BIOLOGICAL AND MOLECULAR CHARACTERISTICS OF *BEAN COMMON MOSAIC VIRUS* ISOLATES CIRCULATING IN COMMON BEAN IN IRAN

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SUMMARY

Bean common mosaic virus (BCMV) is one of the most widespread and economically important seed and aphid-transmitted viruses. During 2010-2011, a total of 2410 symptomatic bean (*Phaseolus vulgaris*) leaf samples were collected from nine provinces of Iran and checked by DAS-ELISA (double antibody sandwich enzyme-linked immunsorbent assay), resulting in detection of BCMV in 655 samples. Following biological purification, the reaction of 16 differential bean cultivars (belonging to 11 host groups) against five geographical BCMV isolates revealed the occurrence of five different BCMV pathogroups, PG- I, II, IV, V and VII, which were new for Iran. The response of five commonly cultivated bean genotypes against three BCMV pathotypes (I, II and IV) was evaluated by semi-quantitave ELISA and two genotypes, Chiti karaj and Jules, were characterized as highly sensitive and tolerant genotypes, respectively. Pathogenicity of three different BCMV pathotypes on five bean genotypes used in this study revealed that they all belong to host group 1. Amplification of the 3'-terminal region (comprising coat protein region and partial 3'-UTR) of four BCMV isolates was performed by reverse transcription polymerase chain reaction (RT-PCR). Phylogenetic analysis of nucleotide and deduced amino acid sequences of coat protein genes did not differentiate BCMV isolates by host, location and pathogenicity group.

Keywords: BCMV, coat protein gene, pathogenic group, phylogeny.

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INTRODUCTION

Common bean (*Phaseolus vulgaris* L., Fabaceae), as an important protein component of the human diet, is consumed all over the world (Drijfhout, 1978). *Bean common mosaic virus* (BCMV) belongs to the genus *Potyvirus*, family *Potyviridae* (Adams *et al*., 2011) and causes economically serious crop losses in common bean. BCMV causes mosaic, dwarfing, leaf curling and chlorosis in bean plants (Morales, 1998) and is one of the most widespread and important seed and aphid-transmitted viruses on *P. vulgaris* (Pasev *et al*., 2013; Bos, 1971). During recent past years the taxonomy of BCMV has undergone major changes. Unlike the other viruses, the first level of biological subdivision for BCMV is the pathogenicity group or pathogroup (PG). BCMV strains were categorized into seven pathogroups (PGs) based on their reaction across 11 host groups (HGs) of differential bean cultivars (Drijfhout, 1978; Drijfhout *et al*., 1978). Resistance to different BCMV strains is controlled by the dominant *I* gene and/or with combinations of several recessive genes (*bc-u*, *bc-1*, *bc-1*^{2}, *bc-2*, *bc-2*^{2} and *bc-3*) (Kelly *et al*., 1995; Strausbaugh *et al*., 1999). Differentiation of pathogroups is achievable only by symptomatic reaction of *P. vulgaris* genotypes (Drijfhout, 1978), although it is likely that RT-PCR and restrictotype map could be applicable for some pathogroups (Xu and Hampton, 1996). Based on current classification, Drijfhout pathogroups I, II, IV, V and VII now comprise BCMV, whereas pathogroups III (strain NL-8) and VI (strains NL-3 and NL-5) comprise *Bean common mosaic necrosis virus* (BCMNV) (Mink *et al*., 1994). Recently, the new pathogroup, PG-VIII has been introduced with a novel BCMV isolate 1755a. This isolate displayed a novel pattern of interactions with resistance genes in *P. vulgaris* with ability to overcome *bc-2* and *bc-3* alleles in common bean (Feng *et al*., 2015). Some earlier known distinct legume infecting potyvirus species like *Blackeye cowpea mosaic virus* (BlCMV), *Azuki bean mosaic virus* (AzMV) and *Peanut stripe virus* (PStV) are now considered as strains of BCMV based on their coat protein sequences (McKern *et al*., 1992, 1994; Rybicki and Shukla, 1992; Ward *et al*., 1992).

