PRESENCE OF BCMV AND BCMNV IN FIVE DRY BEAN-PRODUCING STATES IN MEXICO

[PRESENCIA DEL BCMV Y BCMNV EN CINCO ESTADOS PRODUCTORES DE FRIJOL EN MEXICO]

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SUMMARY

A survey was conducted to assess the frequency of BCMV and BCMNV in five of the main dry bean producing states in Mexico during the spring-summer 2009 and fall-winter growing seasons 2009-2010. States included in the survey were Nayarit, Sinaloa and Sonora in the pacific west coast, Veracruz in the gulf coast and Guanajuato in central Mexico. A total of 338 samples were collected and analyzed by RT-PCR with specific primers for each viral species. Forty-four samples (13%) gave positive reaction for BCMV, 70 (21%) for BCMNV and 30 (9%) were positive for both viral species, 164 (48%) were negative for both viruses and 30 (9%) could not yet be defined. As for cultivars, Azufrado Higuera (Nueva Granada race) grown at Sinaloa showed the highest frequency (33%) of BCMV, whereas Negro Jamapa (Mesoamericana race) from Nayarit displayed highest frequency (50%) of BCMNV. In these two states the percentage of positive samples for either viral species was 80%. In addition, in cultivar Negro Jamapa mixed infections of both viruses were detected. Results point out a high risk of viral infection with seed movement across states, particularly since both viral species are seed transmitted and in the states at the pacific west coast, large seed lots are produced during the fall-winter season.

Key words: BCMV; BCMNV; bean plants viruses

RESUMEN

Se realizó un muestreo en campo para estimar la frecuencia de la ocurrencia de BCMV y BCMNV en cinco estados productores de frijol en México durante los periodos de cultivo primavera-verano y otoño-invierno en el 2009 y 2009/2010. Los estados incluidos en el estudio fueron Nayarit, Sinaloa y Sonora en la costa del pacífico, Veracruz en la costa del golfo y Guanajuato en el centro de México. Se colectó un total de 338 muestras que se analizaron por RT-PCR con iniciadores específicos para cada una de las dos especies virales. Cuarenta y cuatro muestras (13%) resultaron positivas para el BCMV, 70 (21%) para el BCMNV, 30 (9%) fueron positivas para ambos virus, 164 (48%) fueron negativas para ambos virus y otras 30 (9%) no han sido definidas aún. En referencia a cultivares, Azufrado Higuera (raza Nueva Granada), de Sinaloa, mostró la frecuencia más alta (33%) de BCMV, mientras que Negro Jamapa (raza Mesoamericana) de Nayarit, mostró la más alta (50%) de BCMNV. En estos dos estados el porcentaje de muestras positivas para cualquiera de las dos especies virales fue de 80%. Adicionalmente se detectaron infecciones mixtas en el cultivar Negro Jamapa. Los resultados indican el alto riesgo de infección con el movimiento interestatal de semillas ya que ambos virus son transmitidos por este medio y en los estados de la costa occidental de pacífico se producen grandes cantidades de semilla durante el ciclo de otoño-invierno.

Palabras clave: BCMV, BCMNV, virus de plantas de frijól

INTRODUCTION

In Mexico common bean grown during the spring-summer season is located at the semiarid and central highlands, 96% of 1.4 million Ha under rainfed conditions (SIAP, 2012). At the Bajio subregion and central highlands, BCMV can reduce seed yield and infect the seed that might be used in subsequent plantings. During the fall-winter season dry beans are grown in the humid and dry tropic regions. In the first region, mostly grown on residual moisture and in the second, under irrigation. In the first region that includes the southeast states of Veracruz and Chiapas, BCMNV is more prevalent as well as in Nayarit in the dry tropics. In Sinaloa, BCMV along with the 'calico viral disease' can damage the bean crop. Losses due to viral diseases can fluctuate between 20 to 100%. In Mexico BCMV has been reported to damage production ranging from 30 to 80% (Chew *et al.*, 2010), however reported losses due to BCMNV are scarce.

