of plants tested, number of insects per assay plant, and the experimental conditions.

S. lineatus is an important insect pest of faba bean in Morocco, and the adults are encountered there during the whole life cycle of the crop (Chairi, 1989). Although the characteristic feeding damage they cause is not economically important, it now appears that the species is potentially important in the spread of BBMV in nature. Moreover, our results show that this insect is able to transmit the virus from faba bean to the other important food legumes lentil and pea. We could not achieve vector transmission to three breeding lines of chickpea, possibly because the insect does not like feeding on this legume species, but we could detect BBMV in naturally infected chickpea plants.

The transmission efficiency of the other weevils was not evaluated because of the limited numbers of individuals available. Nevertheless, *Apion radiolus* seems of potential importance in the transmission of BBMV. A short IAP is enough for the species to

transmit the virus. Some *Apion* species have been reported as vectors of BBMV. *A. vorax* has been reported from Britain (Cockbain, 1983) with a transmission rate of 20%, and *A. arrogans* from Syria (Makkouk and Koumari, 1989). Virus transmission by *Apion* spp. has been shown earlier by Cockbain (1971), who found that the weevils of the genera *Apion* and *Sitona* are vectors of broad bean stain and broad bean true mosaic comoviruses. Later, Gerhardson and Pettersson (1974) reported that *A. apricans* Hbst. and *A. varipes* Germ. transmit red clover mottle comovirus.

This is the first report on *Apion radiolus, Hypera variabilis, Smicronyx cyaneus*, and *Pachytychius strumarius* as vectors of BBMV, and the first record of the curculionid genera *Hypera*, *Smicronyx*, and *Pachytychius* as virus vectors. This gives further evidence that the members of the Curculionidae are important as virus vectors. Compared to *S. lineatus*, other curculionids are not abundant on faba bean in Morocco, are of no economic importance, but appear now potentially important as vectors of BBMV. Moreover, species of the genera *Apion* and *Sitona* might also be vectors of the comoviruses occurring on faba bean in Morocco, i.e. broad bean stain and broad bean true mosaic viruses, as reported in Britain (Cockbain, 1971). Since BBMV is seed transmitted in faba bean (Fortass and Bos, 1992), the vectors reported here may well play a determinant role in spreading the virus from crop plants infected from seed and from other infected legumes.

Among the 102 samples of the legume weeds *Trifolium alexandrinum, Medicago scutellata, Medicago hispida*, and *Vicia sativa* collected in faba-bean fields, none was found harbouring the virus. Although a large number of legume weeds were found susceptible to mechanical inoculation and were thus reported as potential hosts of BBMV (Makkouk *et al.*, 1988), none of the ones tested in the present study were found to host the virus. More sampling and testing is needed, however. In contrast, common bean and the food legumes pea, chickpea, and lentil were found naturally infected by BBMV. The virus was suspected to occur naturally in food legumes other than faba bean since it has already been found pathogenic to these food legume species and several other fodder and weed legumes upon mechanical inoculation (Makkouk *et al.*, 1988; Fortass and Bos, 1992). Moreover, some isolates of BBMV were found to be more pathogenic on

chickpea, lentil, or pea, than on faba bean (Fortass and Bos, 1992), implying therefore that BBMV should be regarded as a food-legume virus rather than a mere faba-bean virus.

This is the first report on the natural occurrence of BBMV in crops of chickpea, lentil, pea, and common bean. Further surveying of food legumes other than faba bean for this virus would undoubtedly reveal its natural occurrence in these crops in other food legume producing regions in the world. Earlier research (Makkouk *et al.*, 1988; Fortass and Bos, 1992) and the present investigations on incidence, natural occurrence in a number of legume crops, and seed and weevil transmission of BBMV in Morocco and other countries in West Asia and North Africa, have clearly shown the virus to be of long-neglected agricultural importance in the region. Moreover, the seed transmission of BBMV in chickpea and pea (Fortass and Bos, 1992) and probably in other legumes, and its transmissibility from faba bean to other food legumes by *Sitona lineatus*, make this virus a threat to food legume improvement programmes.

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CHAPTER 5

Luteoviruses infecting faba bean in Morocco; characterization by serology, polymerase chain reaction, and molecular hybridization

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SUMMARY

A number of Moroccan faba-bean samples with symptoms suggestive of luteovirus infection were serologically tested using polyclonal antisera to BLRV, BWYV, and SCRLV, and two monoclonal antibodies which discriminate between BWYV and BLRV. Several serological reaction patterns were obtained and this points towards a large variation among the luteovirus isolates under study. None of these isolates could be clearly identified as one of the known legume luteoviruses, although a number of them behaved BWYV-like serologically. The potential of the polymerase chain reaction in detecting these luteoviruses was investigated, and a set of oligonucleotide primers was designed which specifically amplified a 535-bp fragment of the coat protein gene of known luteoviruses and of all Moroccan isolates tested. In nucleic acid hybridization tests, the field isolates showed homology in nucleotide sequence among them and with BLRV, but not with BWYV. In polyacrylamide gel electrophoresis, a purified Moroccan isolate was found differing from BLRV and BWYV in coat-protein migration.

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INTRODUCTION

Luteoviruses characteristically cause yellowing and reddening symptoms and often leaf curling and plant stunting in many plant species, and considerable reduction in yield of a wide range of crops (Rochow and Duffus, 1981; Waterhouse *et al.*, 1988). The identification of these viruses was based initially on information on host ranges, symptoms, aphid transmission, and serological affinities. Later, it was understood that many of the luteovirus names were synonymous (Casper, 1988; Waterhouse *et al.*, 1988). The International Committee on Taxonomy of Viruses now recognizes 10 definitive luteoviruses and 12 possible members (Francki *et al.*, 1991). Various approaches have been used to differentiate the luteoviruses and to evaluate their interrelationships. Among these are host-range and vector specificity (Johnstone *et al.*, 1984), serology (D'Arcy *et al.*, 1989; Martin and D'Arcy, 1990), nucleic-acid hybridization (Martin and D'Arcy, 1990), and polymerase chain reaction followed by restriction-enzyme analysis of the amplified coat-protein gene (Robertson *et al.*, 1991). Proper identification is essential not only in taxonomy, but also in breeding for resistance.

A number of luteoviruses infecting faba bean have been reported from different parts of the world. Three of them, bean leafroll virus (BLRV) (Quantz and Völk, 1954), beet western yellows virus (BWYV) (Duffus, 1964), and subterranean clover red leaf virus (SCRLV) (Wilson and Close, 1973), have been reported to occur naturally on the crop. Other reported faba-bean luteoviruses are considered synonymous of one of these. Pea leafroll virus, legume yellows virus, and Michigan alfalfa virus are synonymous of BLRV, while soybean dwarf virus is identical to SCRLV (Francki *et al.*, 1991).

When surveying faba bean (*Vicia faba* L.) for viruses in Morocco, samples with symptoms suggestive of luteovirus infection were collected. Serological testing revealed various reaction patterns indicative of the involvement of different luteoviruses or luteovirus strains (Fortass and Bos, 1991). In order to further identify these luteoviruses, a number of isolates were collected from different areas. This paper reports on further characterization of faba-bean luteoviruses from Morocco by serology, polymerase chain reaction, and molecular hybridization.

MATERIALS AND METHODS

Sample collection and virus isolates

Twenty faba-bean plants with symptoms suggestive of luteovirus infection were collected from the main faba-bean growing areas in Morocco during 1992. They were kept under aphid-proof conditions in the greenhouse at the Centre Regional de la Recherche Agronomique, Settat, Morocco.

Leaf samples of alfalfa (*Medicago sativa*) infected with BLRV (Ashby and Huttinga, 1979), *Physalis floridana* infected with either BWYV (Van den Heuvel *et al.*, 1990) or potato leafroll virus (PLRV) (Van der Wilk *et al.*, 1989), and *Trifolium subterraneum* infected with SCRLV (Johnstone *et al.*, 1982) were used as positive controls throughout the experiments. Faba-bean plants either healthy or infected with broad bean mottle virus (BBMV) or bean yellow mosaic virus (BYMV) were used as negative controls.

Serological testing

The collected samples were tested for luteovirus infection in double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA), using polyclonal antisera as described by Clark and Adams (1977), and in triple-antibody sandwich ELISA (TAS-ELISA) using monoclonal antibodies (MAbs) according to Van den Heuvel *et al.* (1990).

Sample preparation. Samples were prepared by grinding 0.5 g leaf material in 7 ml sample buffer (0.02 M sodium phosphate buffer pH 7.4, containing 0.15 M sodium chloride, 2% polyvinylpyrrolidone, 0.05% Tween 20, and 0.2% ovalbumin).

DAS-ELISA. The polyclonal antisera to BLRV, BWYV, and SCRLV were kindly provided by L. Katul (Institute for Biochemistry and Plant Virology, Braunschweig, Germany), J.E. Duffus (U.S.D.A. Agricultural Research Station, Salinas, California, USA), and G.R. Johnstone (Tasmanian Department of Agriculture, Tasmania, Australia), respectively. The γ-globulin fraction was partially purified by ammonium sulphate precipitation as described by Clark and Adams (1977), and conjugated with alkaline phosphatase according to Avrameas (1969). The γ-globulins were used at a concentration of 1 µg/ml for coating the plates, and the enzyme conjugates were diluted 1:1000 in case

of BLRV and SCRLV, and 1:2000 in case of BWYV.

TAS-ELISA. The γ-globulins to BWYV were used as trapping antibodies as in DAS-ELISA, and two MAbs, WAU-A12 and -A24 which discriminated between BLRV and BWYV (Van den Heuvel *et al.*, 1990) were used as detecting antibodies at 6,000- and 1,000-fold dilutions, respectively. Alkaline phosphatase-conjugated goat anti-mouse antibodies (Sigma) were applied at a 1,000-fold dilution to detect the immobilized MAbs.

In either type of ELISA, all samples including virus-free controls were tested in triplicate. Reagents were applied at 100 µl per well. The presence of alkaline phosphatase was monitored after adding 1 mg/ml of para-nitrophenyl phosphate disodium salt in 10% diethanolamine, pH 9.8. A sample was considered positive if the mean absorbance value measured at 405 nm was higher than the mean value of the negative control plus three times the standard deviation (critical optical density, OD_c).

cDNA synthesis and polymerase chain reaction (PCR)

Primers. In the initial experiments, a set of luteovirus-specific oligonucleotide primers designed by Robertson *et al.* (1991) was used. The downstream primer Lu4 (5' GTCTACCTATTTGG 3') corresponds to bases 4084 to 4097 of BWYV-RNA (Veidt *et al.*, 1988), and bases 583 to 597 of BLRV-RNA (Prill *et al.*, 1990). The upstream primer Lu1 (5' CCAGTGGTTRTGGTC 3'), degenerate at one position, pairs with bases 3562 to 3576 of BWYV-RNA. The length of the predicted PCR product is 535 bp.

RNA extraction and cDNA synthesis. RNA was extracted from 0.2 g of leaf material by grinding the tissue in liquid nitrogen, followed by extraction at 65°C with a mixture (1:1) of phenol and extraction buffer (0.1 M glycine pH 9.5, 0.1 M NaCl, 10 mM EDTA). After centrifugation, the supernatant was extracted with phenol:chloroform, followed by chloroform extraction. Subsequently, the RNA was ethanol precipitated. The pellets were washed with 70% ethanol, vacuum-dried and resuspended in 20 µl of RNAse-free water.

Annealing of the primer was carried out by heating 10 µl extracted RNA with 50 pmol Lu4 for 5 min at 95°C, incubating at 42°C for 10 min, and chilling on ice. The cDNA was synthesized by adding an equal volume of a mixture containing 100 mM Tris-HCl,

pH 8.3, 150 mM KCl, 6 mM $MgCl_2$, 10 units/µl of M-MLV reverse transcriptase (Gibco BRL), 2.5 mM of each dNTP, and 5 units RNasin. The reaction mixture was incubated for 1 h at 37°C and for 10 min at 65°C, and subsequently stored at -20°C until needed.

DNA amplification. A total volume of 40 µl PCR mixture contained 5µl of the cDNA preparation, 2.5 mM of each dNTP, 5 pmol each of both primers Lu4 and Lu1, 2.5 units of Super *Taq* DNA polymerase (HT Biotechnology LTD), 50 mM Tris-HCl, 50 mM KCl, 7 mM $MgCl_2$, 0.2 mg/ml BSA, and 16 mM $(NH_4)_2SO_4$. The samples were overlaid with 50 µl mineral oil and placed in a DNA thermal cycler (Perkin Elmer Cetus) programmed to give one cycle at 95°C (1 min), 46°C (2 min), and 72°C (10 min), followed by 45 cycles at 94°C (1 min), 46°C (1 min), 56°C (1 min), and 72°C (2 min), and a final cycle of 94°C (1 min), 46°C (2 min), and 72°C (10 min). The PCR products (10 µl each) were analysed by electrophoresis in 1% agarose gels stained with ethidium bromide.

Nucleic-acid hybridization tests

Radioactive labelled probes were generated to the 535 bp PCR products of BWYV, BLRV, and field isolate V using a random priming kit (Boehringer, Mannheim). About 100 ng of the total PCR products of BWYV, BLRV, and of isolate V were run on a 1% low melting point agarose gel. The 535 bp fragments were cut out of the gel and purified by two phenol extractions followed by ethanol precipitation. The pellets were resuspended in 30 µl sterile water. Ten µl of the extracted DNA was applied in labelling reactions according to the manufacturer's instructions. The labelled probes were purified by removing non-incorporated [α-^{32}P]dATP using a Sephadex G50 column.

Southern blotting. The PCR products obtained from extracts derived from selected field samples and from the PLRV, BWYV, and BLRV positive controls as well as the healthy control were subjected to agarose gel electrophoresis, and transferred to Hybond-N membrane (Amersham). Prehybridization and hybridization were performed as described by Sambrook *et al.* (1989). The hybridization was carried out at 65°C, and was followed by washing the blots at 42°C in 2x SSC, 1x SSC, and 0.1x SSC containing

0.1% SDS, before exposure of the membrane to X-ray film (Kodak).

Polyacrylamide gel electrophoresis (SDS-PAGE)

Field isolate V was purified from faba bean cv. Aguadulce as described by Van den Heuvel *et al.* (1990). Purified isolate V as well as BWYV and BLRV were subjected to SDS-PAGE in 12% acrylamide according to Laemmli and Favre (1973). After electrophoresis, the gel was stained in Coomassie Brilliant Blue.

RESULTS

Serological testing

The reactivities of the field samples to the MAbs and polyclonal antisera used in ELISA are summarized in Table 1. All samples reacted with at least one antiserum or one MAb, implying therefore that they were luteovirus-infected. Based on the reactivities of the samples with the polyclonal antisera to BLRV, BWYV, and SCRLV in DAS-ELISA, different patterns could be distinguished. They ranged from a reaction to all the three antisera used (sample B) to no reaction to any of the polyclonal antisera (samples I, Y, and Z). The majority of the samples reacted with BWYV antiserum, whereas only four and seven samples reacted with BLRV and SCRLV antisera, respectively.

