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Incidence and transmission of *Faba bean necrotic yellows virus* (FBNYV) in Spain

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Abstract

Information on the distribution of *Faba bean necrotic yellows virus* (FBNYV) in Spain was gathered by sampling different legume crops (faba bean 'Muchamiel', forage faba bean, pea and chickpea) in four regions (Murcia, Andalucía, Castilla y León, and Extremadura). The virus was detected by TAS-ELISA in faba bean 'Muchamiel' and peas only in the Murcia region. A portion of 272 bp within the C5 component of a FBNYV from Murcia was amplified by IC-PCR and directly sequenced. The Spanish FBNYV was 93.75% identical to two previously sequenced isolates of FBNYV from Syria and Egypt. Periodic samplings of faba bean 'Muchamiel' over one growing season (Oct. 1999 to Jan. 2000) in Murcia provided no clear evidence of any spread of the disease either within or beyond the affected fields. Mixed infections of FBNYV and *Tomato spotted wilt virus* (TSWV), *Bean leaf roll virus* (BLRV) and *Bean yellow mosaic virus* (BYMV) were commonly observed. The necrotic symptoms developed on the leaf borders were more pronounced in these mixed infections. Transmission experiments using a Spanish isolate as inoculum source and the aphid species *Acyrthosiphon pisum* and *Aphis craccivora* showed that both are efficient FBNYV vectors. In these experiments, the faba bean cvs. 'Aguadulce', 'Muchamiel' and 'Valenciana' were all susceptible to FBNYV.

Additional key words: IC-PCR, legume virus, mixed infections, sequencing, TAS-ELISA, viruliferous aphids.

Resumen

Incidencia y transmisión del virus del amarilleamiento necrótico del haba (FBNYV) en España

Para obtener información de la distribución del virus del amarilleamiento necrótico del haba (*Faba bean necrotic yellows virus*, FBNYV) en España, se realizaron muestreos extensivos en cuatro comunidades autónomas españolas (Andalucía, Castilla y León, Extremadura y Murcia), en diferentes cultivos de leguminosas (haba 'Muchamiel', habas forrajeras, guisantes y garbanzos). Los análisis realizados por ELISA-TAS demostraron que FBNYV estaba presente solo en habas y guisantes de Murcia. Se amplificó por IC-PCR un fragmento de 272 pb del componente C5 del FBNYV aislado de un haba de Murcia, cuya secuencia fue 93,75% idéntica a dos aislados previamente secuenciados de Siria y Egipto. Muestreos periódicos en campos de haba 'Muchamiel' de Murcia durante una campaña (octubre 1999 a enero 2000) no evidenciaron claramente la dispersión de la enfermedad. Se encontraron frecuentes infecciones mixtas de FBNYV con el virus del bronceado del tomate (TSWV), el virus del enrollado de la judía (BLRV) y el virus del mosaico amarillo de la judía (BYMV) y los síntomas necróticos desarrollados en los bordes de las hojas fueron más pronunciados en las infecciones mixtas. Experimentos de transmisión utilizando un aislado español del FBNYV como inóculo, mediante los pulgones *Acyrthosiphon pisum* y *Aphis craccivora,* pusieron de manifiesto la eficiencia de éstos como vectores del FBNYV, y que los cultivares de haba 'Aguadulce', 'Muchamiel' y 'Valenciana' fueron susceptibles a la infección por FBNYV.

Palabras clave adicionales: ELISA-TAS, IC-PCR, infecciones mixtas, pulgones virulíferos, secuenciación, virus de leguminosas.