BCMV and BCMNV are two strongly related pathogens infecting bean plants. Up until 1992, they were considered as two serotypes, A and B, but now they are considered as two separate species from the same Genus (*Potyvirus*) and Family (*Potyviridae*) (Morales (1998). The separation into species initially based on the serological reactivity was further confirmed by different properties of the virus (Berger *et al.*, 1997) such as the molecular weight and peptidic profile of the capsid protein (McKern, 1992, Huguenot *et al.*, 1994), as well as the cytological effects (Vetten *et al.*, 1992), produced on the infected tissue and general responses on the infected plant (Kelly, 1997). Nucleotide sequence backup for this taxonomic demarcation has also been reported by Saiz *et al.* (1994). Both viral species are seed transmitted (Hall, 1991), making their control more difficult with the use of residual seed from previous harvests and due to the exchange of seed between users from different localities. Both practices have an implication on the presence and diversity of BCMV and BCMNV. Symptoms produced by both viral species are very similar and they include: mosaic, stunt, chlorosis and leaf deformation. At higher temperatures systemic necrosis may be observed, depending on one of the seven pathogroups affecting the crop. BCMV and BCMNV are categorized as pathogroups according to a classical study by Drijfhout (1978) and Kelly (1997). This division is based on the response of different bean cultivars to viral isolates due to the genetic composition of the set of cultivars used. One important response, systemic necrosis, is a hypersensitive reaction to the necrotic viral strains as studied by Collmer *et al.* (1996). It starts with either pinpoint lesions or veinal necrosis appearing as "cross road" or pinpoint spots, on certain genetic composition of the host plant; i.e, the presence of the dominant I gene with combination of the recessive bc genes.

In Mexico, initial reports only make reference to the presence of BCMV in the federal states of Puebla (Diaz-Plaza *et al.*, 1992), Guanajuato (Montes-Rivera and Arévalo-Valenzuela, 1985), Veracruz (López-Salinas *et al.*, 1994) and Sonora (Jiménez-García and Nelson, 1994). A subsequent work showed the prevalence of BCMV over BCMNV in Mexico mostly at the central states, whereas BCMNV proliferated toward the eastern tropical states (Flores-Estévez and Silva-Rosales, 2000; Flores-Estévez *et al.*, 2003). In this report, the frequency of both species is reported in terms of the different cultivars sampled in five different major dry-bean producing federal states in Mexico. Also, the implications of germplasm movement as a factor shaping the distribution of both viral species are discussed. Some possible explanations are given to understand the presence of both viral species in black seeded cultivars.

MATERIALS AND METHODS

Sample collection

Field samples were obtained from commercial and experimental bean fields collected in five federal states in Mexico representing four agricultural systems: irrigated and rainfed crop during winter-spring and spring-summer seasons (Guanajuato); irrigated crop during fall-winter season (Sinaloa and Sonora); residual moisture crop at the fall-winter season (Nayarit) and rainfed and residual moisture crops during summer and fall-winter seasons at the humid tropics (Veracruz). In order to obtain uniformly collected samples and recorded data, a registration manual was implemented specifically for this project, containing main visual criteria guidelines on virus symptoms (Figure 1), and main strategies for sample collection such as the gathering of the widest diversity of symptomatic varieties in as many cultivated fields as possible, in order to increase viral diversity and collection of bean plants in a defined perimeter around a source of infection. A total of nine samples per infection foci, within a hectare were initially spotted, eight of which were symptomatic and one asymptomatic plant. Symptomatic plants were collected at equidistant plows. A total number of 338 samples were collected (Table 1), in about 24 localities and from about 35 bean cultivars and photographed with a registration number to generate a database for the frequency account of both viral species in the generated collection.

Table 1. Cultivars sampled by state.