Over the past several years, virus surveys conducted in bean cultivated areas of Iran has demonstrated the concern of BCMV (Kaiser and Mossahebi, 1974; Shahraeen *et*

Table 1. Sampling locations and BCMV infection rate in symptomatic samples of *Phaseolus vulgaris.*

Geographical location	Symptomatic samples No.	BCMV infected No.	BCMV infection rate (%)
Alborz	1100	0	0
Tehran	160	89	55.62
Hamedan	512	392	76.56
Kurdistan	220	114	51.81
Qazvin	96	21	21.87
Zanjan	57	20	35.08
Gilan	43	7	16.27
Isfahan	38	12	31.57
Khuzestan	184	0	0
Total	**2410**	**655**	**27.17**

al., 2005; Naderpour *et al*., 2010; Dizadji and Shahraeen, 2011; Musavi *et al*., 2014). A sound knowledge on BCMV and its strains is a vital prerequisite for applying reliable and economic control method by host breeding for resistance, but little is known about BCMV strains and pathogroups in Iran. The aim of this study was to determine pathogroup(s) of geographical BCMV isolates based on their biological and molecular characteristics and to evaluate the reaction of five widely grown bean cultivars to three BCMV pathotypes.

MATERIALS AND METHODS

Sampling and DAS-ELISA plant materials. During 2010-2011, bean growing fields in nine different provinces of Iran (Table 1), as the most important bean growing centers in Iran, were surveyed and a total of 2410 bean leaf samples showing mosaic, leaf rolling and vein banding symptoms were collected. Symptomatic samples were assayed for the presence of BCMV by DAS-ELISA as described by Clark and Adams (1977) using polyclonal antibodies against BCMV (DSMZ-AS-0242) following the manufacturer's instructions. Fresh leaf tissues of inoculated plants were extracted (1:10) using PBST-PVP (phosphate buffered saline, 0.5% Tween 20, 2% polyvinylpyrrolidone) and used in double antibody sandwich enzyme-linked immunsorbent assay (DAS-ELISA). The absorbance of each well was measured at 405 nm (OD_{405}) by ELISA-reader (Beckman AD 340S; Beckman Coulter, Fullerton, CA, USA). Samples were considered positive only if the absorbance value was more than three times of negative control.

Virus isolates, maintenance and bioassays. Among ELISA positive samples of surveyed provinces, five BCMV isolates (Ir-S31, Ir-H60, Ir-Q51, Ir-Z1 and Ir-D7, from five different geographic origins) (Table 2) were randomly selected. The isolates were biologically purified by three serial single local lesion transfers on *Chenopodium quinoa* using BCMV infected plant leaf extract in phosphate buffer and propagated on *Phaseolus vulgaris* cv. Stringless Green Refugee (SGR) for further studies. Symptoms of selected BCMV isolates were determined following mechanical inoculation of five test plants species from four families (Table 3). The inoculated plants were tested serologically by DAS-ELISA at 25 days post-inoculation (dpi) and kept for one month in a presumably insect-free glasshouse with controlled conditions to develop disease symptoms.

Table 2. Assignment of pathogroups to BCMV isolates identified in this study.

Isolate[a]	Original host	Geographical location	Pathogenic group
Ir-D7	*P. vulgaris*	Tehran (Damavand)	V
[Ir-H60]	*P. vulgaris*	Hamedan	IV
[Ir-Q51]	*P. vulgaris*	Qazvin	I
[Ir-S31]	*P. vulgaris*	Kurdistan (Sanandaj)	II
Ir-Z1	*P. vulgaris*	Zanjan	VII

[a]Isolates used in biological studies with bean genotypes assay are given in brackets.

Table 3. Reaction of test plants to inoculation with five BCMV isolates.

Test plants	Ir-D7	Ir-S31	Ir-H60	Ir-Z1	Ir-Q51
C. amaranticolor	-	-	-	-	-
C. quinoa	LL	LL	LL	LL	LL
P. vulgaris cv.SGR	M/LR	M/LR	M/LR	M/LR	M/LR
N. benthamiana	M	M	M	M	M
V. unguiculata L. Walp	-	-	-	-	-

LL, Local Lesion; LR, Leaf Rolling; M, Mosaic.