Introduction

Faba bean necrotic yellows virus (FBNYV; genus *Nanovirus*) causes severe yield losses and crop failure in food and fodder legumes in Western Asia and North Africa (Makkouk *et al.,* 1992, 1994; Franz *et al.*, 1995; Makkouk and Kumari, 2000). The virus has a wide host range—58 host legume species have been identified (Katul *et al.,* 1993; Franz *et al.,* 1997). FBNYV is persistently transmitted by aphids, most efficiently (in laboratory tests) by *Acyrthosiphon pisum* (Harris) and *Aphis craccivora* (Koch). *Acyrthosiphon pisum* is

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considered the most efficient vector of Syrian FBNYV isolates and *A. craccivora* the most important vector under field conditions (Franz *et al.*, 1995, 1998). The virus has a multipartite genome consisting of several circular ssDNA components that are similar in size (about 1 kb) and individually encapsidated in small isometric particles about 18 nm in diameter (Katul *et al.,* 1993). The main host is the faba bean (*Vicia faba* L.). Early-infected plants remain stunted, showing leaf yellowing followed by necrosis and plant death.

The presence of FBNYV was reported for the first time in Spain a few years ago (Babín *et al.,* 2000). The present paper examines the distribution of FBNYV in the main legume-producing areas of Spain and its transmission efficiency by two aphid vectors.

Material and Methods

To determine the presence or absence of FBNYV in the main legume-producing areas of Spain, fields of faba bean cv. Muchamiel (*Vicia faba* L.), forage faba bean (*Vicia faba* L.), pea (*Pisum sativum* L.) and chickpea (*Cicer arietinum* L.) were surveyed in four regions (Andalucía, Castilla y León, Extremadura and Murcia) during the period 1998-2001 (Fig. 1). Samples were collected from plants showing virus-like symptoms, such as leaf rolling, yellowing, necrosis, and stunted growth. The number of samples collected in each field depended on the number of symptomatic plants observed.

Figure 1. Castilla y León (1), Extremadura (2), Andalucía (3) and Murcia (4) regions surveyed to determine the presence or absence of FBNYV in legume fields.

The region was considered FBNYV-positive if this virus was detected in at least one of the visited fields.

FBNYV detection was performed with triple antibody sandwich ELISA (TAS-ELISA) (Franz *et al.,* 1996) using polyclonal antibody 577 (diluted 1:400) and a mix of monoclonal antibodies (diluted 1:500). All antibodies were kindly provided by Dr. J. Vetten, BBA, Braunschweig, Germany. Samples with absorbance values higher than the mean value for non-infected control plants plus three standard deviations were considered positive.

The immunocapture-polymerase chain reaction (IC-PCR) was used to confirm FBNYV infection in some samples. Both IC and PCR were carried out in the same PCR tube as explained in Katul *et al.* (1995). For IC, the tubes were coated with polyclonal antibody 577. FBNYV-specific primers P19 (5'-TTATTGTTAAATGTAATTCACCTAT-3') and P6 (5'-CACTTCAACATAAACTCTG-3'), located in the C5 component (J. Vetten, personal communication), were used in PCR reactions. The PCR amplification products were analysed by electrophoresis on agarose gels.

For sequencing, the PCR products were extracted from the agarose gel and purified with the Bioclean DNA purification kit (Biotools S.A., Madrid, Spain) according to the manufacturer's instructions. The amplified fragment (272 bp) was directly sequenced in both directions using an automated DNA sequencer (Applied Biosystem, Foster City, CA, USA). The primers used for PCR amplifications were used for the sequencing reaction. A sequence similarity search was performed against the EMBL and GenBank nucleotide databases using Fasta software (Pearson and Lipman, 1988) at URL: http://www.ebi.ac.uk/fasta33/index.html.

To study the progress of the disease caused by FBNYV over a single faba bean growing season in the Murcia region alone (from October to January), four plots in different locations were selected: Cartagena, Los Martínez, Torrepacheco and Roldán. The selected plots were about 0.5 ha in size in faba bean fields > 5 ha. Fifty samples were collected at monthly intervals during the October 1999-January 2000 growing season from plants (in the central area of the plot) showing symptoms of virosis. Five asymptomatic plants were also collected in each plot as negative controls. The samples were preserved in separate bags at –20ºC until use.