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Cultivar/States	Guanajuato	Nayarit	Sinaloa	Sonora	Veracruz
Aluyori			8		
Azufrado Criollo		9			
Azufrado Higuera 1				18	
Azufrado Higuera 2			54		
Azufrado Noroeste			17		
Azufrado Peruano 87				5	
Azufrado Regional 87				5	
Azufrado Reg. Criollo			18		
Bayo Berrendo		9			
Bayo Blanco				5	
Bayo Madero	1				
CIAT 103-25		8			
Criollo Negro					1
Criollo Vaina Blanca y Morada					2
Criollo Vaina Morada					9
ELS 15-55		7			
FJB 08046	1				
Flor de Junio	5				
Flor de Junio Marcela	18	10			
Flor de Mayo	4				
Flor de Mayo Anita	12				
Flor de Mayo Bajío	1				
Flor de Mayo Dolores	2				
Negro 8025	3				
Negro Chapingo		9			
Negro Guanajuato		7			
Negro Huasteco 81					5
Negro Jamapa	7	46			6
Negro Papaloapan					3
Negro San Luis Criollo	1				
Pinto Durango	6				
PTB 08005	1				
Rosa de Castilla 62	6				
Zac. 524/8025/vax-4-2	9				
	77	105	97	33	26

cDNA synthesis and RT-PCR analysis

Total leaf RNA was extracted with TRIzol™, according to the manufacturer instructions. After quality verification with "GelRed" staining in a 1% non-denaturing agarose gel, RNA was quantified and stored at -80 °C until cDNA synthesis.

Total RNA was used as a template for RT-PCR (reverse transcription followed by polymerase chain reactions) using specific primers directed toward the

coat protein cistron to obtain products of 890 bp and 740 bp for BCMV and BCMNV respectively as previously described by Flores-Estévez *et al.* (2003). For each sample 1 ng up to 5 µg total RNA was used plus 1 µL of 10 µM oligo $(dT)_{18}$ and 1 µL of dNTP Mix (10 mM each) in a volume of 12.5 µL with sterile, distilled water. Reaction tubes were incubated at 65 °C for 5 minutes and quickly chilled on ice. Then 4 µL of 5x First-Strand Buffer, 2 µL of 0.1 M DTT and 1 µL de RNaseOUT™ (40 U/µL) were added to each tube. The tubes were shaken gently and incubated at 42 °C for 2 minutes. Finally 0.5 µL of SuperScript™ RT (200 U/µL) were added per reaction tube and mixed with the aid of a micropipet. The tubes were incubated at 42 °C for 50 minutes and then heated at 70 °C for 15 minutes to inactivate the reverse transcriptase.

PCR amplification was performed using between 0.5 and 1 µL of cDNA in 20 µL reaction volume containing 2 µL of 10x PCR buffer without Mg^{2+}, 0.4 µL of 10 mM dNTP mixture, 0.6 µL of 50 mM $MgCl_2$, 1 µL of primer mix (10 µM of each nucleotide) and 0.2 µL of *Taq* DNA Polymerase (5 U/µL). The contents of the tubes were mixed with the aid of a pipet. cDNA synthesis of the coat protein cistron of BCMV and BCMNV was carried out with a initial denaturation at 94 °C for 1 minute followed by 30 cycles each of: 15 seconds of denaturation at 94 °C, 30 seconds of annealing at 63 °C and 40 seconds of elongation at 72 °C. A final elongation extension was done at 72 °C for 10 minutes in a thermal cycler (Applied Biosystems). The PCR fragments were verified in a 1 % non-denaturing agarose gel after "GelRed" staining. They were then cloned and sequenced by capillary electrophoresis in Cinvestav, Sede Irapuato, at LANGEBIO Unit. The sequence analysis and alignment were performed using the ClustalW option from the Geneious ™ software package.