All samples except Ad reacted positively with the MAbs WAU-A12 and -A24 in TAS-ELISA, even those which were negative in DAS-ELISA. All samples reacted moderately with WAU-A24, whereas half of them were strongly detected by WAU-A12, among which three were positively identified in DAS-ELISA with the BLRV antiserum.

The reaction patterns of the different isolates were found independent of the geographical area from which the samples originated.

Detection by PCR

In preliminary PCR experiments with BLRV, BWYV, and PLRV, the pair of primers Lu1 and Lu4 were found to specifically amplify sequences from PLRV and BWYV, but

not from BLRV or the field isolates. By comparing the nucleic acid sequences of the coat protein genes of BWYV (Veidt *et al.*, 1988) and BLRV (Prill *et al.*, 1990), a new upstream primer was designed i.e. VW66 (5' TACCAGTCCGTTCTGG 3') which pairs with bases 3562 to 3576 of BWYV-RNA, and with bases 62 to 76 of BLRV-coat protein gene. Using this primer, a PCR product of about 530 bp was obtained from all luteovirus-infected control plants, but not from healthy controls (Fig. 1). No bands in the agarose gels were observed when applying the PCR procedure on extracts from faba-bean samples infected with either a bromovirus (BBMV) or a potyvirus (BYMV), indicating that the set of primers Lu4/VW66 recognized specifically luteovirus sequences.

Table 1. Reactivity[a] of the faba-bean samples showing luteovirus-like symptoms in DAS- and TAS-ELISA.

	DAS-ELISA			TAS-ELISA	
Sample Designation	BWYV	BLRV	SCRLV	A12	A24
B	0.29	0.56	0.42	2.85	0.60
A	0.31	0.48	-[b]	1.33	0.50
D	0.31	0.62	-	0.34	0.39
T	0.30	0.53	-	1.47	0.48
S	0.45	-	0.70	2.95	0.60
F	0.31	-	0.42	2.36	0.42
V	1.12	-	0.55	1.54	0.54
Ena	0.27	-	0.66	0.55	0.46
E	0.43	-	-	0.61	0.46
G	0.33	-	-	0.87	0.43
M	0.35	-	-	0.37	0.43
N	0.38	-	-	0.46	0.35
O	0.33	-	-	0.60	0.34
Q	0.34	-	-	1.12	0.36
X	0.85	-	-	1.41	0.42
Ad	0.34	-	-	-	0.87
Dyt	-	-	0.58	0.48	0.42
I	-	-	-	2.90	0.49
Y	-	-	-	0.62	0.37
Z	-	-	-	0.93	0.40
OD_c	0.25	0.22	0.38	0.19	0.27
PLRV	0.76	-	-	3.00	1.90
BWYV	1.97	-	-	2.45	0.62
BLRV	-	1.15	-	-	0.93
SCRLV	0.28	-	1.28	nt[c]	nt

[a]: Mean optical density at 405 nm
[b]: Mean optical density did not exceed the critical optical density (OD_c)
[c]: Not tested

When field samples of faba bean showing luteovirus-like symptoms, and selected on the basis of the geographical origin, were subjected to PCR with the primer set Lu4/VW66, a major PCR product, of approximately the same size as was obtained with extracts from BLRV- and BWYV-infected plants, was visualized in agarose gel (Fig. 2). For some samples, additional bands of different sizes were also amplified.

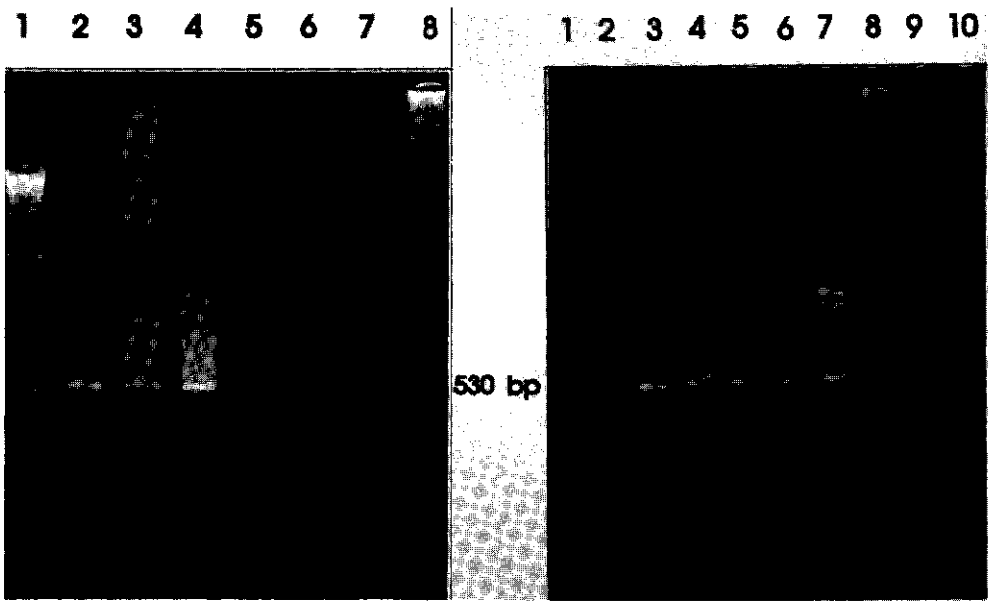

Fig. 1. Agarose electrophoresis of PCR products. Lane 1, DNA size markers (GIBCO BRL); lanes 2 to 6, PLRV, BWYV, BLRV, BYMV, and BBMV, respectively; lanes 7 and 8, healthy controls.

Fig. 2. Agarose electrophoresis of PCR products. Lane 1, DNA size markers; lanes 2 to 3, BLRV, BWYV, lanes 4 to 8, luteovirus field samples D, V, Ena, Dyt, and Ad, respectively; lanes 9 and 10, healthy controls.

Nucleic-acid hybridization tests

Four of the field isolates varying in serological reaction and showing strong signals in PCR were selected for the hybridization tests. Figure 3 shows that the 535 bp PCR product of BWYV hybridized with itself and with the PCR product of BLRV, but no cross hybridization was obtained with the PCR products of any of the field isolates. The probe derived from the 535 bp PCR product of BLRV strongly tagged the homologous PCR product, and showed a weak cross reaction with BWYV as well as with the field isolates (Fig. 4). The southern blot of the PCR products tested with the labelled 535 bp PCR product of the isolate V shows that it detected the PCR products from BLRV and from the field isolates tested, but not from BWYV (Fig. 5). Strikingly, these results indicate that the PCR products derived from the field isolates all shared a substantial sequence homology with BLRV but not with BWYV, which was not expected from the serological data.

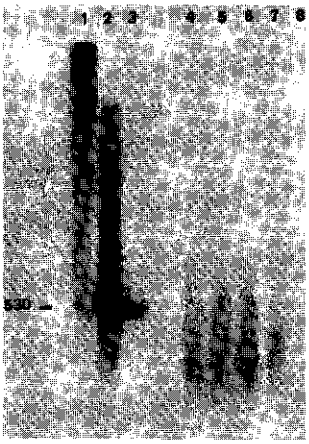

Fig. 3. Southern blot of PCR products hybridized with a probe to the 530 bp PCR product from BWYV. Lanes 1 to 3, PLRV, BWYV, and BLRV; lanes 4 to 7, luteovirus field samples D, Ena, V, and Dyt, respectively; lane 8, healthy control.

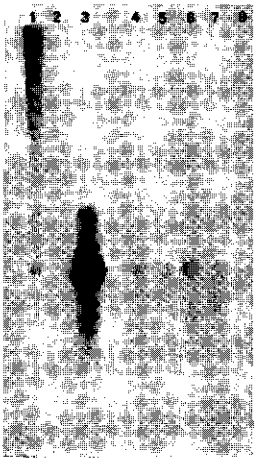

Fig. 4. Southern blot of PCR products hybridized with a probe to the 530 bp PCR product from BLRV. Lanes 1 to 3, PLRV, BWYV, and BLRV; lanes 4 to 7, luteovirus field samples D, Ena, V, and Dyt, respectively; lane 8, healthy control.

SDS-PAGE

Since the PCR does not detect mixed infections, and in order to elucidate this possibility for the field isolate V, the migration pattern of the coat protein of isolate V was compared with that of BLRV and of BWYV. Figure 6 shows that the coat protein of the isolate V migrated differently from the coat proteins of BWYV and BLRV which also differed mutually. This excludes a possibility of mixed infection of BLRV and BWYV in the field sample V.

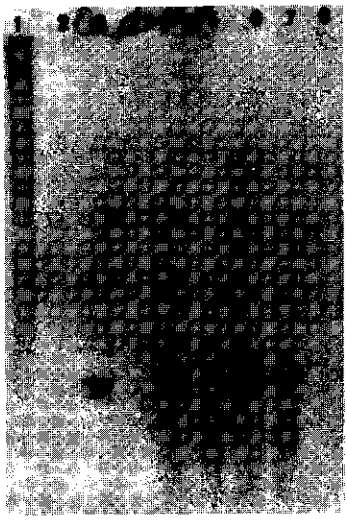

Fig. 5. Southern blot of PCR products hybridized with a probe to the 530 bp PCR product from the field isolate V. Lanes 1 to 3, PLRV, BWYV, and BLRV; lanes 4 to 7, luteovirus field samples D, Ena, V, and Dyt, respectively; lane 8, healthy control.

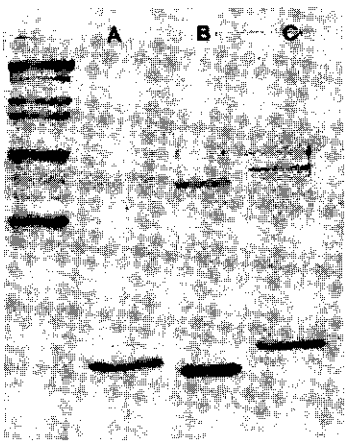

Fig. 6. Migration patterns in SDS-PAGE of the luteovirus isolate V (A), BWYV (B), and BLRV (C) after staining with Coomassie Blue. Left line, protein markers.

DISCUSSION

The twenty field samples of faba bean showing symptoms indicative of luteovirus infection were found reacting in ELISA with at least one of the antisera or MAbs used, confirming therefore that they were infected with a luteovirus or a complex of luteoviruses. Based on the reactivities with the polyclonal antisera used, most of the samples reacted with the antiserum to BWYV, while some samples showed cross reactions with two or even three antisera, and one sample scored negative in DAS-ELISA. Various serological relationships among the members of the luteovirus group have been reported (Waterhouse *et al.*, 1988). It is therefore difficult to serologically identify most of the Moroccan isolates as one of the three legume luteoviruses. This is even more so, since the reaction patterns of our isolates obtained with polyclonal antisera were very diverse.

On the basis of the intensities of the reactions with the MAbs WAU-A12 and -A24, as defined by Van den Heuvel *et al.* (1990), two clusters of isolates can be differentiated. The first cluster includes isolates which reacted strongly to WAU-A12 and moderately to -A24 (B, I, and F, for instance), and should then be considered as BWYV-like isolates. The second cluster groups isolates which reacted moderately to both WAU-A12 and -A24, and on this basis, they appear different from both BWYV and BLRV. However, many of the isolates which reacted in a similar fashion as BWYV were also detected by antisera to BLRV and SCRLV. Since the three antisera did not or only marginally cross-react with their respective positive controls in reciprocal assays (Fig. 1), it may well be that we are not only dealing with BWYV isolates but also with other possibly new luteoviruses or luteovirus strains. However, affinity determination utilizing only two epitopes is likely to be too narrow a basis for virus identification. More serological studies are undoubtedly needed, and the diversity of reaction patterns points to considerable variation of the viruses under study.

In PCR, the set of primers Lu4/Lu1 previously used by Robertson *et al.* (1990) to detect PLRV, BWYV, and different serotypes of barley yellow dwarf virus, could not be applied for BLRV. The pair of primers we designed (Lu4/VW66) detected PLRV,

BWYV, and BLRV in inoculated control plants, as well as luteovirus(es) in faba-bean field samples. The sensitivity of the PCR compared to other diagnostic techniques has been reported (Hadidi *et al.*, 1993). It offers the additional advantage of providing amplified DNA fragments that would generate sequence information to be used to characterize unknown isolates.

To evaluate the suitability of the restriction enzyme analysis of the amplified PCR fragments in differentiating luteoviruses, as reported earlier (Robertson *et al.*, 1990), some experiments were initially conducted with the BWYV-derived PCR product. The restriction pattern obtained, following digestion of the 535 bp PCR product by *Sau* 3AI, was different from that predicted from the known nucleotide sequence (Veidt *et al.*, 1988). This is probably due to the variability of the virus, and this makes the differentiation of genetically noncharacterized luteoviruses by this approach unreliable, especially when dealing with field isolates.

The hybridization tests involving the amplified 535 bp PCR products showed that the four field isolates D, Ena, Dyt, and V share nucleotide homology in their coat protein gene with BLRV, but not with BWYV. This conflicts with the serological data. The isolate V, for instance, appears serologically BWYV-like while no cross hybridization with BWYV could be detected. That isolate differs in migration pattern of the coat protein in SDS-PAGE from BLRV and BWYV, and this excludes the possibility of a mixed infection of both BLRV and BWYV. This further supports the supposition of the involvement of (a) different luteovirus(es) adapted to faba bean.

Luteoviruses form a continuum of serologically related members with overlapping host ranges (Waterhouse *et al.*, 1988). As pointed out by Ashby and Johnstone (1985), we can envisage the possibility of evolution of different members of the group from an ancestral type as a result of the feeding preference of different aphid species, and BWYV is likely to be the ancestral type since its host range is wide, and it is transmitted by a large number of aphid species. Host preferences of some aphid species may have favoured the selection and maintenance of luteovirus variants, thus evolving into a faba-bean luteovirus sharing some properties with both BLRV and BWYV. *Aphis craccivora*, the prevailing aphid in faba-bean fields in Morocco, could have played a crucial role in this

proces. However, characterization studies of some of the collected isolates, including the nucleotide sequences of their coat protein genes, will give further insight in the identification of the faba-bean luteoviruses.

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CHAPTER 6

Identification of potyvirus isolates from faba bean, and the relationships between bean yellow mosaic virus and clover yellow vein virus

M. FORTASS, L. BOS, and R.W. GOLDBACH

SUMMARY

Clover yellow vein virus (CYVV) isolate H and the related potyvirus isolates E178, E197 and E242 could be distinguished from bean yellow mosaic (BYMV) isolates by their wider host range among non-legume test plant species, the peculiar enlargement of the nucleolus in infected plants, and the larger size of their coat protein as evidenced by slower migration in SDS-PAGE. Serologically, they are qualitatively indistinguishable in electro-blot immunoassay (EBIA) also with antibodies specific to the N-terminal part of BYMV-B25 coat protein, implying therefore that CYVV and BYMV coat proteins contain identical amino-acid sequences in the N-terminal region.