To detect mixed infections, FBNYV-positive samples were analysed for other viruses that commonly infect faba bean crops in Spain (Fresno *et al.,* 1997): *Bean leaf*

roll virus (BLRV), *Beet western yellows virus* (BWYV), *Bean yellow mosaic virus* (BYMV), *Potyviruses* and *Tomato spotted wilt virus* (TSWV). BLRV was identified using an IC-RT-PCR method (Ortiz *et al.,* 2005) with a monoclonal antibody (diluted 1:400) (kindly provided by Dr. Vetten). BWYV, *Potyvirus* and TSWV were identified by ELISA using the commercial antibodies anti-BWYV 07009 (Loewe, dilution 1:200), anti-poty (Agdia, dilution 1:200), and anti-TSWV BR01 (Loewe, dilution 1:200) respectively. Samples that were *Potyvirus* positive were analysed by IC-RT-PCR (Ortiz, 2003), using a pair of BYMV-specific primers for the coat protein gene P1984 (5'-CAAGGTGAGTGGACAATGATGG-3') and P1985 (5'-GAGAGAATGATACACATACTGAA-3'). PCR products were purified on Bioclean columns (Biotools S.A) and cloned into the plasmid vector pGem-T Easy (Promega, Madison, WI, USA). Ligation, transformation and selection of recombinant clones were carried out according to the manufacterer's recommended protocol (T-vector cloning brochure, Promega). The DNA sequence of both strands of the cloned PCR products was then determined using an automated DNA sequencer (Applied Biosystem, Foster City, CA, USA). Sequence analysis was performed using Fasta software (Pearson and Lipman, 1988).

The transmission efficiency of FBNYV by aphids was tested with *Acyrthosiphon pisum* (green biotype) collected from lentil (*Lens culinaris* Medik.) fields in Salamanca (Spain), *Acy. pisum* (pink biotype), and *Aphis craccivora* obtained from Dr. A. Franz (IPO Wageningen, The Netherlands). One virus-free aphid clone of each species was obtained starting from a single adult on a faba bean seedling under greenhouse conditions (20.5 ± 5°C, photoperiod 12 ± 4 h). Adult aphids were transferred to young seedlings every week to maintain the clone. The transmission experiment was performed in a growth chamber (FISONS Mannerl 60063/THTL) at a temperature of 22 ± 3ºC and with a 16 h photoperiod. The insects used for transmission were the offspring of 10 adults that had been kept on virus-free plants for five days. FBNYV-infected faba bean 'Muchamiel' plants collected from Murcia and found positive by IC-PCR were used as a source of virus.

Aphis craccivora, the most important FBNYV vector under field conditions (Franz *et al.,* 1998) and *Acy. pisum*, the most efficient vector of Syrian isolates (Franz *et al.,* 1995), were evaluated for their ability to transmit FBNYV to three Spanish faba bean cultivars: 'Aguadulce', 'Muchamiel' and 'Valenciana'. The acquisition (AAP) and inoculation access feeding periods (IAP) were 72 h and 108 h respectively. During the AAP the plants were covered with glass cylinders to avoid the escape of the insects and contamination by other aphid species. The insects were then transferred onto healthy seedlings of faba bean 'Muchamiel', 'Valenciana' and 'Aguadulce'. Five aphids were placed on each plant for an IAP of 108 h (see above). The plants were assayed for viral infection 45 days later by TAS-ELISA and IC-PCR. Healthy faba bean seedlings and virus-free aphids were used as negative controls.

Results

Over the original four-year (1998-2001) survey of faba bean cv. Muchamiel, forage faba bean, pea and chickpea fields, 2348 symptomatic plants were analysed by TAS-ELISA and IC-PCR. FBNYV was detected in 85 samples from the Murcia region, but only from faba bean cv. Muchamiel fields and a single pea field.