RESULTS

A total of 338 samples were collected at the federal states of Guanajuato, Nayarit, Sinaloa, Sonora and Veracruz (Table 1). The database from the collection is available at (www.frijol.inifap.gob.mx). RT-PCR reactions were carried out for all the samples and the presence or absence of the corresponding 740 and 890 bp bands was indicative of the presence of BCMNV and BCMV, respectively (Figure 2). Some of the bands (9% of them) were difficult to interpret since the product was not clearly visible, possibly due to poor RNA quality coming from a leaf tissue on suboptimal conditions and were therefore registered as not defined in this report, and left out for further hybridization analyses in a different study. Almost half of the samples were scored as negatives (48%). Less than half of all samples (34%) were either positive for BCMV (13%) or BCMNV (21%), or for both viral species (9%). There were more positive samples having BCMNV (21%) than BCMV (13%). Besides single infections, by any of BCMV or BCMNV, mixed infections were detected in 30 out of 144 positive samples equivalent to a 20%, this is a higher percentage than the 12% obtained by Flores-Estévez *et al.* (2003). Neither on that occasion, nor at this time, mixed infections were found in Veracruz and Guanajuato (Table 1 in reference 15) .In this study, mixed infections were found on Sinaloa and Nayarit (Table 2). Interestingly, as opposed to the 2003 study where mixed infections were found in light seed-colored cultivars, in this work, mixed infections were found in black seeded cultivars like Negro Jamapa from Nayarit, however samples of the same cultivar from Veracruz did not have BCMV. Sinaloa and Nayarit had the highest percentage of mixed infections. Sinaloa had the highest frequency of BCMV whereas Nayarit the highest of BCMNV (Table 2).

Table 2. Occurrence of BCMV and BCMNV by Federal State.

Federal States	BCMV positives	BCMNV positives	BCMV and BCMNV positives	Negatives	Not defined	Total
Guanajuato	7 (9%)	3 (4%)*	-	43 (56%)	24 (31%)	77
Nayarit	-	43 (41%)	12 (11%)	46 (44%)	4 (4%)	105
Sinaloa	34 (35%)	7 (7%)	18 (19%)	36 (37%)	2 (2%)	97
Sonora	3 (9%)	3 (9%)	-	27 (82%)	-	33
Veracruz	-**	14 (54%)	-	12 (46%)	-	26
Total	44 (13%)	70 (21%)	30 (9%)	164 (48%)	30 (9%)	338

*Percentage of incidence was expressed as the number of incidence divided by the total number of samples for a particular State.

**- no positives were found for the searched virus.

Table 3. BCMV and BCMNV in single or mixed infections (in different bean cultivars) in Sonora and Nayarit

Cultivar	Race	Frequency					Total samples
		BCMV positive	BCMNV positive	BCMV and BCMNV positive	Negatives	Not defined	
Azufrado Higuera 2	Nueva granada	18 (33%)*	3 (6%)	11 (20%)	22 (41%)	-	54
Negro Jamapa	Mesoamericana	-	23 (50%)	10 (22%)	13 (28%)	-	46
Azufrado Reg. Criollo	Mesoamericana	14 (78%)	-	-	4 (22%)	-	18
Azufrado Noroeste	Nueva Granada	1 (6%)	3 (18%)	7 (41%)	6 (35%)	-	17
Flor de Junio Marcela	Jalisco	-	2 (20%)	-	8 (80%)	-	10
Azufrado Criollo	Mesoamericana	-	4 (44%)	1 (12%)	4 (44%)	-	9
Bayo Berrendo	Mesoamericana	-	7 (76%)	1 (12%)	-	1 (12%)	9
Negro Chapingo	Mesoamericana	-	6 (67%)	-	3 (33%)	-	9
Aluyori	Nueva Granada	1 (12%)	1 (12%)	-	4 (50%)	2 (26%)	8
CIAT 103-25	Mesoamericana	-	-	-	8 (100%)	-	8
ELS 15-55	Mesoamericana	-	-	-	5 (71%)	2 (29%)	7
Negro Guanajuato	Mesoamericana	-	1 (14%)	-	5 (72%)	1 (14%)	7