The faba-bean virus isolates from Sudan, Syria and the Netherlands could be identified as BYMV isolates especially adapted to faba bean. All of them were weakly pathogenic to *Phaseolus* bean with the exception of SV205, assuming an intermediate position between *Phaseolus*-bean isolates, with low pathogenicity to faba bean, and faba-bean isolates, usually having low pathogenicity to *Phaseolus* bean. Strains of BYMV are thus hard to delimit.

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INTRODUCTION

The potyviruses are the largest and economically most important group of plant viruses (Hollings and Brunt, 1981a, 1981b; Milne, 1988). The current taxonomy of this group is very unsatisfactory due mainly to much overlap and varying degrees of relationships between members of the group in serology (Beemster and Van der Want, 1951; Bercks, 1960a, 1960b) and biological properties such as host range (Bos, 1970). The lack of reliable taxonomic criteria has often made it difficult to distinguish between strains of one potyvirus and even between different potyviruses (Francki, 1983; Milne, 1988).

The literature concerning the BYMV subgroup of potyviruses, in particular BYMV, CYVV, and pea mosaic virus (PMV), contains many reports of variation and overlap in host range, symptom expression and serological properties. Uncertainty exists as to whether these are distinct viruses, or serotypes or strains of the same virus (Barnett *et al.*, 1985). For example, some investigators consider BYMV and CYVV to be synonymous (Jones and Diachun, 1977), while others consider them to be separate viruses (Bos *et al.*, 1974; Bos *et al.*, 1977; Lindsten *et al.*, 1976). For some authors, CYVV, BYMV and PMV are three different viruses (Reddick and Barnett, 1983; Barnett *et al.*, 1985, 1987), while others consider them different serotypes of BYMV (Jones and Diachun, 1977).

In recent years, Shukla and coworkers (Shukla and Ward, 1988), claimed to be able to differentiate between distinct potyviruses and their strains on the basis of the amino-acid sequence homologies between their coat proteins. Distinct members of the potyvirus group were found to exhibit sequence homologies ranging from 38 to 71%. In contrast, strains of individual viruses exhibited sequence homologies of 90 to 99% and had very similar N-terminal lengths and sequences. These findings throw doubt on the continuum phenomenon first demonstrated by Bos (1970) and later supported by Hollings and Brunt (1981b), Harrison (1985), and Milne (1988), and which under the name "continuum hypothesis" (Shukla and Ward, 1988) has been used to explain the unsatisfactory taxonomy of the potyvirus group. Moreover, recent findings demonstrate that the N-terminal part of the coat protein, which can be removed from intact particles by treat-

ment with lysyl-endopeptidase, constitutes the most immunodominant region of potyvirus particles. Virus-specific epitopes appear to be located in the N-terminus, whereas potyvirus-group-specific epitopes are contained in the core protein region (Shukla *et al.*, 1988). Consequently, a method of affinity chromatography has been developed to obtain antibodies directed towards the epitopes on the N-termini of coat proteins of potyviruses and these antibodies have been used to specifically detect potyviruses and their strains (Shukla *et al.*, 1989a). Such specific antibodies have been used to revise the taxonomy of the potyviruses infecting maize, sorghum and sugarcane in Australia and the United States (Shukla *et al.*, 1989b).

Bean yellow mosaic virus (BYMV) was one of the viruses most frequently isolated from faba bean (*Vicia faba* L.) in West Asia and North Africa, particularly Egypt, Sudan, Syria and Tunisia during a survey within the framework of a collaborative project between IPO, Wageningen, and ICARDA (International Centre for Agricultural Research in the Dry Areas), Aleppo, Syria (Makkouk *et al.*, 1988). Earlier research at IPO had revealed the existence of highly different strains of BYMV, biological overlap of BYMV with clover yellow vein virus (CYVV), and the possible existence of strains of BYMV, or of a separate virus, especially adapted to faba bean (Bos *et al.*, 1974). When attempts were made to identify a range of new potyvirus isolates from faba bean, the problem of variation within the cluster of legume potyviruses (Bos, 1970) recurred. We have therefore compared some representative new faba-bean isolates with isolates earlier identified as BYMV or CYVV, and did so for their biological properties and serology also employing virus-specific antibodies directed towards N-terminal parts of coat proteins (Shukla *et al.*, 1989a). Proper characterization is not only required for reliable diagnosis and naming, but also has a bearing on breeding for resistance and the introduction of resistant crop genotypes for disease control.

MATERIALS AND METHODS

Virus isolates and maintenance

The isolates included in this study and their origin are listed in Table 1. The isolates, stored in desiccated infected leaf material over calcium chloride, were revived in their original hosts. CYVV-H, earlier obtained from Hollings and Nariani (1965), and the neotype B25 of BYMV are included as reference viruses.

Table 1. List of virus isolates studied.

Isolate code	Original host	Supplier or reference
Isolates earlier identified[a] as		
BYMV		
typical bean strain		
B25	French bean	Bos *et al.* (1977)
pea yellow mosaic strain		
E198	pea	Bos *et al.* (1977)
pea necrosis strain		
E197	pea	Bos *et al.* (1974)
E199	pea	Bos *et al.* (1974)
CYVV		
CYVV-H	white clover	Hollings and Nariani, 1965
E178[b]	pea	Bos *et al.* (1977)
E242[b]	pea	Beczner *et al.* (1976)
Isolates not yet or incompletely identified		
BSMV-TN	French bean	L. Bos and N. Huijberts
SV205-85	faba bean (Syria)	K.M. Makkouk (ICARDA)
SV231-85	faba bean (Syria)	,,
SuV128-88	faba bean (Sudan)	,,
SuV270-88	faba bean (Sudan)	,,
Vf15	faba bean (Netherl.)	Bos *et al.* (1974)
Vf42	faba bean (Netherl.)	L. Bos

[a] See also Beczner *et al.* (1976) and Bos *et al.* (1977).
[b] Isolates first described as pea necrosis virus, later found to be CYVV (Bos *et al.*, 1977).

Virus purification

All isolates were purified from pea (*Pisum sativum* 'Koroza') by method two of Reddick and Barnett (1983).

Antiserum preparation

The antiserum to BYMV-B25 has been prepared by immunizing a rabbit with two intravenous injections of 0.5 mg freshly purified virus at a two-day interval. A third injection consisting of 1 mg of freshly purified virus, mixed with an equal volume of Freund's incomplete adjuvant, was given two weeks later. The blood was collected three weeks after the last injection.

Purification of γ-globulins

The γ-globulin fraction from the antiserum to B25 was purified according to Clark and Adams (1977).

Host-range studies

Some legume and non-legume hosts found earlier (Bos *et al.*, 1974) to react differentially to BYMV isolates were mechanically inoculated, and the symptoms produced were recorded at different times during development. At least three plants per species were inoculated. In case of absence of symptoms, inoculated and non-inoculated leaves were tested separately for the presence of virus by double antibody sandwich ELISA using E178 antiserum and alkaline-phosphatase conjugate to detect the CYVV-like isolates E178, CYVV-H, E197 or E242, and E198 antiserum and alkaline-phosphatase conjugate for the remaining isolates. These two antisera had in tentative tests proved to discriminate between the two groups of isolates.

Pathogenicity on *Phaseolus*-bean

Since BYMV was originally described as a *Phaseolus*-bean virus and effect on crops and crop cultivars are of paramount importance to plant pathologists, interactions of the isolates with *Phaseolus*-bean cultivars were studied in detail. Eight plants per cultivar

and isolate were inoculated and symptoms were recorded after five weeks. To take both incidence of infection and symptom severity into account, the effect on genotypes was assessed by a severity index (SI; Silbernagel and Jafri, 1974):

$$SI = \frac{(0xN_0 + 1xN_1 + 2xN_2 + 3xN_3 \ldots\ldots + XxN_X) \times 100}{N_t \times (N_c - 1)}$$

in which severity of symptoms is estimated per plant according to a scale ranging from 0 to X, and

N_0 = the number of plants in class 0, etc.,

N_t = the total number of plants, and

N_c = the number of disease categories.

The resulting SI may vary between 0 and 100. It indicates the vulnerability of the cultivar or genotype, and may be used as a reciprocal indicator of disease resistance. Since the effect of the isolates on foliage seemed not to parallel that on pods, the severity index (SI) was applied for both separately. (a) Leaf symptoms were classified into 7 categories, viz.

0, no symptoms; 1, local symptoms only; 2, mild systemic mosaic; 3, clear mosaic; 4, mosaic and growth reduction or plant stunting; 5, tip necrosis and/or leaf drop; 6, plant death. (b) Pod symptoms were grouped into 4 categories, viz. 0, healthy; 1, weak symptoms; 2, visible lesions; 3, abnormal in size and shape; 4, no pods formed.

Light microscopy

The inclusion bodies induced by the different isolates were investigated in epidermal strips from young stems and petioles of faba bean as described earlier (Bos, 1969), using a mixture of phloxine and methylene blue in Christie's solution as a dye.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE of purified virus preparations was carried out according to Laemmli and Favre (1973) using a Bio-Rad Mini Protean II apparatus. The stacking and separating gels contained 4% and 12% acrylamide, respectively. About 3 ug of virus preparation dissolved in an equal volume of Laemmli sample buffer and boiled for 3 min, was applied per slot. After electrophoresis, the gel was stained in Coomassie Brilliant Blue. The protein markers (Boehringer) used were phosphorylase B (92.5 K), bovine serum albumin (67 K), ovalbumin (45 K), carbonic anhydrase (29 K), sorghum trypsin inhibitor (21 K), and cytochrome C (12.5 K).

Preparation of coat-protein core and purification of N-termini-specific antibodies

N-terminal peptide regions of the coat protein were removed from intact particles of BYMV-B25 and their specific antibodies were purified as described by Shukla *et al.*, (1988). The conditions were modified as follows: 10 mg of virus in 0.05 M potassium phosphate buffer pH 8.0 and 12 ug lysyl-endopeptidase (Boehringer Mannheim B.V., NL) were used per mg virus for 1 h at 25°C in a water bath, instead of 6 mg of enzyme and 30 min incubation, and 0.1 M borate saline buffer (BSB) pH 8.4 instead of BLOTTO for washing the antiserum through the affinity-chromatography column.

Electro-blot immunoassay (EBIA)

The electro-blot immunoassays were performed after SDS-PAGE and electro transfer of the peptides onto nitrocellulose membranes. For each isolate, 3 ug purified virus was used per slot of the gel. The immunoassay was performed by transferring the membranes to Petri dishes containing a blocking agent, 5% horse serum in phosphate-buffered saline (PBS). After incubation on a shaker for 2 h, excess blocking agent was removed by washing the membranes three times in PBS containing 0.1% Tween-20. The membranes were then transferred to Petri dishes containing the γ-globulins at 2 mg/ml in BSB, incubated for 2 h on a shaker and washed as before, and thereafter transferred to Petri dishes containing the sheep anti-rabbit alkaline phosphatase conjugate (Sigma Chemicals), diluted 1:100 in PBS containing 0.1% Tween-20 and 0.5% bovine serum albumin.

After 2 h of incubation on a shaker, the membranes were washed as described before and incubated for 15 min in a preparation of the substrate Nitroblue-tetrazolium according to the manufacturer's (Sigma) directions. The prestained protein markers are as mentioned in the SDS-PAGE Section.

RESULTS

Host range and symptomatology

The results of host-range tests are summarized in Table 2. This shows that the isolates E178, CYVV-H, E197 and E242 clearly differ from the remaining isolates by the necrotic local lesions induced in *Chenopodium amaranticolor*, the systemic necrotic lesions produced in *C. quinoa*, their ability to systemically infect pea 'Vitalis', which is immune to BYMV, and the systemic necrosis induced in 'Koroza'. These four isolates further differ in their wider host range, notably comprising the non-legume species *Cucumis sativus* 'Gele Tros', *Nicotiana debneyi* and *Nicotiana clevelandii*, found not to react with the other isolates.

The bean isolates B25 and BSMV-TN appear more adapted to *Phaseolus* beans. They infected all bean cultivars tested, BSMV-TN being especially virulent on this host. They did not induce any visible local reaction in faba bean 'Compacta', but this host reacted with a vein chlorosis and very mild green mosaic. In pea 'Koroza' they induced a mild systemic green mosaic.

The isolate E198 differs from the bean isolates by its systemic mottling induced in faba bean 'Compacta' and its clear yellow mosaic in pea 'Koroza'.

The isolate E199 seems intermediate between the above necrotic isolates and the rest of the isolates in that it induced necrotic reactions in faba bean 'Compacta' and pea 'Koroza', while it failed to infect the non-legumes other than *Chenopodium* species.

The faba-bean isolates, at which the study was especially aimed, induced only chlorotic local lesions in both *Chenopodium* species tested, but did not infect the other non-legume species and induced a variable systemic mottling in faba bean 'Compacta'.

Table 2. Summary of host–plant reactions

Host plants	Isolates													
	B25	BSMV-TN	E198	E199	E197	CYVV-H	E178	E242	SV-205	SV-231	SuV-128	SuV-270	Vf15	Vf42
Chenodopium amaranticolor	LS	LS	LS	L–	L^n–	L^n–	L^n–	L^n–	L–	Ls	L–	L–	L–	Ls
Chenopodium quinoa	L–	L–	L–	L–	L^nS^n	L^nS^n	L^nS^n	L^nS^n	L–	L–	L–	L–	L–	L–
Cucumis sativus 'Gele Tros'	––	––	––	––	L^n–	(––)	L^n–	L^n–	––	––	––	––	––	––
Nicotiana debneyi	––	––	––	–s	L–	L–	L–	L–	––	––	––	––	––	–s
Nicotiana clevelandii	––	––	–s	––	Ls	LS	LS	Ls	––	–s	––	––	––	––
Phaseolus vulgaris														
'Almere'	LS	LS	LS	L^nS	L^nS^n	L^nS	LS	L^nS^n	LS	LS	L^n–	L^nS	L^n–	L^nS
'Bataaf'	LS	L^n	LS	L^nS	L^n–	L^nS^n	L^nS^n	L^n–	LS	LS	L–	L–	L–	L^nS
'Double White Princess'	L^nS	L^n	L^n–	L^n–	L^nS^n	L^n–	L^nS^n	L^nS^n	LS	LS	L–	LS^n	LS	L^n–
'Flotille'	LS	LS	LS	LS	LS	––	––	––	LS	––	––	––	L–	L–
'Great Northern 123'	L^ns	L^ns	––	L^n–	–s	L^ns	––	–s	–s	––	L^n–	––	––	L^n–
'Lasso'	LS	LS	––	––	––	––	––	––	––	––	––	––	––	––
'Michelite'	L^nS	L^nS^n	L^nS	–S	L^nS	L^nS^n	L^n–	–S	–S	–S	L^n–	––	L^nS	L^nS
'Top crop'	LS	LS^n	LS	LS	LS	L^nS	L^nS^n	LS	LS	L–	L–	LS	L–	L^nS
Pisum sativum														
'Dark Skin Perfection'	––	––	––	––	––	––	–S	––	––	––	––	––	––	––
'Koroza'	LS	LS	LS	L^nS	L^nS^n	L^nS^n	L^nS^n	L^nS^n	LS	LS	LS	LS	LS	LS
'Vitalis'	––	––	––	––	–S	–S	–S	–S	––	––	––	––	––	––
Vicia faba 'Compacta'	–S	–S	LS	L^nS^n	LS	LS	L^nS	L^nS^n	LS	LS	LS	LS	LS	LS

L Local symptoms; *S* systemic symptoms; *s* latent systemic infection; – no infection as tested by ELISA; *n* necrotic reaction
In brackets, results differing from those of Bos et al. [10]

They varied considerably in the type of mosaic produced in pea 'Koroza' and in their ability to infect the bean cultivars tested as well as in the type of symptoms induced in these cultivars.