To confirm FBNYV infection, the PCR-amplified fragment (272 bp) of a faba bean 'Muchamiel' from Murcia was sequenced. The nucleotide sequence was deposited in the GenBank Nucleotide Sequence Database under the accession number DQ830990. FASTA analysis showed that the sequence of 272 bp within the C5 component was 93.75% identical to two previosly sequenced isolates of FBNYV of Syrian and Egiptian origin (acc. nos. Y11408 and AJ132183, respectively) and only 79.78% and 64.57% identical to other Nanoviruses as *Milk vetch dwarf virus* (acc. AB044387*)* and *Subterranean clover stunt virus* (acc. L47332), respectively.

Table 2, which refers to the one year survey of the progress of the disease in the Murcia region alone, shows that FBNYV was present in three of the four plots. The virus was not recovered from any of the 200 plants sampled during the four samplings of the plot in Cartagena, nor from any asymptomatic plant collected as a negative control. Although FBNYV was present in the three remaining plots, the number of infected plants remained similar on each of the four sampling dates shown, and no spread of FBNYV to the plot at Cartagena was observed. This indicates that there was no spread of FBNYV within or between the plots during the 1999-2000 growing season.

With the exception of the samples collected from the Los Martínez plot in October, where an infection rate

Table 1. Faba bean plants infected with FBNYV in the Murcia region

Sampling month	Plot 1 Cartagena	Plot 2 Los Martínez	Plot 3 Torrepacheco	Plot 4 Roldán
October	0/50[1]	19/50 (38%)	9/50 (18%)	4/50 (8%)
November	0/50	7/50 (14%)	5/50 (10%)	2/50 (4%)
December	0/50	8/50 (16%)	3/50 (6%)	3/50 (6%)
January	0/50	7/50 (14%)	3/50 (6%)	9/50 (18%)

[1] Plants were analysed by TAS-ELISA. Data show the number of FBNYV-positive plants/number of plants sampled.

of 38% was observed (Table 1), all samplings yielded infection rates of < 18%. This small number of FBNYV-positive plants coincided with the small number of plants showing symptoms of yellowing in the field.

In addition to FBNYV, a second virus was detected in some FBNYV-positive plants (*Potyvirus* and TSWV), and occasionally a mixture of two other viruses (TSWV/BLRV and TSWV/*Potyvirus)* (Table 2). To identify the potyvirus in the samples that reacted with the broad-spectrum anti-*Potyvirus* monoclonal antibody, IC-RT-PCR was performed using a pair of BYMV-specific primers. In the electrophoretic analysis of all samples analysed, bands of the expected size corresponding to 525 nt were observed. The presence of BYMV was confirmed by both sequence and Fasta analysis of the amplified cDNA (acc. no. AM286741). The sequence of 525 bp within the capsid protein gene was 96.95% identical to BYMV (acc. no. AB097089). The necrotic symptoms on the leaf borders were more pronounced in mixed infections.

TAS-ELISA and IC-PCR showed that both *A. craccivora* and *Acy. pisum* efficiently transmit the Spanish FBNYV isolate tested (Table 3). The three faba bean cultivars 'Aguadulce', 'Muchamiel' and 'Valenciana' were all susceptible. The FBNYV symptoms observed on the faba bean plants under the present experimental conditions were similar to those observed in the field, but less severe, perhaps due to the presence of mixed infections in the field.

Discussion

FBNYV was first reported from Syria in 1988 and subsequently was recorded in many Arab countries of West Asia and North Africa as Syria, Jordan, Ethiopia, Egypt, Algeria and Morocco (Katul *et al*., 1993; Franz *et al.*, 1996). In the 1991/1992 growing season, the virus occurred on an epidemic scale on faba beans in Egypt to complete crop failure (Makkouk *et al.,* 1994). In Spain FBNYV was first detected in 1997 in a faba bean sample collected at Balearic Islands and in 34 samples collected during 1997-1999 in Murcia (Babín *et al.,* 2000). The present work reports that recent surveys in the main Spanish legume-producing areas showed FBNYV to be present only in Murcia.