*Percentage of incidence was expressed as the number of incidence divided by the total number of samples for a particular variety.
Reg. stands for Regional

Almost half of the collected samples did not contain any of the viral species reflecting the sampling of plants without any symptoms (healthy), as part of the survey plan; or plants with virus-like symptoms (mosaics, choloris or leaf deformations), probably due to toxemias caused by insects feeding from phloem sap or else, by fungi and bacteria infected plants. These pathogens may cause similar infection symptoms as those caused by BCMV and BCMNV making it difficult to differentiate in the field, which one is causing the infection. This is why incidence and severity are not reliable parameters in this study and were not an addressed issue here.

Thirty-four bean lines or cultivars were collected in the five sampled federal states mentioned before. Lines from the different states from which samples were collected are shown in Table 3. Guanajuato was the state where more cultivars were collected due to the presence of INIFAP experimental station where periodic evaluation of national germplasm normally takes place. Eight different cultivars were collected in Nayarit, six in Veracruz, and four in Sinaloa and Sonora. The highest sample number was from Negro Jamapa and Azufrado Higuera 2 cultivars, followed by Flor de Junio Marcela, Azufrado Higuera 1, Azufrado Regional Criollo, Azufrado Noroeste, etc. (Table 3).

As mixed infections were found in Sinaloa and Nayarit, a closer examination of the viral species present per cultivar was done for these two states. Five cultivars had both viruses present in mixed infections, these were: Azufrado Higuera 2 (11 out of 54); Negro Jamapa (10 out of 46); Azufrado Noroeste (7 out of 17); Azufrado Criollo and Bayo Berrendo (both with 1 out of 9). As single infections is concerned, nine cultivars were positive for BCMNV: Azufrado Higuera 2 (3 out of 54 samples); Negro Jamapa (23 out of 46); Azufrado Noroeste (3 out of 17); Flor de Junio Marcela (2 out of 10); Azufrado Criollo (4 out of 9); Bayo Berrendo (7 out of 9); Negro Chapingo (6 out of 9); Aluyori (1 out of 8) and Negro Guanajuato (1 out of 7). Lastly, four were positive for BCMV; Azufrado Higuera 2 (18 out of 54); Azufrado Regional Criollo (14 our of 18) Azufrado Noroeste (1 out of 17) and finally, Aluyori, with only one out of 8.