Pathogenicity on *Phaseolus* bean

The Severity Indexes calculated for the symptoms on leaves and pods are recorded in Table 3, and an impression of the pathogenicity of most of the isolates on cv. Bataaf is given in Fig. 1. These data, together with those for *Phaseolus* cvs contained in Table 2, show that the *Phaseolus*-bean isolates B25 and BSMV-TN are pathogenic on all *Phaseolus*-bean cultivars tested, with BSMV-TN being the most pathogenic in its effects on both leaves and pods. The cv. Lasso reacted to these two isolates only. The other isolates did not show any symptom on leaves or pods (Table 3) of this cultivar, and they could not be detected by ELISA in either inoculated and non-inoculated leaves (Table 2).

In contrast, the faba-bean isolates appeared to be less pathogenic on *Phaseolus*-bean cultivars and their effect on pods was low. An exception was SV205, which was relatively pathogenic on 'Almere', 'Bataaf' and 'Flotille'. In pathogenicity on *Phaseolus* beans this isolate appears to be intermediate between *Phaseolus*-bean isolates and faba-bean isolates.

The CYVV-like isolates (CYVV-H. E178, E197, and E242) and to some extent E199 were necrotic on most *Phaseolus*-bean cultivars.

Light microscopy

All isolates investigated were found to induce granular cytoplasmic inclusions in epidermal cells of faba bean 'Compacta' (Fig. 2A,B). The isolates E178, CYVV-H, E197 and E242 induced additional striking nucleolar enlargements sometimes nearly filling the nuclear space (Fig. 2B).

Table 3. Severity index (SI), on a 0 to 100 scale, of the virus isolates on leaves and pods of six *Phaseolus*-bean cultivars.

Isolates	Bean cultivars					
	Almere	Bataaf	Flotille	Michelite	Lasso	Topcrop
B25	67[a]/2[b]	67/60	76/20	87/60	67/0	67/40
BSMV-TN	67/20	98/80	67/60	96/80	67/20	77/69
E198	67/40	50/40	50/20	42/30	0/0	67/41
E199	50/40	58/30	50/40	58/28	0/0	50/41
E197	50/58	50/20	50/40	67/40	0/0	50/41
CYVV-H	67/50	85/100	0/0	100/100	0/0	87/93
E178	50/44	50/75	0/0	1/3	0/0	97/100
E242	50/40	67/80	0/0	94/75	0/0	83/81
SV205	67/60	67/60	50/20	15/9	0/0	50/21
SV231	17/0	40/28	17/0	19/8	0/0	17/1
SuV128	17/0	50/20	0/0	6/0	0/0	17/1
SuV270	50/20	50/28	0/0	0/0	0/0	50/21
Vf15	17/0	50/40	17/0	6/0	0/0	17/1
Vf42	17/0	50/20	4/8	10/8	0/0	37/1

[a] Severity index on leaves, [b] Severity index on pods.

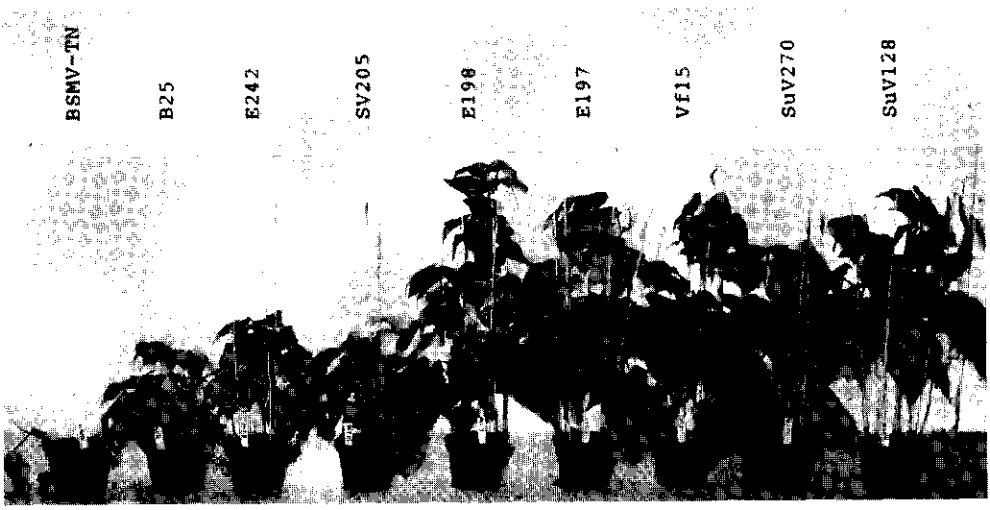

Fig. 1. Reaction of *Phaseolus* bean 'Bataaf' to most of the isolates studied, 38 days after inoculation.

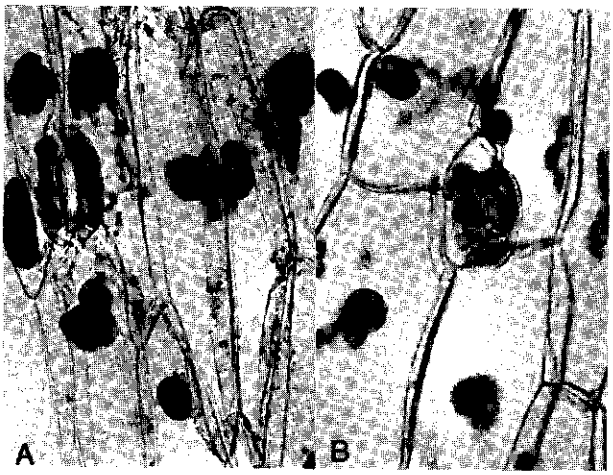

Fig. 2. Light micrographs of granular inclusion bodies in epidermal cells of petioles of faba bean infected with SuV128 (A) and E197 (B) (ca. x 220). Staining with phloxine and methylene blue.

SDS-PAGE

The migration pattern of the coat proteins of the different isolates is shown in Fig. 3. It appears that the coat proteins of E178, CYVV-H, E197 and E242 migrated slower and thus have a higher molecular weight (about 36 K) than those of all other isolates (34 K). The higher protein bands represent the intact coat protein of the isolates, while the lower ones are breakdown products as already reported earlier by others (Shukla *et al.*, 1989a).

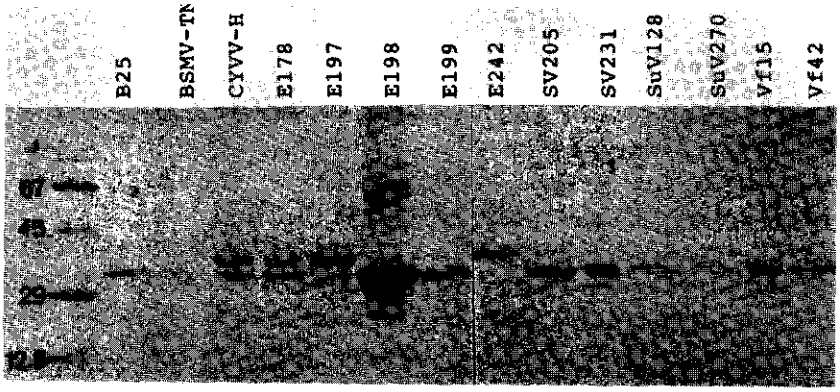

Fig. 3. Migration patterns in SDS-PAGE of the protein bands of purified preparations of the isolates indicated after staining with Coomassie Blue. Numbers at the left indicate the M_r of the marker proteins in the left lane.

EBIA

Twelve ug of lysyl-C-endopeptidase per mg virus was found sufficient for complete removal of the N-terminus of BYMV-B25 coat protein as judged by SDS-PAGE. The antibodies collected after passage through the affinity-chromatography column were found to be specific for the N-terminus, as they no longer reacted in EBIA with PVY^{n} nor with B25 coat protein devoid of the N-terminus.

The reaction of the isolates studied in EBIA with unfractionated antibodies to B25 is shown in Fig. 4A. The antibodies to B25 reacted with all the isolates and with two unrelated potyviruses included as controls, viz. bean common mosaic virus (BCMV-NL5), a potyvirus infecting legumes, and onion yellow dwarf virus (OYDV), a non-legume potyvirus. The antibodies reacted weakly to OYDV, BCMV-NL5, E242, E197 and to CYVV-H.

When using the antibodies specific to the N-terminus of B25 (Fig. 4B), no reaction was recorded with the controls, while these antibodies still reacted with all the isolates under investigation, and with the same intensities as the unfractionated antibodies did.

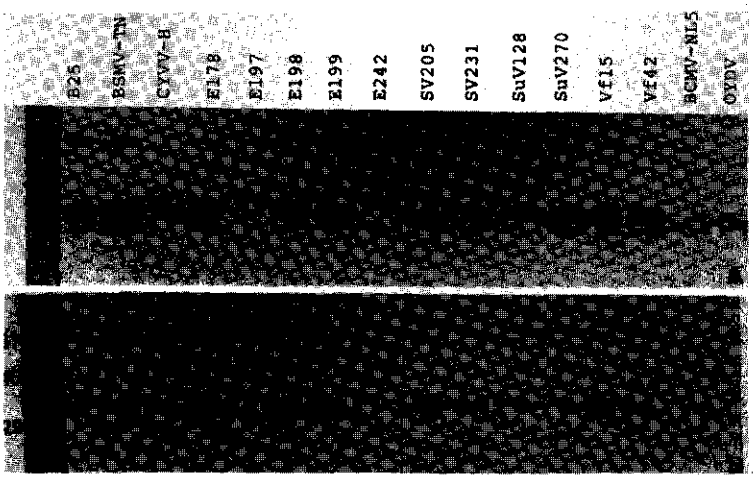

Fig. 4. Reactivities in EBIA of the 14 virus isolates studied, with BCMV-NL5 and OYDV as controls, with unfractionated antibodies to BYMV-B25 (A) and antibodies to the N-terminus of the coat protein of BYMV-B25 (B). Numbers at the left indicate the M_r of the prestained marker proteins in the left lane. N.B. By accident, E198 was also loaded in the slot of E178. In other EBIA experiments, E178 reacted like other CYVV isolates.

DISCUSSION

Based on host range, inclusion-body type, and pattern of coat-protein migration in SDS-PAGE, the isolates studied can be grouped into CYVV-like isolates (E178, CYVV-H, E197 and E242) and BYMV-like isolates (the remaining isolates). But based on the reaction to the BYMV N-terminal antibodies, all isolates studied should be considered strains of BYMV according to Shukla's hypothesis (Shukla *et al.*, 1988), unless they share restricted epitopes causing an unexpected paired relationship (Shukla and Ward, 1989a, 1989b). The latter is a distinct possibility since these serological data do not corroborate biological and physico-chemical data which, however, cannot be ignored.

Our biological results clearly indicate that the CYVV-like isolates have a wider host range among non-legume species than the BYMV-like isolates. These biological differences were earlier reported by Bos *et al.* (1977) and later confirmed by Lisa and Dellavalle (1983), and are further corroborated by the fact that CYVV regularly infects white clover while BYMV usually does not (Bos *et al.*, 1977; Barnett *et al.*, 1985). The two groups of isolates are distinct pathologically, and the differences in host range indicate different ecological potentials. Pathological differences are further supplemented by striking differences in nucleolar abnormalities as revealed by light microscopy, although Edwardson (1974) has classified BYMV and CYVV in subdivision II based on the type of cytoplasmic inclusion bodies in host tissues as revealed by electron microscopy. In his review, however, nucleolar abnormalities were not taken into account. All CYVV-like isolates studied showed the highly characteristic nucleolar enlargements earlier described (Bos, 1969; Bos and Rubio-Huertos, 1969; Bos *et al.*, 1977). With E178 the enlarged nucleolus is usually covered with long needles often penetrating through the nuclear membrane (Bos and Rubio-Huertos, 1969). This phenomenon seems to be of diagnostic value.

The pathogenicity tests have shown CYVV to be harmful to several *Phaseolus*-bean cultivars. Although the virus is mainly nown to infect *Trifolium* spp. in nature, especially white clover (Hollings and Stone, 1974), it has been reported to severely damage climbing types of *Phaseolus vulgaris* in North-West Italy (Lisa and Dellavalle, 1983).

The present results largely agree with those of earlier reports (Beczner *et al.*, 1976; Bos *et al.*, 1977) with the exception that isolate E197, earlier considered a necrotic strain of BYMV, now appears to be a CYVV strain according to inclusion-body type, host range and size of coat protein. The present results also confirm the earlier grouping of the pea mosaic isolate E198 as a pea (yellow) mosaic strain of BYMV (Bos *et al.*, 1974) characterized by yellow mosaic symptoms in pea and poor pathogenicity to *Phaseolus*-bean genotypes. E199 was also confirmed to be a necrotic strain isolate of BYMV (Bos *et al.*, 1974) instead of CYVV. Immunity now detected in 'Lasso' to all four isolates of CYVV tested is promising.

The two Dutch faba-bean isolates Vf15 and Vf42, the Syrian isolates SV205 and SV231, as well as the Sudanese isolates SuV128 and SuV270 differ from pea mosaic isolates in showing low pathogenicity in pea (mild green symptoms), low pathogenicity in *Phaseolus*-bean genotypes, and clear pathogenicity in faba bean. SV205 seems intermediate between faba-bean isolates and *Phaseolus*-bean isolates in its also being rather pathogenic in bean. Therefore, although faba-bean isolates of BYMV appear to be especially adapted to faba bean, there appears to be no clear-cut distinction between groups of isolates which would allow a clear discrimination between strains according to their original hosts .

The slower migration of the coat protein of the CYVV-like isolates in SDS-PAGE reveals that their molecular weight (ca. 36 K) is higher than the 34 K reported for some BYMV strains (Huttinga, 1975). This slower migration of the CYVV coat protein has been shown earlier (Shukla *et al.*, 1988), and is another clearly distinctive intrinsic feature.