The study of progress of disease caused by FBNYV in four faba bean fields in Murcia by analysing samples at monthly intervals showed that the infection rate was generally < 18% in faba bean during the 1999-2000 growing season. This can be explained by the weekly application of insecticides to control thrips, the vector for TSWV – a virus widely present in Murcia (Jordá *et al.*, 2000). Such treatments also reduce the population of aphids. This affects the spread of persistently transmitted viruses, such as FBNYV, more strongly than that of non-persistently (NP) transmitted viruses, for example *Potyviruses*. In the case of the latter, the aphid loses inoculativity after the first inoculation has taken place, therefore control is not

Table 2. Mixed virus infections in faba bean plants from three locations in the Murcia region

Sampling month	Plot 2 Los Martínez	Plot 3 Torrepacheco	Plot 4 Roldán
October	FBNYV / BYMV 6/19[1]		
	FBNYV / BLRV / TSWV 1/19	FBNYV / TSWV 2/9	0/4
November	FBNYV / BYMV 2/7	FBNYV / TSWV 2/5	FBNYV / TSWV / BYMV 1/2
December	FBNYV / BYMV 3/8		
	FBNYV / BLRV 1/8	FBNYV / BYMV 2/3	FBNYV / TSWV / BYMV 2/3
January	FBNYV / TSWV / BYMV 1/7		
	FBNYV / TSWV 1/7	FBNYV / TSWV 1/3	FBNYV / TSWV 2/9

[1] Total number of mixed infections/number of plants infected with FBNYV. The areas were sampled during the 1999-2000 faba bean cv. Muchamiel growing season.

Table 3. Efficiency of aphid species in transmitting FBNYV to different faba bean cultivars

Faba bean cultivars	*Acyrthosiphon pisum* experiments		*Aphis craccivora* experiments	
	1	2	1	2
Aguadulce	15/20[a]	13/20	11/20	12/20
Muchamiel	12/20	10/20	9/20	10/20
Valenciana	13/20	11/20	11/20	10/20

The acquisition and inoculation access feeding periods were 72 and 108 h respectively. [a] Number of infected plants/number of plants inoculated.

as effective as that exerted over viruses spread in a circulative persistent (CP) manner in which the aphid is infective throughout its lifetime and can continue infecting other plants. In addition, CP viruses have an acquisition and latency period ranging from hours to several days before they can infect a new host. In contrast, NP viruses require only seconds to minutes (Hull, 2002). These requirements can affect dispersion both within fields and to neighbouring fields. Fear for the spread of FBNYV to other Spanish regions still exists, because weekly application of insecticides is not a normal practice in legumes crops in Spain.

Vector transmission of viruses is a complex process involving the virus, the vector, the plant, and environmental conditions (Bourdin *et al.,* 1998). In line with that reported by Franz *et al.* (1997, 1998), the present results show that *Acy. pisum* and *A. craccivora* efficiently transmit FBNYV (with *Acy. pisum* a slightly more efficient vector in all three reports). These results differ from those obtained by Katul *et al.* (1993) who reported *A. craccivora* to be an inefficient vector of Syrian isolates of FBNYV. The difference in these reports may be due to the isolate used: Katul *et al.* (1993) transmitted a Syrian isolate, Franz *et al.* (1997) an Egyptian isolate, and in the present study a Spanish isolate was used. This seems to indicate that transmission efficiency depends upon the virus isolate, as proposed by Franz *et al.* (1997).

As conclusion, though FBNYV is present in legumes of East and South Mediterranean countries, until now has been only found in Spain on the European continent. In this country, it has been detected only in the Eastern Mediterranean regions of Balearic Islands and Murcia. The present work shows that at present, FBNYV is endemic in Murcia and not spread to other Spanish legume-producing areas.

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