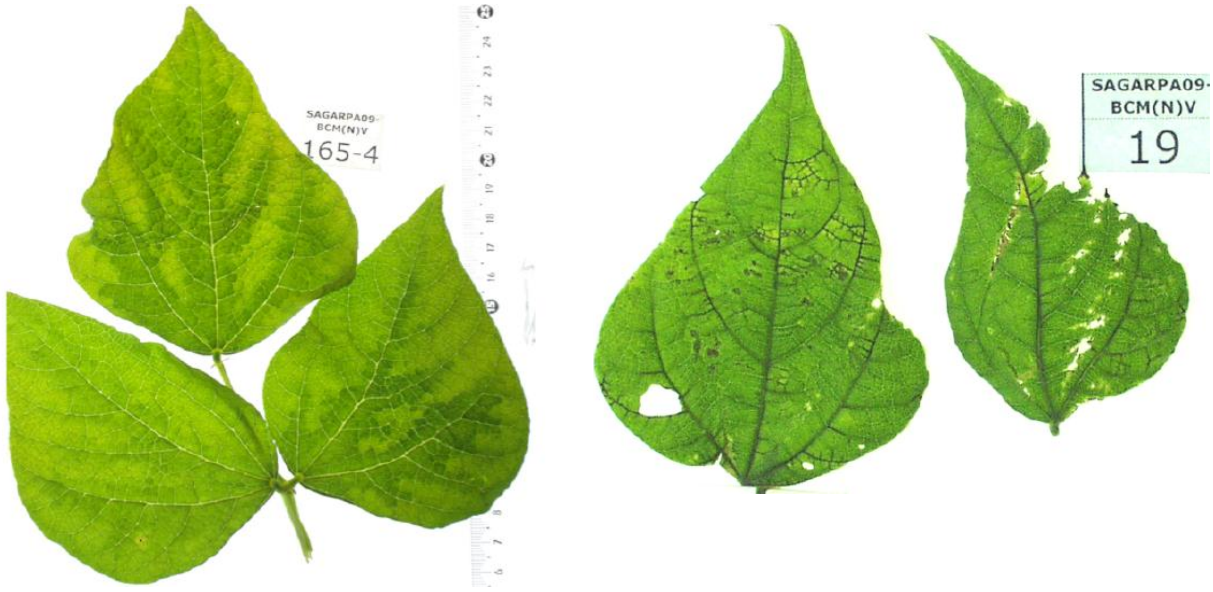

Figure 1. Two examples of the 338 collected samples from the different states under survey in this work (Guanajuato, Sonora, Sinaloa, Nayarit and Veracruz). One sample (left), shows typical symptoms of BCMV with dark green thick areas circumventing primary and secondary veins. BCMNV symptoms are seen as areas where the minor veins become necrotic resulting in an apparent net or cross-road appearance (right).

In short, in Nayarit and Sinaloa, where most of Azufrados and Negros come from, mixed infections were detected. BCMNV was found in all sampled states in single infections whereas BCMV was found in Guanajuato, Sinaloa and Sonora.

DISCUSSION

The presence of the species of the bean common mosaic virus was analyzed, namely BCMV and BCMNV, within 338 samples collected in the states of Guanajuato, Nayarit, Sinaloa, Sonora and Veracruz. Both species were detected in a targeted survey on symptomatic plants. This is why at least one of the viral species was detected in most of the 24 localities sampled. On a previous work, the presence of BCMV and BCMNV was monitored in Mexico (Flores-Estévez *et al.* (2003) in 2003. At that time, sixteen federal states were surveyed but no samples from Sonora, Sinaloa and Nayarit were included. In the present survey, in these three States, BCMNV was present but BCMV was absent in the states of Nayarit and Veracruz. The absence of BCMV in Veracruz was also observed in the survey of 2003. It is in this state that black seed samples from the Mesoamericana race (Singh *et al.* (1991) were permissive for BCMNV. One salient difference with that first survey is that in this work, mixed infections in black bean cultivars such as Negro Jamapa were found, although only in Nayarit. Such an event was not recorded before; mixed infections were only detected in light seed-colored materials.

The question remains as to how this black seeded cultivar, Negro Jamapa, has acquired BCMV. It is possible that the necrotic species are more abundant in dry and humid tropical climates due to the climate *per se* and to alternate hosts within the year. Other possibility would be that in the breeding process, while developing this cultivar, the presence of different combinations of the *bc* type recessive resistance genes was allowed along with the *I* resistance gene providing resistance for BCMV but not to BCMNV in mixed infections. In fact, this cultivar is a multiline cultivar made up by the intermixing of eleven lines (Rosales-Serna *et al.* (2004). Interstate movement of this cultivar might have allowed the presence of the BCMV species on seeds where high viral pressure occurs (places where this species are prevalent).