In EBIA, the reactivity of all the isolates studied and of BCMV-NL5 and OYDV to the unfractionated antibodies to BYMV-B25 is due to the cross-reacting antibodies directed to epitopes located in the core protein, as reported by Shukla *et al.*, (1988). However, the reactivity of these isolates to the B25 N-terminal antibodies, which did not recognize the controls BCMV-NL5 and OYDV does not necessarily establish that they are strains of BYMV, unless serological affinity should be considered the conclusive criterion for virus classification. The BYMV-like isolates on the one hand and the

CYVV-like isolates on the other remain biologically distinguishable and are distinct in the size of their coat protein. The intensity of the reaction in EBIA might be used as a quantitative criterion for differentiation of both viruses.

The reaction of the CYVV isolates with the antibodies directed to the N-termini of BYMV-B25 coat protein suggests that the two viruses share epitopes in the N-terminal region. This has recently also been shown for Australian strains of BYMV and CYVV, where a sequence of four amino acids in the N-terminal region were found identical in the two viruses (Shukla and Ward, 1989b). Moreover, it has been reported that only few amino-acid residues in an epitope are responsible for antibody production and binding (Geysen *et al.*, 1988). Clearly, two different potyviruses that have an identical amino-acid sequence at the N-terminal region cannot be differentiated by antibodies specific for that region. This was also found earlier for Johnson grass mosaic virus and watermelon mosaic virus 2, the former virus infecting monocotyledons and the latter dicotyledons (Shukla *et al.*, 1988).

It seems therefore that the N-terminal-specific antibodies are of little use to distinguish between the two viruses. Work is meanwhile going on to sequence the coat proteins of both viruses to verify whether they possess common sequences in the N-terminal region of their coat proteins. As it has already been pointed out (Shukla and Ward, 1989a, 1989b), the problem of paired serological relationships may be more acute with potyviruses infecting legumes, and polyclonal antibodies to defined synthetic peptides may be required to discriminate them.

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CHAPTER 7

Identification and taxonomy of the bean yellow mosaic virus subgroup of potyviruses; developments and diagnostic application

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SUMMARY

The potyviruses belonging to the bean yellow mosaic virus (BYMV) subgroup are more closely interrelated than they are to other potyviruses. As a consequence, the current serological assays are insufficient for reliable diagnosis. The recent accumulation of genome sequence data of potyviruses may provide a more rational basis for the identification and classification of potyviruses in general. This chapter reviews the developments in the identification and taxonomy of the BYMV subgroup of potyviruses and discusses the exploitation of the molecular data in the polymerase chain reaction (PCR) for diagnostic purposes. A preliminary study using the PCR has shown the possibility to distinguish between BYMV and clover yellow vein virus.

INTRODUCTION

The bean yellow mosaic virus (BYMV) subgroup of the genus *Potyvirus* (Family *Potyviridae*) includes BYMV, clover yellow vein virus (CYVV), pea mosaic virus (PMosV), and sweet pea mosaic virus (Randles *et al.*, 1980; Barnett *et al.*, 1987). These viruses are serologically related in varying degrees, and each of them consists of a number of strains or variants which differ mainly in biological properties. Attempts were

made to classify these viruses by classical approaches such as symptomatology, host-range studies, serology, and nucleic acid hybridization (Barnett *et al.*, 1987; Bos *et al.*, 1974; Jones and Diachun, 1977; Reddick and Barnett, 1983). These studies showed that the legume-infecting potyviruses are closely related to each other and that there is no single parameter allowing their unambiguous discrimination. In general, no sharp borderline separating individual potyviruses could be drawn, but a continuum of variants or strains seems to exist (for a historical review see Bos, 1992). However, the recent molecular studies appear to provide a more rational basis for the identification and classification of potyviruses. This paper reviews the recent developments in the identification and taxonomy of the bean yellow mosaic virus subgroup of potyviruses, and provides some results of preliminary experiments on the use of the available molecular data to design oligonucleotide primers that differentiate between BYMV and CYVV in the reverse transcriptase-polymerase chain reaction (PCR).

Amino acid sequence of the coat protein

The increased knowledge of the coat protein (CP) structure of potyviruses has considerably contributed to the characterization of potyviruses and the discrimination between individual potyviruses and their strains. Distinct viruses were found to exhibit CP-sequence homologies ranging from 38 to 71%, compared to 90 to 99% for related strains, which also show similar N-terminal lengths and sequences (Shukla and Ward, 1988). Moreover, the N-terminal part of the CP was found to contain virus-specific epitopes, while the potyvirus-group-specific epitopes are located in the core protein region (Shukla *et al.*, 1988). The use of antibodies directed towards the N-terminal part of the CP proved helpful in elucidating the taxonomic status of the potyviruses infecting maize, sorghum, and sugarcane (Shukla *et al.*, 1989), and of the bean common mosaic virus-subgroup of potyviruses (Khan *et al.*, 1990). However, when this approach was applied to members of the BYMV-subgroup, it was found that CYVV, which is biologically distinct from BYMV and has a lower migrating coat protein, reacted in electro-blot immunoassay with antibodies directed towards the N-terminal part of the

coat protein of BYMV (Fortass *et al.*, 1991). This implies that the two distinct viruses share common epitopes in the N-termini of their CPs, and that the serology using antibodies specific to the N-terminus of the CP is not of aid in discriminating between BYMV and CYVV. This type of serological cross reaction was also reported for two other distinct viruses, Johnson grass mosaic virus and watermelon mosaic virus 2 (Shukla *et al.*, 1988).

Information on the amino acid sequences of the N-terminus of the CP brought more insight. Uyeda *et al.* (1991) reported an amino acid homology between the N-termini of the CPs of CYVV and BYMV ranging from 39 to 47% and considered them distinct viruses, since strains of the same virus differ only in few amino acids in the N-terminal region of the CP (Shukla *et al.*, 1988). Later, it was reported that the homology between the N-termini of the CPs of CYVV and BYMV strains is clustered at the immediate N-terminus where 17 amino acids are conserved (Bryan *et al.*, 1992). The existence of such an area of homology in the N-terminus explains the reported cross reactivity between BYMV and CYVV when using the N-terminus directed antibodies (Fortass *et al.*, 1991).

Such called 'unexpected paired serological relationships' (Ward and Shukla, 1991) limit the general use of the N-terminal serology as a parameter for classifying potyviruses. Furthermore, a degree of homology between amino acid sequences of CPs of two isolates laying midway between the homology found between distinct potyviruses and that of related strains, makes that the borderline separating distinct viruses would probably fade as information on sequences of more isolates becomes available. Such an intermediate degree of homology (83%) has already been reported for watermelon mosaic virus 2 and soybean mosaic virus N (Yu *et al.*, 1989). Shukla and Ward (1989) have drawn attention to some genetic events which could affect the amino-terminal region of the coat proteins of potyviruses, and consequently complicate the use of N-terminal serology in the identification of potyviruses. Examples of such events are, e.g., internal deletions in the CP gene for strains of plum pox virus (Maiss *et al.*, 1989) and watermelon mosaic virus (Yu *et al.*, 1989), and a sequence diversity of yet unknown origin in the N-terminal of the CPs of sugarcane mosaic virus strains (Frenkel *et al.*, 1991).

Nucleotide sequence of the CP gene and of the 3'-nontranslated region of the genome

Information on the nucleotide sequence of the CP-coding region and the of the 3'-nontranslated region (3'-NTR) of RNAs has been reported to further assist in identifying and classifying potyviruses. The degree of homology of the 3'-NTR ranges from 83 to 99% between strains, whereas the sequences of distinct potyviruses have identities in the range 39 to 53% (Frenkel *et al.*, 1989). When comparing the nucleotide sequences of the CP genes and of the 3'-NTR of BYMV-S and CYVV-B, Tracy *et al.* (1992) reported a homology of the CP gene of 77% (which is above the value found for distinct viruses), and a 65% homology in the 3'-NTR (which is below the value range found for strains of a potyvirus). Furthermore, polymerase chain reaction probes corresponding to the 3'-NTR of CYVV and BYMV hybridized strongly only with the strains of their respective viruses but not with other potyviruses including PMosV. The authors concluded that BYMV, CYVV, and PMosV should be regarded as distinct potyviruses. A similar conclusion regarding the taxonomic relationship between BYMV and CYVV had earlier been reached by Uyeda *et al.* (1991) who reported homology in the nucleotide sequence of the 3'-NTR of 76% between a Japanese isolate of CYVV and two strains of BYMV (BYMV-CS and GDD), while the homology between the CP coding genes was found to be 73 and 69%. The available data on the amino acid sequences of the CP and the nucleotide sequences of the 3'-NTR of BYMV and CYVV isolates are summarized in Table 1. The amino acid sequence homology of the CP varies from 87 to 94% among the isolates of BYMV, and from 88 to 93% among those of CYVV. On the other hand, the homology of BYMV isolates to CYVV isolates ranges from 70 to 77%. Such a range is intermediate between that of distinct potyviruses and that of related strains. The same holds for the homologies of the nucleotide sequences of the 3'-NTRs between both viruses (Table 1).

The two viruses, though distinct, are thus more closely related to each other than to other potyviruses of the genus, and an isolate with known nucleotide sequence can easily be identified as either a BYMV or a CYVV strain. High-performance liquid

chromatographic peptide profiling of tryptic digests of the coat proteins of a number of isolates from the BYMV-subgroup recently allowed McKern *et al.* (1993) to classify PMosV and white lupin mosaic virus as strains of BYMV. This appears in conflict with the conclusions of Tracy *et al.* (1992) who considered PMosV a distinct virus based on hybridization tests involving the 3'-NTR. However, nucleotide sequencing of the CP gene and the 3'-NTR will undoubtedly clarify the taxonomic status of PMosV and of sweet pea mosaic virus, another closely related virus.

Table 1. Amino acid sequence homology of coat proteins (a) and nucleotide sequence homology of 3'-NTR (b) between BYMV and CYVV isolates. Data from Uyeda (1992).

		BYMV				CYVV		
		CS	Danish	GDD	S	30	B	NZ
BYMV	CS	-/-	89[a]/94[b]	88/86	87/90	73/75	77/74	77/73
	Danish		-/-	94/92	89/94	71/77	76/76	73/74
	GDD			-/-	89/88	70/73	74/74	73/71
	S				-/-	71/74	77/73	73/72
CYVV	30					-/-	92/98	88/92
	B						-/-	93/92
	NZ							-/-

Classification and diagnosis based on PCR: preliminary study

The recently accumulated sequence data converge towards the distinction of BYMV and CYVV as separate viruses and corroborate their biological distinction. Sequencing techniques are thus powerful tools to identify an isolate as a BYMV or a CYVV strain and indicate the degree of relationships between such viruses. The genome nucleotide sequence is an intrinsic property of a virus, such data are more easily accessible (than

other properties) once they are deposited in a data base. The value of serology is still limited, but McKern *et al.* (1993) observed that an amino acid sequence in the C-terminal part of the CP of 'PMosV' and BYMV is conserved and differs from that of CYVV strains. Such sequences would generate antibodies that may aid in assigning isolates from the BYMV subgroup as strains of BYMV or CYVV. Moreover, this variable region of the genome may also be used to design primers which would discriminate between BYMV and CYVV in the polymerase chain reaction as described below.

Four BYMV isolates (the neotype B25, a Syrian isolate from faba bean SV205, a necrotic isolate from pea E199, and a Moroccan isolate N5) and two CYVV isolates (the type member CYVV-H and a pea isolate E242) identified earlier on the basis of migration pattern in polyacrylamide gel electrophoresis, and on biological and cytopathological properties (Fortass *et al.*, 1991; Fortass and Bos, 1991) were used in this study. A pair of oligonucleotide primers was designed by comparing the known CP gene sequences of BYMV and CYVV (Boye *et al.*, 1990; Bryan *et al.*, 1992; Hammond and Hammond, 1989; Takahashi *et al.*, 1990; Tracy *et al.*, 1992; Uyeda *et al.*, 1991). The downstream primer V 24 (5' ATACGAACACCAAGC 3'), located in the 3' end of the CP gene, corresponds to bases 804 to 818 of the Danish BYMV-RNA (Boye *et al.*, 1990), and the upstream primer V 18 (5' GGAATGCAACTGAGAGG 3'), located in the core of the CP, pairs with the bases 542 to 558 of the same virus (Figure 1). The expected size of the amplified fragment is 276 bp.

RNA was extracted from leaves of infected pea plants as described by Chomczynski and Sacchi (1987). The cDNA was synthetized by heating 10 µl extracted RNA with 40 pmol primer V 24 for 3 min at 95°C, and for 42°C for 10 min, followed by addition of an equal volume of a mixture containing 100 mM Tris-HCl, pH 8.3, 150 mM KCl, 6 mM $MgCl_2$, 10 units/µl of M-MLV reverse transcriptase (Gibco BRL), 2.5 mM of each dNTP, and 5 units RNasin. The reaction mixture was incubated for 60 min at 37°C and for 10 min at 65°C. Amplification of the 276 bp fragment was carried out in 40 µl PCR mixture containing 4 µl cDNA, 5 mM of each dNTP, 15 pmol of each primer, 2 units of

Taq polymerase, 50 mM Tris-HCl, 50 mM KCl, 7 mM $MgCl_2$, 0.2 mg/ml BSA, and 16 mM $(NH_4)_2SO_4$. The samples were overlaid with 50 µl mineral oil and placed in a DNA thermal cycler giving one cycle at 94°C for 1 min, 56°C for 1 min, and 72°C for 30 s, followed by 33 cycles at 94°C for 30 s, 56°C for 1 min, and 72°C for 30s, and a final cycle at 94°C for 30 s, 56°C for 1 min, and 72°C for 3 min. Aliquots (10 µl) of the reaction mixtures were analyzed on 1% agarose gels stained with ethidium bromide (1µg/ml).

```
                  521                                                V18
BYMV- Danish      CAGAAGTTGCAGAAGCCTACATTGAAAAGAGGAATGCAACAGAGAGGTACATG
    - GDD         ..................................C.................
    - S           ..C.............T..T..A..G..A.......................
    - CS          ..C.G.....T.....T..T..A..G..............T........T...
CYVV- B           .CC.G.CA..C...T.A.....A..GTTC.....CT................
    - 30          .CC.G..A..T...T.A.....A..GTTC.A...ACTC.ACAGAGTT..T...
    - NZ          .CC...CA.....GT.A....A...TTC......TGC..T..A.........

                  772                                                V24
BYMV- Danish      GCAGGAGATGTCAATCGTGATATGCACACCATGCTTGGTGTTCGTATTTAG
    - GDD         ....................................................
    - S           ....................................................
    - CS          ....................................................
CYVV- B           ...AAT.....T..CA.GA.C....ATCA...TGC...A.CA..AT.C...
    - 30          ...AAT.....T..CA.GA.C.....TCA...TGC...A.CA..AT.....
    - NZ          ...AAT........CA.AA.......TCAT..TGC.....C...CT.C...
```

Fig. 1. Alignement of the nucleotide sequences (from selected areas used in designing the primers V18 and V24) of the coat protein genes of BYMV and CYVV isolates. Data were from the following sources: BYMV-CS (Takahashi *et al.*, 1990), -Danish (Boye *et al.*, 1990), -GDD (Hammond and Hammond, 1989), -S (Tracy *et al.*, 1992), and CYVV-30 (Uyeda *et al.*, 1991), -B (Tracy *et al.*, 1992), -NZ (Bryan *et al.*, 1992). Numbers indicate nucleotide positions in the coat protein gene of BYMV-Danish.