Pathogroup determination of BCMV isolates. Pathogroup of five BCMV isolates was determined following mechanical inoculation of each isolate onto a standard set (Table 4) of bean differential cultivars (Drijfhout, 1978). The cotyledons of ten plants of each differential cultivar were mechanically inoculated using crude sap (1 g of fresh leaves ground in 10 ml of 0.01 M sodium phosphate buffer, pH 7, containing active charcoal) from systemically infected tobacco plants. Three plants of each differential cultivar were mock inoculated with buffer only as control. Inoculated plants were maintained in a glasshouse with a 16 h photoperiod at 20 to 25°C. The reaction of inoculated plants were indexed daily for virus symptoms for 30 dpi and their infection status was checked by DAS-ELISA on apical leaves at 21 dpi. When systemic infection with conspicuous mosaic was detected, the plant was recorded as susceptible. If they did not show any systemic symptoms which was verified by DAS-ELISA, they were recorded as resistant. Moreover, plants with no symptoms but positive reaction in DAS-ELISA were recorded as tolerant. Plants with systemic or partial necrosis were distinguished as susceptible with temperature-dependent necrosis phenotype (above 30°C).

Table 4. The reaction of differential bean cultivars to five Iranian BCMV isolates.

Differential cultivar			BCMV isolate				
Host group	**Differential cultivar**	**Resistance genes**	**Ir-D7**	**Ir-H60**	**Ir-Q51**	**Ir-S31**	**Ir-Z1**
1	Dubbele Witte	*bc-u*	+	+	+	+	+
1	The Prince	*bc-u*	+	+	+	+	+
1	Stringless Green Refugee (SGR)	*bc-u*	+	+	+	+	+
1	Sutter Pink	*bc-u*	+	+	+	+	+
2	Imuna	*bc-u*, *bc-1*	$+_t$	+	−	$+_t$	+
3	Redlands GreenLeaf B (RGB)	*bc-u*, *bc-1*^{2}	−	+	−	−	+
4	Sanilac	*bc-u*, *bc-2*	+	−	−	−	−
5	Pinto UI 114	*bc-u*, *bc-1*, *bc-2*	+	−	−	−	−
6	Great Northern UI 31	*bc-u*, *bc-1*^{2}, *bc-2*^{2}	−	−	−	−	+
6	Monroe	*bc-u*, *bc-1*^{2}, *bc-2*^{2}	−	−	−	−	+
7	IVT7214	*bc-u*, *bc-2*, *bc-3*	−	−	−	−	−
8	Widusa	*I*, *bc-u*	−	n_+	−	−	−
9a	Jubila	*I*, *bc-u*, *bc-1*	−	n_+	−	−	−
9b	Top Crop	*I*, *bc-u*, *bc-1*	−	n_+	−	−	−
10	Amanda	*I*, *bc-u*, *bc-1*^{2}	−	−	−	−	−
11	IVT 7233	*I*, *bc-u*, *bc-1*^{2}, *bc-2*^{2}	−	−	−	−	−

+, susceptible, systemic mosaic; −, resistant, no systemic infection; $+_t$, tolerant, no symptoms, virus detected by ELISA; n_+, susceptible, systemic or partial temperature-dependent necrosis (above 30°C).

Table 5. Designed primer pairs to amplify 3'-end genome of BCMV isolates in this study.

Primer	Sequence (5'-3')	Tm (°C)	Position	Product size	Amplified region
BCMV-F1	TGGGGGTATCCTGAACTGCTCCA (23mer)	66.4	8648-8670	758 bp	Partial NIb + Partial CP
BCMV-R1	TGACGGAGTGTTGGCTTTGCAT (22mer)	62.1	9384-9405		
BCMV-F2	CCGGATGTGAATGGCACATGGGT (23mer)	66.4	9308-9330	685 bp	Partial CP + complete 3'-UTR
BCMV-R2	GGAACAACAAACATTGCCGTGGCT (24mer)	65.3	9969-9992		

Reaction patterns of five domestic bean genotypes to three BCMV isolates. The response of five bean genotypes (Chiti karaj, Derakhshan, Red karaj, Akhtar and Jules) which are widely cultivated in Iran, to BCMV isolates were assayed based on infection rate and accumulation level of virus. To evaluate the reactions of bean genotypes three geographical BCMV Ir-S31, Ir-H60 and Ir-Q51 isolates (belonging to three different pathogroups) were inoculated on bean cotyledons. The experiment was carried out as factorial based on randomized complete block design (RCBD) with 15 plants (five plants, three replications) for each genotype/BCMV isolate combination. As a control, ten plants of each bean genotype were mock inoculated with inoculation buffer only.