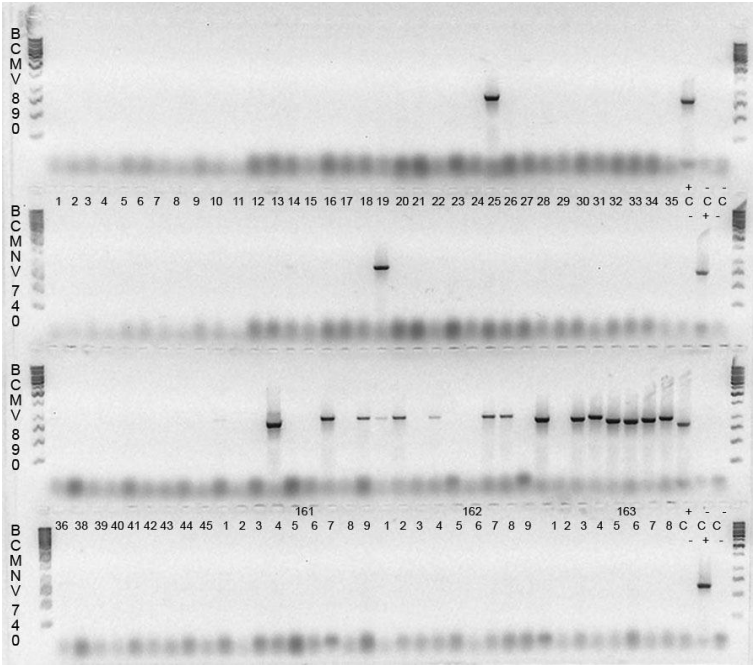

Figure 2. Representative photograph of a 1% agarose gels with the amplified RT-PCR products for the coat protein (CP) for either BCMV or BCMNV as shown on the left side of each gel indicating the expected band size for each viral species. Positive and negative controls as represented by + and – symbols respectively.

Another possibility is that the presence of the necrotic species facilitates the presence (replication) of the non-necrotic species, an equivalent simile to synergism. However, replication rates would need to be measured before the proper use of this term in this system (black seeded cultivar with mixed infections). The last possibility would be that the presence of the *I* gene in the cultivar Negro Jamapa can cause a permissive state for the presence of BCMV at the average temperatures in Nayarit providing that a pressure of this virus prevails in this state as compared to Veracruz.

However, more studies would be needed to understand the presence of both species in black seeded germplasm putatively having the BCMV-resistance *I* gene. Further studies are being conducted to characterize the pathogroups of BCMV and BCMNV present in Mexico and also to try to understand the permissiveness of both species in black seeded cultivars. Due to its geographical position, the Bajío region is a potential region with the high risk of occurrence of necrotic strains if there is an indiscriminate introduction of contaminated seed from Nayarit and Sinaloa, mostly during the winter-spring season. Also, at the Bajío region due to high temperatures during the winter-spring irrigated crop, temperature-dependent necrotic strains might have resulted with the hypersensive reaction in plants without the I gene.

The prevalence of BCMV found among the Azufrados, improved cultivars (Nueva Granada race) and landraces (Mesoamerican race) at Sinaloa, might indicate the presence of effective genes against BCMNV but less towards BCMV. Another possibility is that suboptimal temperatures during the winter crop cycle at this northern state is not favorable to the presence of BCMNV.

Unfortunately, since a high percentage of farmers buy certified seed at each planting season, the high prevalence of BCMV found in most samples of Azufrado cultivars in Sinaloa suggest that seed production system does not comply with all the requirements for the production of clean-disease-free seed and/or the re-use of contaminated grain as seed. This result also indicates that effective resistant genes against BCMV need to be incorporated by breeding into the popular cultivars grown at these two states.

CONCLUSIONS

The high presence of negative samples found in this research indicates that around half of the visual scores given on the symptoms of BCMV in the field, are wrong, therefore their presence must be defined by other means such as the RT-PCR technique used here. In Mexico, both BCMV and BCMNV are present in some of the main bean growing areas. At the pacific coastal areas at Sinaloa and Sonora BCMV is

prevalent whereas at Nayarit and at the lowlands of Veracruz, BCMNV is more abundant.

A high prevalence of BCMNV was found at the state of Nayarit, mostly on black seeded cultivar Negro Jamapa and of BCMV at Sinaloa on Azufrado type cultivars.

Mixed infection with both BCMV and BCMNV were found in the black seeded cultivar Negro Jamapa grown at Nayarit during the fall-winter season.

Since both viral species BCMV and BCMNV, are seed transmitted, there is a high risk of epidemics at the states of Sinaloa and Nayarit, respectively, and into other states through the movement of seed across states.

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