Figure 2 shows that a PCR product of the expected size was present in samples infected with each of the BYMV isolates tested, but not in samples infected with either CYVV-H or E242, implying therefore that the set of primers used recognized specifically BYMV sequences, but not the two CYVV isolates tested. Although the upstream primer is located in a conserved region among potyviruses, the downstream primer is in a variable region, and would already discriminate between potyviruses in the cDNA synthesis step.

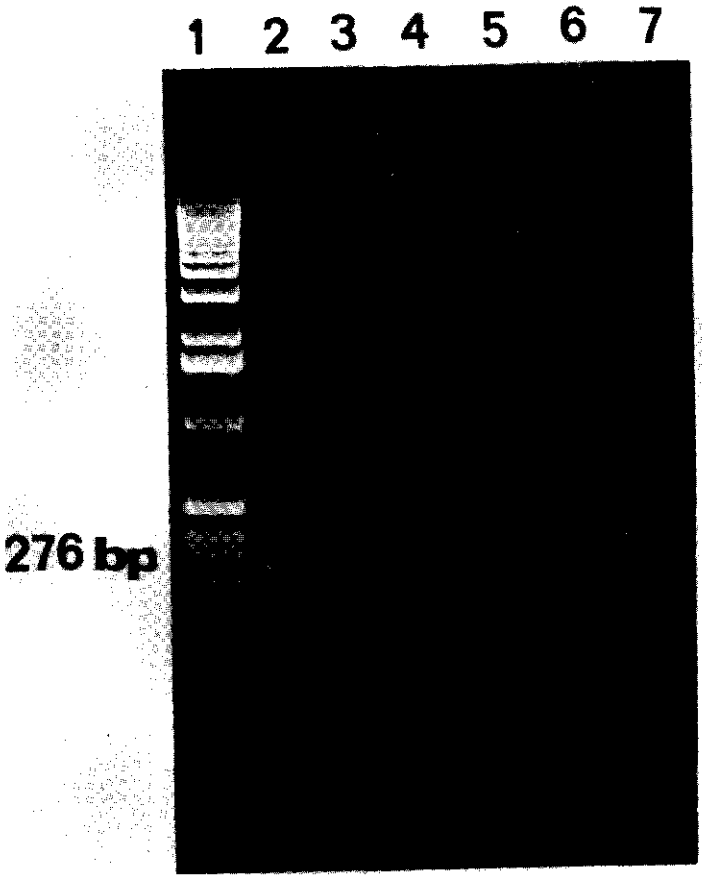

Fig. 2. Agarose electrophoresis of PCR products. Lane 1, DNA size markers (Gibco BRL); lanes 2 to 5, BYMV isolates B25, SV205, E199, and N5; lanes 6 and 7, CYVV isolates CYVV-H and E242.

CONCLUSION

Whereas serological assays are common practice in the detection and diagnosis of plant viruses, the reported cross-reactivity of antibodies raised against coat proteins of distinct potyviruses urges for more refined diagnostic tools to distinguish these economically important viruses. Even the use of specific antibodies, raised against the N-terminal domain of the potyviral coat protein is not satisfactory, as demonstrated by the cross-reactivity between BYMV and CYVV isolates (Fortass *et al.*, 1991). Genome sequencing is time- and money-consuming, is not practicable everywhere, and therefore not suitable for rapid diagnosis of field isolates. Diagnostic procedures can however be developed based on (partial) genome sequence data. Their exploitation in designing primers to be used in PCR would be a rational basis for rapid identification of field isolates. However, the specificity of the primers should be tested for a large number of known isolates, and would probably improve as information on more sequences of closely related, but biologically distinct, potyviruses becomes available.

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CHAPTER 8

General discussion and conclusions

The virus survey conducted in 1990 covered the main faba-bean growing areas in Morocco. From the 240 samples collected on the basis of virus-like symptoms, at least nine viruses were revealed using electron microscopy, biological indexing, and serology (Chapter 2). Some of these had already been reported in Morocco (El Maataoui and Fischer, 1976), but pea enation mosaic virus, pea seed-borne mosaic virus, broad bean true mosaic virus, and the luteoviruses were found for the first time in the country. Earlier reports were based on incidental findings or sample testings. The present survey is the first one carried out systematically, and therefore presents more reliable information on incidence and importance of these viruses. Broad bean mottle virus (BBMV) previously considered of limited distribution in Morocco (Fischer, 1979) was found to be remarkably prevalent. Knowledge about luteoviruses in faba bean is even completely new in Morocco. These viruses were found to occur in unexpectedly high incidences. They are likely to have been present for many years already, but overlooked since the symptoms they cause greatly resemble those of drought or mineral deficiencies. In contrast, broad bean stain virus (BBSV) and bean yellow mosaic virus (BYMV) earlier found prevalent (Fischer and Lockhart, 1976; Fischer, 1979), were now encountered in low incidence. For still unknown reasons, certain viruses may also rapidly emerge, as is the case with the newly described faba bean necrotic yellows virus (Katul, 1992) which has developed into an alarming epidemic in 1992 in Egypt (Bos, personal communication). This indicates that the incidence of viruses on a crop, largely depending on conditions, is a dynamic process. Therefore, continuous monitoring of the field situation is needed in order to keep on the alert as to actual importance.

Virus importance is not only determined by incidence, but also by time of infection and severity of symptoms. Several viruses, especially those causing mild mosaics, when

moving late into a crop, or when gradually spreading within the crop, may not be very harmful. But the assimilation-reducing capacity of viruses like BBMV, but especially the luteoviruses, may be great. Yield losses ranging from 41 to 76% have been reported to be caused by BBMV on faba bean (Fezzaz and Bourbah, 1979). The effect of luteoviruses is often dramatic, paricularly after early infections (Kaiser, 1973). Experiments to assess losses caused by viruses, planned for 1992, unfortunately failed because of poor crop growth due to excessive drought, as well as by the early and severe infestations of the plots by luteoviruses. Several of the viruses found on faba bean in Morocco are also of great potential economic importance for ecological reasons to be discussed below.

A scientifically interesting problem with great practical implications for surveying and control, which soon emerged and gradually developed during the surveying, is that of virus identification (characterization and recognition). Several virus isolates were hard to recognize because they resembled viruses that are incompletely characterized in the literature, or because they were deviant strains of known viruses. This particularly held for the potyviruses, such as the viruses related to BYMV, but even more so for the luteoviruses. Information on virus variation also is of considerable importance to breeding for resistance since genetic plant resistance usually is virus specific and often even strain specific.

For example, during biological indexing of the survey samples, a variation in symptom severity on test plants was detected among BBMV isolates. Comparison of seven selected Moroccan isolates with isolates from Algeria, Sudan, and Tunisia, revealed a pathogenic variability of BBMV on genotypes not only of faba bean, but also of a number of other food legumes such as pea, chickpea, and lentil (Chapter 3). Clusters of isolates differing in virulence were distinguished, although they reacted similarly with antisera to a Moroccan and a Syrian isolate. This indicates also the importance of supplementing serological testing with biological indexing in surveys in order also to detect possible pathogenic variability which is of practical importance.

Variation is well known for the potyvirus group and there is a continuum of viruses

around BYMV (Bos, 1970, 1992), a virus found of potential importance on faba bean in Morocco because of its wide legume host range, seed transmission and its occurrence in different growing areas. It was therefore studied for its relationships to clover yellow vein virus (CYVV), known for its necrotic effect on faba bean (Hollings and Stone, 1974). The two viruses could be distinguished by selected host range including non-legume species, by cytopathology, and by migration patterns of their coat proteins in polyacrylamide gel electrophoresis (Chapter 6). Results with conventional polyclonal antisera remain confusing, and even the new techniques claimed to be successful for discrimination between potyviruses (Shukla *et al.*, 1989) using antisera to epitopes in the more specific N-terminal part of the coat protein failed to distinguish between BYMV and CYVV (Chapter 6). However, by elaborate biological and cytopathological techniques, and by coat protein migration in gel electrophoresis, the potyviruses detected in the 1990 survey were confirmed to be BYMV and not CYVV. This indicates that there is a need to develop more simple and rapid tools to reliably identify these viruses. Recent molecular studies, including amino acid sequences of coat proteins and nucleotide sequences of RNAs of potyviruses, appear to provide a more rational basis for the identification and classification of potyviruses in general. Literature data are presented on the use of the sequence information in the polymerase chain reaction (PCR) in order to distinguish BYMV and CYVV. By aligning the known sequences of the coat-protein gene of strains of CYVV and BYMV, a set of primers were designed, and preliminary experiments showed that this pair of primers discriminates between both viruses in the PCR (Chapter 7). However, the specificity of these primers should be further tested for a large number of isolates, and would probably improve as information on sequences of more biologically distinct isolates becomes available. Since the genomic sequence is an intrinsic property of a virus, diagnostic procedures should be based on it to become more reliable.

The problem of variation was also obvious when dealing with the luteoviruses. Recognition of the different luteoviruses described so far continues to create difficulties. In the literature, a number of legume luteovirus diseases have been described but their causes, even when ascribed to specific viruses, have not always been completely

identified. Even antisera used are often to viruses of uncertain taxonomic status. When a number of Moroccan faba-bean samples with symptoms suggestive of luteovirus infection were screened in DAS-ELISA with polyclonal antisera to bean leafroll virus (BLRV), beet western yellows virus (BWYV), and subterranean clover red leaf virus (SCRLV), and in TAS-ELISA with two monoclonal antibodies reported to discriminate between BLRV and BWYV (Van den Heuvel *et al.*, 1990), different reaction patterns were obtained, and several samples reacted with more than one antiserum (Chapter 5). They could therefore not be conclusively identified with known legume luteoviruses. A pair of designed oligonucleotide primers were found to specifically amplify in PCR a 535-bp fragment of the coat protein gene of the standard luteoviruses tested as well as of the Moroccan isolates. They should then be used for detecting luteoviruses in faba bean and possibly in other food legumes. In molecular hybridization tests, selected Moroccan field isolates showed nucleotide sequence homology among them and with BLRV, but not with BWYV, although they were serologically different (Chapter 5). These results point to variation among the Moroccan luteovirus isolates and the possible involvement of (a) new luteovirus(es) adapted to faba bean. More studies including genome sequencing of selected Moroccan isolates are certainly needed in order to identify the luteoviruses involved.

Disease control, which for viruses mostly is preventive, requires detailed information on the ecology of the pathogen involved. BBMV, reported to be widespread in West Asia and North Africa (Makkouk *et al.*, 1988), and now found to be one of the most prevalent viruses in Morocco, was therefore studied for its ecology. It was found to have a rather wide host range among legume species, and to be more dramatic on several of them than on faba bean by causing necrosis and premature death (Chapter 3). Such symptoms can easily be mistaken for other causes including fungal infections, and this may explain why the virus has never been reported from these crops so far. During these studies, BBMV was detected in naturally infected plants of chickpea, lentil, pea, and even of common bean (*Phaseolus vulgaris* L.) (Chapter 4), and it is likely to be more prevalent in legumes other than faba bean than now found. Among the potential hosts found in host-

range studies, several perennial legumes were noticed (Makkouk *et al.*, 1988), in which, however, the virus was not detected to occur naturally in Morocco following limited testing. Nevertheless, such perennials may well play an important role in perennation of the virus and as natural sources of infection. A major source of infection, however, are plants infected from the seed. Seed transmission, first found by Murant *et al.* (1974) for BBMV in mixed infection with BYMV, as later confirmed by Makkouk *et al.* (1988), is now corroborated for the virus when occurring on its own (Chapter 3). BBMV was also found seed transmitted in chickpea and pea. This certainly plays an important role in the dissemination of the virus, especially in countries such as Morocco where certified seeds are not available but part of the harvest is used as seed for the next growing season. This may lead to a build-up of the virus within the farm and permit an easy (though unknown) dissemination of the virus within the region or even the country, of course depending on availability, population density, and efficiency of vectors.

Information on the curculionid weevils *Apion radiolus*, *Hypera variabilis*, *Pachytychius strumarius*, and *Smicronyx cyaneus*, in addition to the earlier reported (Borges and Louro, 1974) *Sitona lineatus*, as vectors of BBMV is new. Among these, *S. lineatus* L. was found to be an efficient vector, and can also transmit the virus from faba bean to lentil and pea (Chapter 4). This vector will undoubtedly play a role in the dissemination of BBMV from faba-bean fields to the late-sown food legumes such as lentil, and within (and from) fields where plants developing from infected seeds are present. Early infestations of faba-bean fields by *S. lineatus* and other potential curculionid vectors makes that weevil control should be considered.

The existence of isolates which are more pathogenic on other food legumes than on faba bean, seed transmission of this virus also in chickpea and pea, and its vector transmission from faba bean to pea and lentil and possibly to other species, make that BBMV should be regarded as a food-legume virus rather than a virus occurring naturally on faba bean only. But the same holds for most other viruses of faba bean, and this indicates that in investigating these viruses for practical purposes, they should not be studied in a single crop but in a broader ecological context.

Especially in countries where knowledge about cultural hygiene is poor, the use of

genetically resistant cultivars, and, if no such cultivars are available yet, breeding for resistance are major methods of control. Attention was therefore paid to the interaction between BBMV isolates and genotypes of faba bean as well as of other important food legume species. When a number of promising breeding lines and accessions of the main food legumes, obtained from the Genetic Resources Unit of the International Center for Agricultural Research in the Dry Areas (ICARDA), were tested with the BBMV isolates, they were all found vulnerable to the isolates investigated (Chapter 3). This would mean that if such germplasm would be used on a large scale for genetic improvement in the country, vulnerability of the crop to BBMV might increase, then further amplifying the importance of the virus. Such phytosanitary risks of the introduction of alien genetic material for crop improvement has, for example, been well demonstrated by the coming to the fore of the endemic rice yellow mottle virus in tropical Africa in imported Asian genotypes of rice (John *et al.*, 1986). In order to avoid introduction of genetic vulnerability, it is therefore essential to test alien legume germplasm for vulnerability to BBMV, and other actually or potentially important endemic viruses, prior to introduction into new recipient areas or countries. Since several of the viruses are highly variable, the careful choice of appropriate isolates in screening food-legume germplasm for resistance to BBMV is of paramount importance.