All inoculated plants were maintained in above mentioned glasshouse conditions. Virus detection was carried out by DAS-ELISA at 21 dpi, as described before. Based on the ELISA results, the infection rate (the number of infected plants/number of inoculated plants) of each genotype/BCMV isolate combination was determined. The accumulation level of BCMV in infected plants of each combination was determined by semi-quantitative DAS-ELISA method as described previously (Mazier *et al*., 2004) using 1:10 dilution of leaf extracts, where the relationship between OD_{405} and the viral concentration was linear based on a preliminary calibration curve. Plants infection rate (%) and BCMV accumulation level data were statistically analyzed by two-way ANOVA (Proc. GLM) followed by Student-Newman-Keuls (SNK) test at $\alpha = 1\%$ to estimate the differences among the variants using SAS 9.1 software (SAS Institute Inc., Cary, NC, USA).

RNA extraction and RT-PCR amplification. Total RNA from healthy and BCMV infected *Nicotiana benthamiana* leaves was extracted using RNeasy Plant Mini Kit (Qiagen, Germany) according to the manufacturer's instructions and used in RT-PCR reactions. Two specific oligonucleotide primer pairs (Table 5) were designed based on BCMV cowpea isolate R (GenBank accession No. AJ312437) genome using Primer3 (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) to amplify genomic 3'-end (including 3'-UTR, coat protein and partial NIb regions) of five BCMV isolates (Ir-S31, Ir-H60, Ir-Q51, Ir-D7 and Ir-Z1). First-strand cDNA was synthesized using total RNA as template in 20 µl reaction mixture containing 5 µl total RNA, 4 µl 5× RT-PCR buffer, 0.5 mM dNTPs, 5 mM DTT, 20 U RNAse inhibitor (Fermentas, Lithuania), 5 pM of each reverse primers separately (BCMV R1 and BCMV R2) and sterile distilled water. The RT mix was incubated at 72°C for 3 min (for enzyme inactivation) and immediately incubated on ice and 100 U M-MuLV reverse transcriptase (Fermentas) was added to each reaction. The reactions were incubated at 42°C for one h in thermocycler (Palm cycler, CG1-96, Cobett research, Australia). PCRs were carried out in 25 µl reaction mixtures containing 5 µl cDNA, 2.5 µl of 10× PCR-buffer, 4 mM $MgCl_2$, 2 pM of

Table 6. Characteristics of 36 BCMV isolates used in phylogenetic analysis in this study.

Strain/Isolate	Origin	Host	Pathogroup	Accession No.
Ir-D7	Iran	*Phaseolus vulgaris*	V	KU051675
Ir-H60	Iran	*P. vulgaris*	IV	KU051676
Ir-S31	Iran	*P. vulgaris*	II	KU051674
Ir-Z1	Iran	*P. vulgaris*	VII	KU051677
1755a	USA	*P. vulgaris*	VIII	KT175570
AAU	India	Cluster bean		JX420811
CH1	Chlie		IV	L19539
D-1	India	*P. vulgaris*		GQ456169
GGSSA	South Africa	*Cyamopsis tetragonolaba*		AF045066
GGSUS	USA	*C. tetragonolaba*		AF045065
Golestan	Iran	*P. vulgaris*		KF670145
HB	China	Hyacinth bean		KC478389
K2	India	*P. vulgaris*		FJ157246
L-2	India	*P. vulgaris*		FJ387162
Lorestan6	Iran	*P. vulgaris*		KC969187
MB	China	Mung bean		KC832502
Mexican	Mexico			L11890
MS1	Australia	*Macroptilium atropurpureum*		EU761198
N17		*P. vulgaris*		U37075
Neishabour1	Iran	*P. vulgaris*		KC969188
NL1	Netherlands	*P. vulgaris*	I	AY112735
NL1	Netherlands		I	KM023744
NL1-n	India	*P. vulgaris*	I	KF114860
NL2			V	L19472
NL4	Colombia	*P. vulgaris*	VII	DQ666332
NY15	USA		V	AF083559
PR1	Puerto Rico		I	L21767
PV0915	Germany	*P. vulgaris*		HG792064
R	China	*Vigna unguiculata*	I	AJ312437
RU1-OR-B	USA		VII	KF919297
RU1-OR-C	USA		VII	KF919298
Soybean	China	Soybean		KC832501
US1	USA		I	L12740
US10	USA		VII	KF919299
US10	USA			U37072
US3	USA	*P. vulgaris*	IV	U37073
US4	USA	*P. vulgaris*	IV	U37074
US5	USA		IV	L19473
US7	USA		II	L19474
Y	China	*V. unguiculata*		AJ312438
BCMNV	Michigan	*P. vulgaris*		NC004047