Seed transmission plays an important role in the ecology of a virus, and legume viruses are notorious for their seed transmissibility (Frison *et al.*, 1990). This holds not only for virus perennation from one season to another, but also for virus dissemination from one region or one country to another. The interregional exchange of seeds of legumes for human consumption (and sowing of part of it) undoubtedly explains the already wide-spread occurrence of for instance BBMV in West Asia and North Africa (Makkouk *et al.*, 1988). The exchange of germplasm of unchecked health status could seriously add to the international spread of the virus. This holds also for other seed-transmitted viruses, as well as for other food-legume growing areas in West Asia and North Africa.

Apart from quarantine in the case of viruses not yet occurring in the country, seed transmission of viruses already present plays a role as a quality factor for commercial

seed. Such 'quality viruses' should be present in such low percentages that no economic damage ensues in crops grown from the seed. This requires seed certification in which the relative absence of seed-transmitted viruses is part of the quality-determining aspects. Further agricultural modernization in Morocco will certainly involve improved seed production by special growers and adequate certification. Seed-transmitted viruses will require special attention, and seed testing will require adequate methods of virus detection, in turn requiring detailed knowledge of the viruses concerned and their strains.

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SUMMARY

A systematic virus survey covering the main areas where faba bean (*Vicia faba* L.) is grown in Morocco was conducted in 1988 and 1990. From the 240 leaf samples collected on the basis of symptoms suggestive of virus infection from 52 fields, the following viruses were detected by means of electron microscopy, biological indexing, and serology, and their incidence and geographical distribution were assessed: alfalfa mosaic virus (AMV), bean yellow mosaic virus (BYMV), broad bean mottle virus (BBMV), broad bean stain virus (BBSV), broad bean true mosaic virus (BBTMV), pea-early browning virus (PEBV), pea enation mosaic virus (PEMV), pea seed-borne mosaic virus (PSbMV), and luteoviruses. BBTMV, PEMV, PSbMV, and luteoviruses had not previously been reported in the country. BBMV, considered earlier of limited distribution, and the luteoviruses were found to be prevalent (in 50 and 56% of the surveyed fields, respectively; and with field incidences of 20 and 33%, respectively), whereas the opposite held for BBSV and BYMV. More detailed studies concentrated on the actually important BBMV and the luteoviruses, and on the potentially important BYMV-like isolates.

The biological indexing of samples revealed considerable variation in symptom severity on test plants among BBMV isolates. Comparison of seven selected Moroccan isolates with isolates from Algeria, Sudan, and Tunisia, revealed a pathogenic variability of the virus on a number of food-legume genotypes. Clusters of isolates differing in virulence could be distinguished as mild, intermediate, and virulent, although they all reacted similarly to the antisera to a Moroccan and a Syrian isolate. When a number of promising ICARDA breeding lines and accessions of faba bean, chickpea, lentil, and pea were tested with the BBMV isolates, different interactions were observed, but all genotypes were found vulnerable to all the isolates investigated, and no immunity could be detected. Some isolates were more pathogenic (often even necrotic) on other food legumes, such as pea and chickpea, than on faba bean. BBMV was found to be seed transmitted in faba bean (at a rate of 1.2%) when occurring on its own, and was detected to be so in chickpea and pea at transmission rates of 0.9 and 0.1%, respectively. Besides

transmission by seed, BBMV was found to be transmissible by the curculionid weevils *Apion radiolus*, *Hypera variabilis*, *Pachytychius strumarius*, *Smicronyx cyaneus*, and the previously reported *Sitona lineatus*. The latter appeared to be an efficient vector since the first bite was sufficient for acquisition and transmission of the virus, virus retention lasted for at least seven days, and the transmission rate was estimated to be 41%. *S. lineatus* turned out to transmit BBMV not only from faba bean to the same species, but also to lentil and pea. When searching for natural sources of the virus by testing of 351 samples of food legumes from 24 fields and 102 samples of wild legumes, it was found to occur naturally in chickpea, lentil, and pea, as well as in common bean (*Phaseolus vulgaris*) in 16, 11, 19, and 16% of the tested samples, respectively; but it was not detected in the samples of wild legumes reported in literature as potential hosts.

The problem of virus variability emerged and gradually evolved during these studies. It was encountered with BBMV showing a variation of isolates. It particularly holds for the cluster of potyviruses related to BYMV, but also for the luteoviruses, where it leads to difficulties in virus identification.

When testing faba-bean samples, showing luteovirus-like symptoms, in DAS-ELISA with polyclonal antisera to bean leafroll virus (BLRV), beet western yellows virus (BWYV), and subterranean clover red leaf virus (SCRLV), and in TAS-ELISA with two monoclonal antibodies discriminating between BLRV and BWYV, various serological reaction patterns were obtained. This pointed to a considerable variation among the luteovirus isolates which could not be identified as one of the known legume luteoviruses. To enable reliable detection of this group of viruses by the polymerase chain reaction (PCR), a pair of designed oligonucleotide primers were found to specifically amplify a 535-bp fragment of the coat-protein gene of known luteoviruses and of all Moroccan isolates tested. In molecular hybridization tests, selected field isolates showed nucleotide sequence homology among them, and with BLRV, but not with BWYV, although some of them behaved BWYV-like serologically. These results support the idea of the involvement of either deviant strains of known luteoviruses or of (a) completely new faba-bean luteovirus(es).

On the other hand, BYMV and the closely related clover yellow vein virus (CYVV)

could be distinguished by host-range studies including non-legume test species, and by cytopathology and polyacrylamide-gel electrophoresis of their coat proteins. Both viruses could not be distinguished by the N-terminal serology claimed to discriminate between potyviruses. Conflicting reports as to the taxonomic status of the potyviruses infecting legumes showed the need to develop more reliable tools to unambiguously identify these viruses. Recent molecular studies, including the elucidation of nucleotide sequences of legume-potyvirus RNAs, appeared to provide a rational basis for the identification and classification of potyviruses in general. The use of such molecular data in PCR for the distinction of BYMV and CYVV was investigated, and preliminary results showed that a pair of primers could be used in PCR to distinguish between both viruses.

SAMENVATTING

Virussen van fababoon (*Vicia faba* L.) in Marokko; inventarisatie, identificatie en ecologische aspecten

Fababoon (*Vicia faba* L.), in Nederland meer bekend als tuin- of veldboon, is in Marokko en andere landen in Noord-Afrika en het Midden-Oosten een sociaal, teeltkundig en economisch belangrijk voedsel- en voedergewas. In Marokko nemen de voedselleguminosen naast granen de tweede plaats in en onder de voedselleguminosen is fababoon met een jaarlijks areaal van 200 000 ha verreweg het belangrijkste gewas. Als toetsplant wordt fababoon veel gebruikt in het virusonderzoek vanwege zijn hoge vatbaarheid en gevoeligheid voor een groot aantal virussen van uiteenlopende plantesoorten. Natuurlijke infectie is in de soort als gewas volgens de literatuur reeds geconstateerd voor een 44-tal virussen, behorend tot tenminste 16 taxonomische groepen. Bij eerder fragmentarisch onderzoek werden in Marokko in fababoon slechts zes virussen aangetroffen. Het nu gerapporteerde onderzoek werd opgezet ter ondersteuning van het nationale fababoneprogramma omdat de informatie over de virussen van fababoon in Marokko nog erg beperkt is. Het vond zijn oorsprong in 1988 in het virologie-samenwerkingsproject tussen het Instituut voor Planteziektenkundig Onderzoek (IPO/DLO) te Wageningen en het 'International Centre for Agricultural Research in the Dry Areas' (ICARDA) te Aleppo, Syrië, in het kader van de toenmalige verantwoordelijkheid van dat instituut voor de gewasverbetering van fababoon in Noord-Afrika en West-Azië. Het onderzoek groeide uit tot een 'sandwich'-programma tussen de Ecole Nationale d'Agriculture (ENA) te Meknes, Marokko, en het IPO en de LUW-Vakgroep Virologie in Wageningen (Hoofdstuk 1).

Allereerst werd in 1988 en 1990 in de fababoneteeltgebieden van Marokko een systematische inventarisatie verricht waarbij in totaal in 52 velden 240 monsters werden verzameld van planten met symptomen die deden denken aan virusinfectie. Bij elektronenmicroscopisch en serologisch onderzoek en beperkte toetsing met

indicatorplanten werden de volgende virussen aangetroffen: bonescherpmozaïekvirus (bean yellow mosaic virus, BYMV), echt tuinbonemozaïekvirus (broad bean true mosaic virus, BBTMV), erwte-enatiemozaïekvirus (pea enation mosaic virus, PEMV), erwterolmozaïekvirus (pea seed-borne mosaic virus, PSbMV), luzernemozaïekvirus (alfalfa mosaic virus, AMV), tuinbonevlekkenvirus (broad bean mottle virus, BBMV), tuinbonezaadvlekkenvirus (broad bean stain virus, BBSV), vergelingsvirussen (luteoviruses) en vroege-verbruiningsvirus van erwt (pea early-browning virus, PEBV). Vaak kwam in eenzelfde veld meer dan één virus voor, soms werden zelfs vier verschillende virussen aangetroffen, en ook kwamen wel twee virussen tegelijk in één plant voor. Van de genoemde virussen waren BBTMV, PEMV, PSbMV en de vergelingsvirussen nog niet eerder in Marokko gesignaleerd. Het wel eerder in het land slechts in beperkte mate gevonden BBMV, en de vergelingsvirussen bleken nu veel voor te komen (in respectievelijk 50 en 56% van de onderzochte velden en met een incidentie tot respectievelijk 20 en 33%). Het tegenovergestelde bleek het geval te zijn met BBSV en BYMV die nu slechts in geringe mate werden aangetoond (Hoofdstuk 2). Het onderzoek werd vervolgens geconcentreerd op de actueel belangrijke virussen van fababoon, te weten het tuinbonevlekkenvirus (BBMV), de vergelingsvirussen (luteovirussen) en het potentieel belangrijke bonescherpmozaïekvirus (BYMV).

De biologische toetsing van monsters toonde tussen isolaten van het BBMV aanzienlijke verschillen aan in hevigheid van de in toetsplanten veroorzaakte symptomen. Daarom werden zeven op symptoomverschillen geselecteerde Marokkaanse isolaten vergeleken met isolaten uit Algerijë, Soedan (een zeer mild isolaat) en Tunesië. Hierbij traden aanzienlijke pathogeniteitsverschillen aan het licht op een beperkt aantal van het ICARDA verkregen veelbelovende genotypen, veelal veredelingslijnen, van de voor de regio belangrijke voedselleguminosen erwt, fababoon, kekererwt en linze. De verschillen bleken vooral te berusten op verschillen in virulentie omdat er geen duidelijke verschillen in virusconcentratie (dus in agressiviteit) konden worden aangetoond, Hoewel de isolaten identiek reageerden met antisera tegen een Marokkaans en een Tunesisch isolaat, konden konden ze op grond van hun interactie met genotypen van voedselleguminosen worden

onderscheiden en gegroepeerd als milde, intermediaire en sterk virulente isolaten. Alle getoetste ICARDA-lijnen van de voedselleguminosen bleken kwetsbaar te zijn voor alle BBMV-isolaten uit Marokko en er werd geen immuniteit gevonden. Een aantal virusisolaten was meer pathogeen, vaak necrotisch, op de voedselleguminosen erwt en kekererwt dan op fababoon. Het elders beschreven Soedanese isolaat is van extreem geringe pathogeniteit in veel plantesoorten; in vier kekererwtgenotypen was de infectie zelfs geheel symptoomloos. In tegenstelling tot wat bekend was uit de literatuur bleek het virus bij fababoon ook met zaad over te kunnen gaan (1,2%) wanneer het alleen voorkomt. Het werd voor het eerst aangetroffen in zaad van erwt (0,1%) en kekererwt (0,9%) (Hoofdstuk 3).

In overdrachtsproeven kon het BBMV, behalve met de reeds langer als vector van het virus bekende bladrandkever *Sitona lineatus*, nu ook worden overgebracht met andere Curculionidae, en wel de snuitkevers *Apion radiolus*, *Hypera variabilis*, *Pachtychius strumarius* en *Smicronyx cyaneus*. De bladrandkever *S. lineatus* bleek bij fababoon een efficiente vector, die het virus al bij de eerste beet kan opnemen en afgeven. Virusretentie hield tenminste zeven dagen aan en 41% van de getoetste exemplaren bleek in staat tot overdracht. Met deze soort kon het virus ook worden overgebracht naar linze en erwt.

Op zoek naar mogelijke besmettingsbronnen van waaruit kevers het virus naar fababoon zouden kunnen overbrengen werden in 1992 in 24 velden met erwt, kekererwt, linze en stamboon (*Phaseolus vulgaris*) 351 monsters voedselleguminosen en 102 monsters wilde leguminosen met mogelijk aan virus toe te schrijven symptomen verzameld en vervolgens serologisch getoetst op infectie met BBMV. Het virus werd inderdaad aangetroffen in alle vier getoetste, natuurlijk geïnfecteerde voedselleguminosen (in respectievelijk 19, 16, 11 en 16% van de monsters van de betrokken geteelde soorten). Ook dit is een nieuw gegeven voor het virus. De waargenomen (veelal necrotische) verschijnselen kunnen gemakkelijk voor afwijkingen van andere oorzaak worden aangezien en ze komen waarschijnlijk in de betrokken gewassen veel meer voor dan tot dusver bekend is. Het virus werd niet aangetoond in de wilde soorten waarvan een aantal volgens de literatuur eerder wel vatbaar was gebleken. Hun rol bij de

natuurlijke instandhouding van het virus kan daarom niet worden uitgesloten (Hoofdstuk 4).

De reeds bij BBMV bestudeerde variabiliteit binnen dat virus speelt vooral ook een rol binnen de groep van luteovirussen en de 'cluster' van min of meer aan bonescherpmozaiekvirus (BYMV) verwante virussen. Dit bemoeilijkte reeds bij de inventarisatie de ondubbelzinnige identificatie (= herkenning van isolaten als reeds beschreven virussen en beschrijving van onbekend lijkende isolaten) en manifesteerde zich spoedig als een belangrijk probleem.

De in fababoon gevonden, niet met sap over te brengen vergelingsvirussen vertoonden bij toetsing in DAS-ELISA verschillende reactiepatronen met polyklonale antisera tegen erwtetopvergelingsvirus ('bean leafroll virus', BLRV), slavergelingsvirus ('beet western yellows virus', BWYV) en 'subterranean clover red leaf virus' (SCRLV), en in TAS-ELISA met twee monoklonale antilichamen waarvan verondersteld wordt dat ze discrimineren tussen BLRV en BWYV. De zo gevonden verschillende vergelingsvirussen konden echter niet worden herkend als één van de uit de literatuur van leguminosen bekende luteovirussen. Het gaat dus om geheel afwijkende stammen van bekende virussen of om geheel nieuwe luteovirussen van fababoon. Om toch een betrouwbare en specifieke detectie van deze 'virussen' mogelijk te maken werd gezocht naar toepassingsmogelijkheden van de polymerasekettingreactie (PCR). Een stel ontworpen oligonucleotide-'primers' bleek een 535-bp-fragment van het eiwitmantelgen van een aantal bekende luteovirussen zowel als van alle getoetste Marokkaanse luteovirussen specifiek te vermeerderen ('amplificeren'). In moleculaire hybridisatieproeven vertoonden geselecteerde veldisolaten, hoewel ze serologisch verschillend waren, onderling en ten opzichte van BLRV nucleotiode-sequentiehomologie, maar niet ten opzichte van BWYV. Deze resulaten versterken de opvatting dat in Marokko één of meer speciale, aan fababoon aangepaste 'nieuwe' luteovirussen voorkomen (Hoofdstuk 5).

De variabiliteit speelt vooral parten bij de identificatie van de min of meer aan BYMV verwante virussen. Voor een betrouwbare herkenning van het potentieel voor Marokko belangrijke BYMV werd speciale aandacht besteed aan de relatie tussen BYMV en het

klavergeelnerfvirus ('clover yellow vein virus', CYVV) dat uit de literatuur bekend is om zijn vaak necrotische reactie op erwt en fababoon. Daartoe werd een aantal BYMV-achtige, reeds eerder gerapporteerde virusisolaten uit de ICARDA-regio (Syrië en Soedan) en uit Nederland vergeleken met een aantal standaardisolaten van het virus (inclusief een erwtemozaïekstam en een erwtenecrosestam) en met een aantal isolaten van het CYVV (inclusief de oorspronkelijke type-stam). CYVV bleek onmiskenbaar van BYMV te verschillen door zijn veel bredere waardplantenreeks (vooral onder de niet-leguminosen), door de lichtmicroscopisch waarneembare, vaak zeer opvallende vergroting van de nucleolus, en door de duidelijk langzamere migratie van het manteleiwitmolecuul in SDS-PAGE. Om de moeilijkheden bij conventionele serologische identificatie te ontlopen werd vooral aandacht besteed aan de toepassing van de sinds kort zeer specifiek geachte antilichamen gericht tegen N-terminale epitopen van het manteleiwit. Met zo'n antiserum tegen BYMV-B25 kon CYVV echter niet van het BYMV worden onderscheiden. Dit houdt in dat de twee verschillende virussen in het N-terminale deel van hun manteleiwit identieke aminozuursequenties bezitten. De geconstateerde continue biologische variatie binnen het virus maakt het moeilijk om hier van scherp gedefinieerde stammen te spreken (Hoofdstuk 6).

De aanhoudende meningsverschillen over de taxonomische onderscheiding van potyvirussen doen de behoefte toenemen aan ondubbelzinnige, snelle en gemakkelijk toepasbare identificatiemethoden. Recente moleculaire studies, vooral met betrekking tot de nucleotidenvolgorde van een groeiend aantal potyvirussen van leguminosen, lijken een meer rationele basis te verschaffen voor de identificatie en classificatie van potyvirussen. Voorlopig onderzoek over het gebruik van zulke gegevens in PCR toonde aan dat het mogelijk is om met een stel 'primers' CYVV en BYMV van elkaar te onderscheiden, maar de specificiteit van deze 'primers' dient met meer isolaten van beide virussen verder te worden getoetst (Hoofdstuk 7).

Voor een doelgerichte bestrijding van virusziekten is het nodig te zijn ingelicht over de identiteit, incidentie en economische betekenis van de onderhavige virussen om te weten waarover we het hebben en hoe belangrijk ze zijn. Ook moet de ecologie van de

belangrijke virussen bekend zijn om daarop de (preventieve) bestrijding te kunnen baseren. Voor de veredeling is het nodig te weten welke virusstammen van belang zijn. Verder houdt introduktie van nieuwe gewasgenotypen door genetische gewasverbetering het risico in van de introduktie van kwetsbaarder rassen, terwijl met het voor veredeling gebruikte genenmateriaal ('germplasm') nieuwe virussen het land kunnen binnenkomen. Zes van de negen of meer tot dusver in fababoon in Marokko voorkomende virussen blijken over te kunnen gaan met zaad. Zaadovergang verklaart het waarschijnlijk reeds lang wijd verbreide voorkomen van de onderhavige virussen in de gehele regio. Het in het kader van de landbouwkundige modernisering te stimuleren gebruik van gecertificeerd commercieel zaaizaad vraagt nauwlettende aandacht voor de gezondheidstoestand van het zaad. Het optreden van virussen in gewassen blijkt ook in fababoon in Marokko wisselend te zijn. Het is daarom gewenst 'een vinger aan de pols te houden' en daarbij niet alleen maar te letten op één afzonderlijk gewas. Bij de bestudeerde virussen gaat het om meer dan alleen maar fababonevirussen (Hoofdstuk 8).

RESUME

Les virus de la fève (*Vicia faba* L.) au Maroc; inventaire, identification, et aspects écologiques

Au Maroc, les cultures légumineuses occupent la seconde place après les céréales. La fève (*Vicia faba* L.) est de loin la principale légumineuse alimentaire, et couvre annuellement une moyenne de 200 000 ha. Son rôle dans les petites éxploitations en tant que composante dans les rotations culturales et en tant que source de proteine n'est plus à démontrer. A l'echelle mondiale, 44 virus ont été rapportés capables d'infécter naturellement la fève, alors que six seulement ont été détectés au Maroc. La présente étude a été entamée dans le cadre du projet de coopération en virologie entre l'Institut de Recherche en Protéction des Plantes (IPO-DLO), Wageningen, Pays-Bas, et le Centre International de Recherches Agricoles dans les Zones Arides (ICARDA), Alep, Syrie, dans le cadre de l'amélioration de la fève dans la région. La recherche s'est développée en un programme 'sandwich' entre le Département de Phytopathologie de l'Ecole Nationale d'Agriculture, Meknès, Maroc, l'IPO, et le Département de Virologie de l'Université Agronomique de Wageningen, Pays-Bas (Chapitre 1).

Des prospections systématiques des virus de la fève ont été effectuées en 1988 et 1990 dans les principales zones de cette culture au Maroc. Un total de 240 plantes suspectes d'être virosées a été prélevé de 52 parcelles. Par le biais de la microscopie éléctronique, l'indexage biologique, et la sérologie, les virus suivants ont été détectés et leur incidence et distribution géographique ont été évaluées: virus de la mosaïque de la luzerne (alfalfa mosaic virus, AMV), virus de la mosaïque jaune du haricot (bean yellow mosaic virus, BYMV), virus de la marbrure de la fève (broad bean mottle virus, BBMV), virus des tâches nécrotiques de la fève (broad bean stain virus, BBSV), virus de la mosaïque vraie de la fève (broad bean true mosaic virus, BBTMV), virus du brunissement précoce du pois (pea early-browning virus, PEBV), virus de la mosaïque enation du pois (pea enation mosaic virus, PEMV), le 'pea seed-borne mosaic virus' (PSbMV), et des

lutéovirus. BBTMV, PEMV, PSbMV, et les lutéovirus n'ont pas été rapportés auparavant au Maroc. BBMV, considéré jadis à distribution limitée, et les lutéovirus se sont révélés économiquement importants (rencontrés dans 50 et 56% des champs prospéctés, respectivement; et à des incidences de 20 et 33%, respectivement), alors que la situation inverse s'applique à BBSV et BYMV (Chapitre 2). Des études approfondies se sont ensuite concentrées sur les virus actuellement importants, en l'occurence BBMV, les lutéovirus, et les isolats ressemblant BYMV et potentiellement importants.

L'indexage biologique des échantillons colléctés a révélé une variation considérable de la sévérité des symptômes occasionnés par les isolats de BBMV. La comparaison de sept isolats Marocains avec des isolats provenant d'Algérie, Tunisie, et Soudan a montré une variabilité pathogénique du BBMV sur un nombre de génotypes de légumineuses alimentaires. Des groupes d'isolats ont été distingués sur la base de la virulence malgré leur réaction similaire à des antisera produits contre deux isolats Marocain et Syrien. Un nombre de lignées prometteuses de fève, petit pois, pois chiche, et lentille provenant de l'ICARDA ont été testées avec des isolats de BBMV. Diverses intéractions ont été observées, mais tous les genotypes se sont révélés vulnérables à tous les isolats utilisés, et aucune immunité n'a été détectée. Certains isolats étaient plus virulents sur d'autres légumineuses alimentaires que sur la fève. BBMV a été trouvé transmis par semence de fève, petit pois, et pois chiche à des taux de transmission respectifs de 1.2, 0.1, et 0.9% (Chapitre 3).

A côté de la transmission par semence, BBMV s'est révélé transmissible non seulement par *Sitona lineatus*, rapporté antérieurement vecteur de ce virus, mais aussi par les coléoptères (Curculionidae) *Apion radiolus*, *Hypera variabilis*, *Pachytychius strumarius*, *Smicronyx cyaneus*. Le premier apparait un vecteur éfficace vue que la première morsure de l'insecte était suffisante pour acquérir et inoculer le virus, la rétention du virus par l'insecte était d'au moins sept jours, et le taux de transmission a été éstimé à 41%. Ce même vecteur était capable de transmettre le BBMV non pas seulement de la fève à la même éspèce, mais aussi à la lentille et au petit pois. Lors de la recherche des sources naturelles d'inoculum, et à partir des 351 échantillons de

légumineuses colléctés, le virus a été detécté dans 11, 19, 16, et 16% des échantillons de lentille, petit pois, pois chiche, et haricot, respectivement. Les symptômes occasionés par le BBMV sur ces éspèces étaient variables, mais souvent nécrotiques et pouvaient être attribués à d'autres agents pathogènes, voire à des causes abiotiques. Sur les 102 échantillons de légumineuses sauvages, rapportées être des reservoirs potentiels dans la nature, aucun d'eux n'a été trouvé hébergeant le BBMV (Chapitre 4).

Le problème de variabilité de virus n'avait pas tardé à apparaître et à se développer lors de cette étude. Il a été déjà rencontré dans le cas de BBMV, mais il concernait particulièrement la gamme de potyvirus reliés au BYMV ainsi que les lutéovirus, et conduisait alors à des difficultés d'identification de virus.

Des échantillons de fève présentant des symptômes suspects d'infection par lutéovirus et testés en DAS-ELISA avec des antisera polyclonaux contre le virus de l'enroulement de la fève (BLRV), le 'beet western yellows virus' (BWYV), et le 'subterranean clover red leaf virus' (SCRLV), et en TAS-ELISA avec deux anticorps monoclonaux trouvés capables de différencier entre BLRV et BWYV, avaient révélé diverses réactions sérologiques indiquant une variabilité au sein des isolats Marocains. Ceux ci ne pouvaient être identifiés aux lutéovirus connus infectant les légumineuses. Pour permettre une détection de ces lutéovirus par la méthode enzymatique en chaine (PCR), une paire d'oligonucléotides 'primers' a été concue, et a permis l'amplification spécifique d'un fragment de 535 bp du gène codant pour l'enveloppe protéique aussi bien chez les lutéovirus standards utilisés que chez les isolats Marocains. Dans les tests d'hybridation moléculaire, des isolats Marocains ont montré une homologie en séquence nucléotidique entre eux et avec BLRV, mais pas avec le BWYV malgré que certains isolats s'étaient comportés comme BWYV dans les tests sérologiques. Ces résultats renforcent davantage l'hpothèse d'éxistence de nouveaux lutéovirus adaptés à la fève (Chapitre 5).

D'autre part, le 'clover yellow vein virus' (CYVV) pouvait être distingué de son apparenté BYMV par une gamme d'hôtes comprenant des éspèces non-légumineuses, par un élargissement éxagéré des nucléoles des cellules infectées, et par une migration lente de son enveloppe protéique dans un gel de polyacrylamide. Les deux virus ne pouvaient pas être différenciés par la sérologie utilisant des anticorps produits contre la partie N-

terminale de l'enveloppe protéique, et rapportée outil de distinction entre les différents potyvirus, indiquant alors que ces deux potyvirus apparentés possèdent des épitopes communs dans la partie N-terminale de leurs enveloppes proteiques. Sur cette base, un nombre de potyvirus isolés des légumineuses alimentaires de la région d'action de l'ICARDA (Syrie et Soudan) et des Pays-Bas ont été identifiés comme étant BYMV ou CYVV, et ceux isolés de la fève au Maroc ont été confirmés être des isolats de BYMV (Chapitre 6). Des rapports contradictoires quant à l'état taxonomique des potyvirus infectant les légumineuses soulèvent le besoin de développer des moyens d'identification plus sûrs. Les récentes études moléculaires, y compris la détérmination des séquences nucléotidiques des ARN de potyvirus infectant les légumineuses, apparaissent offrir une base rationnelle pour l'identification et la classification des potyvirus. L'utilisation de telles données moléculaires en PCR pour distinguer entre BYMV et CYVV a été éxplorée, et les résultats préliminaires ont montré qu'une paire d'oligonucléotides 'primers' pourrait être utilisée pour différencier entre ces deux virus inféctant la fève (Chapitre 7).

En vue de lutter contre les maladies à virus, il est nécessaire de connaître l'identité des virus présents, et d'évaluer leur incidence et importance économique. L'écologie des virus les plus importants doit être cernée et servira de base pour une lutte préventive. La variabilité du virus et l'identification des différentes souches est d'une importance capitale pour les programmes d'amélioration génétique. Dans de tels programmes, l'introduction du germoplasme étranger s'accompagne du risque d'introduction non seulement de virus transmis par semence, mais aussi de la susceptibilité qui se manifestera par des épidémies de virus endémiques. Il est fort possible que la large distribution des virus transmis par semence dans toute la région soit dûe â un échange de semence et germoplasme contaminé. La modernization agricole va sans doute s'accompagner de production de semences améliorées et d'une certification adéquate. Les virus transmis par semence mériteront une attention particulière, et la certification des semences éxigera des méthodes adéquates de détection, qui à leur tour éxigeront une connaissance des virus concernés et de leur souches. L'apparition des maladies à virus

est un phénomène dynamique, par conséquent une surveillance continue (par le biais de prospections) s'impose. Lors des prospections, d'autres légumineuses alimentaires doivent être prises en considération car les virus étudiés ne sont pas inféodés à la fève seule (Chapitre 8).

CURRICULUM VITAE

Mohammed Fortass was born on 24 May 1959 in Karia Ba Mohamed, Taounate, Morocco. He graduated in Agronomy at the 'Institut Agronomique et Vétérinaire Hassan II', Rabat, Morocco in 1983. From July 1983 to January 1985, he completeded his MSc courses in plant pathology at the University of Minnesota, St-Paul, and at the University of Wisconsin, Madison, USA, and obtained his MSc degree at the 'Institut Agronomique et Vétérinaire Hassan II' in 1986.

Since February 1987, he is a permanent staff member as a plant virologist at the Department of Plant Pathology of the 'Ecole Nationale d'Agriculture' (ENA), Meknès, Morocco. From April 1988 until September 1993, he worked for his PhD within a sandwich program involving IPO-DLO (Wageningen), the Department of Virology of the Wageningen Agricultural University, and ENA (Meknès). The results of his research are presented in this thesis.