each forward and reverse primers, 0.2 mM dNTPs and 1.5 U SmarTaq DNA polymerase (Sinaclon, Tehran, Iran). Initial denaturation was performed at 95°C for 5 min, followed by 35 cycles at 94°C for 1 min, 60 and 62°C for 1 min (annealing of primer pairs BCMV F1/ BCMV R1 and BCMV F2/ BCMV R2, respectively), 72°C for 2 min and a final elongation step at 72°C for 10 min. PCR products were analyzed on 1% agarose gel at 80 V with 1× TBE. The gel was stained in ethidium bromide solution (40 µg/ml) for 20 min and visualized with gel documentation (White/Ultraviolet transilluminator-UVP-UK).

Sequencing and phylogenetic analysis. Amplified fragments of BCMV isolates were extracted from the gel using a Vivantis nucleic acid extraction kit (Malaysia) based on manufacturer's instruction and sequenced directly in both directions using the respective PCR primer pairs by Bioneer Inc. (South Korea). The contigs were assembled using Vector NTI Advance™ 11.0 (Invitrogen, CA, USA). Nucleotide sequences of contigs were compared based on the Blastn analysis software with available sequences in GenBank database (Table 6). Both the protein and gene sequences were aligned using Muscle within the MEGA6 sequence analysis package, using the default parameters (Tamura *et al*., 2013). Model evaluation, carried out for the nucleotide and translated amino acid sequences within MEGA 6, showed that the Tamura-Nei (Tamura and Nei, 1993) and Jones-Taylor-Thornton (Jones *et al*., 1992) with gamma distributed rates amongst sites was the best models, respectively. Phylogenetic trees for the nucleotide and translated amino acid sequences were then constructed using maximum likelihood within MEGA6 using the TN93 + G and JTT + G substitution models, respectively. Each model was tested with 1000 bootstraps replicates. To make the calculations tractable the heuristic nearest neighbor interchange was used. *Bean common mosaic necrosis virus*

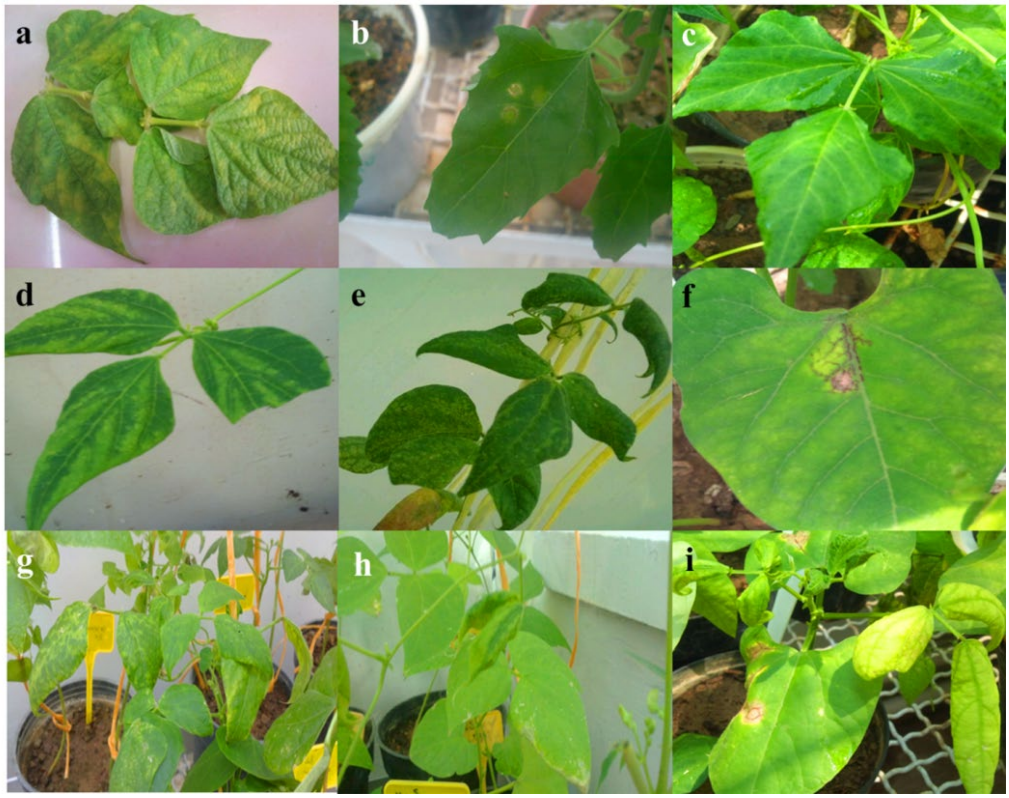

Fig. 1. BCMV symptoms on natural infected (a) and inoculated test plants (b-i). b, Local lesion on *Chenopodium quinoa*; c, Mosaic symptom on 'The Prince' induced by Ir-S31; d, Vein banding induced by Ir-Q51 on 'Sutter Pink'; e, Mosaic and leaf distortion caused by Ir-Z1 on 'SGR'; f, Veinal necrosis induced by Ir-H60 on 'Widusa'; g, Severe mosaic induced by Ir-Z1 on 'Dubbele Witte'; h, Apical leaf rolling and malformation caused by Ir-S31 on 'Sutter Pink'; i, Leaf rolling and yellowing induced by Ir-D7 on 'Imuna'.

(BCMNV, NC-004047) was defined as outgroup. Evolutionary divergence analyses were computed at the amino acid level using the Poisson correction model in MEGA6 software with the complete deletion option (Tamura *et al*., 2013). The rate variation among sites was modeled with a gamma distribution. The distance was calculated as the number of sites at which two sequences being compared were different. There were a total of 75 positions in the final dataset.

RESULTS

Serological and biological assays, BCMV isolates and symptomology. 655 out of 2410 symptomatic leaf samples collected from different bean fields located in nine provinces of Iran were positive for BCMV using DAS-ELISA. The rate of BCMV infection varied from 16.27 to 76.56% in symptomatic samples, excluding Alborz and Khuzestan provinces with no BCMV infection (Table 1). Following biological purification, selected five isolates caused local lesion on *C. quinoa*, mosaic with leaf rolling in *P. vulgaris* cv. SGR and mosaic in *N. benthamiana.* No symptoms developed on *C. amaranticolor* and *V. unguiculata* L. Walp. (Fig. 1). Infection or virus-free status of inoculated test plants was verified by DAS-ELISA at 21 dpi (Table 3).

Analysis of host differential reaction and identification of pathogenic groups. Reaction of 16 differential bean cultivars representing 11 host groups to inoculation with the five BCMV isolates is shown in Table 4. After mechanical inoculation, the reaction of differential bean cultivars revealed that five BCMV isolates used in this study, Ir-Q51, Ir-S31, Ir-H60, Ir-D7 and Ir-Z1, belong to five different pathogenic groups I, II, IV, V and VII, respectively, as described by Drijfhout (1978) (Table 2).

Reaction patterns of five domestic bean genotypes to three BCMV isolates. Reaction patterns of the five bean genotypes against three BCMV isolates are shown in Table 7. Based on the results, infection rate ($F_{4,30}=7.32$, $p=.0003$) and accumulation level (OD_{405}) of the virus ($F_{5,252}=18.56$, $p<.0001$) was significantly different among genotypes. Virus isolate and the interaction between isolate/genotype have no meaningful effect on either infection rate or virus accumulation level. Following mechanical inoculation of bean genotypes with three BCMV isolates, infection rate on genotype Chiti karaj was significantly more than Jules ($F_{4,30}=7.32$, $p=.0003$). Infection rate was high in genotypes Akhtar, Red karaj and Derakhshan. Jules genotype showed meaningfully lowest infection rate than only Chiti karaj, while 20-33% less than other three genotypes. The most severe mosaic

Table 7. BCMV infection rate and accumulation level of virus in inoculated plants of five different genotypes based on semi-quantitative DAS-ELISA at 21 dpi.

Genotypes	BCMV infection rate (%)				BCMV accumulation level (OD_{405}) [a]			
	Ir-Q51	Ir-S31	Ir-H60	Total	Ir-Q51	Ir-S31	Ir-H60	Total
Chiti karaj	80 + 11.55	80.00 + 11.55	60 + 20	73.33 + 8.16 (a)	0.45 + 0.05	0.47 + 0.06	0.45 + 0.08	0.46 + 0.04 (a)
Akhtar	46.67 + 13.33	53.33 + 13.33	66.67 + 6.67	55.56 + 6.48 (ab)	0.27 + 0.06	0.26 + 0.06	0.46 + 0.08	0.33 + 0.04 (b)
Red karaj	53.33 + 6.67	53.33 + 6.67	53.33 + 17.64	53.33 + 5.77 (ab)	0.30 + 0.06	0.31 + 0.06	0.32 + 0.06	0.31 + 0.03 (b)
Derakhshan	33.33 + 6.67	33.33 + 6.67	60 + 23.09	42.22 + 8.46 (ab)	0.20 + 0.05	0.23 + 0.06	0.43 + 0.08	0.29 + 0.04 (b)
Jules	20 + 0.00	33.33 + 6.67	13.33 + 6.67	22.22 + 4.01 (b)	0.15 + 0.04	0.20 + 0.04	0.12 + 0.03	0.16 + 0.02 (c)
control	–	–	–	–	0.07 + 0.00	0.07 + 0.00	0.07 + 0.00	0.07 + 0.00 (c)
Total	46.67 + 6.37(a)	50.67 + 5.81(a)	50.67 + 8.02(a)	–	0.24 + 0.02(a)	0.26 + 0.02(a)	0.31 + 0.03(a)	–

[a] Statistical groups are given in parenthesis.
Data shown are means ± standard errors of the means (SEM).

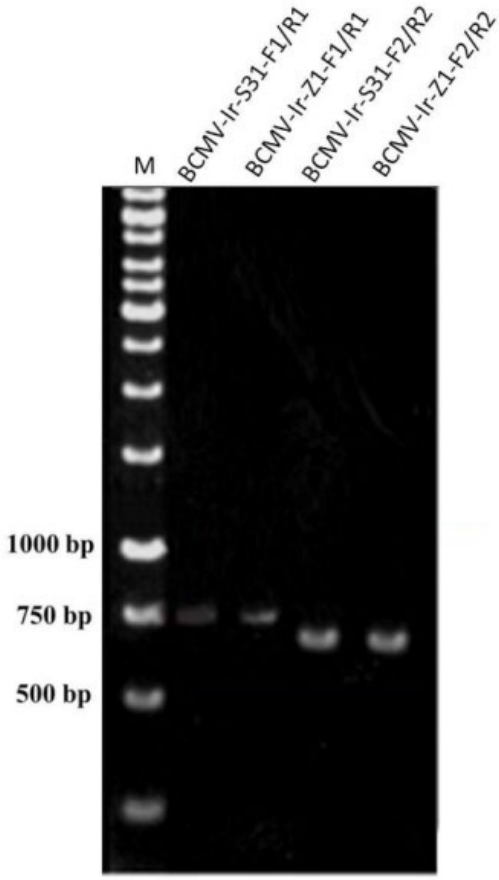

Fig. 2. Agarose gel electrophoresis analysis of RT-PCR products amplified with two primer pairs BCMV F1/R1 and BCMV F2/R2. M, GeneRuler 1kb DNA ladder marker.

and leaf rolling was observed in Chiti karaj plants, mostly resulting in death of infected plants, followed by Akhtar. Mild mosaic and severe vein banding developed in Red karaj, while only mild mosaic and vein banding induced in Derakhshan plants. Almost all inoculated Jules plants showed no symptoms and only very mild mosaic was observed in few ones. Overall, genotypes Chiti Karaj (native) and Jules showed the maximum and minimum rate of the virus detection frequency and accumulation level among assayed genotypes.

RT-PCR, sequencing and phylogenetic analysis. Extracted total RNAs from systemically virus-infected tobacco plants were used as templates for viral cDNA synthesis by RT-PCR. Two predicted 756 and 684 bp DNA fragments were amplified (Fig. 2), using BCMV-F1/R1 and BCMV-F2/R2 primer pairs, respectively, for all isolates, except the isolate Ir-Q51. These primer pairs amplified DNA fragments comprising 227 bp of 3'-terminus of NIb region plus 529 bp of 5'- coat protein region and 428 bp of 3'- CP region plus 256 bp 3'-UTR, respectively. No products were amplified by RT-PCR from healthy plants. Only one fragment with 520 bp in length of Ir-Q51 genome was amplified by using BCMV-F1/R1 which showed 93% identity with US1 and L-2 isolates, while latter primer pair was failed. So, four isolates were used in phylogenetic analysis.

Following sequencing, the overlapping reads were assembled into a contig approximately 1100 bp in length, from genomic 3'-end including CP and 3'-UTR of four BCMV isolates. The CP gene of all isolates contained 864 nt encoding 287 amino acids. The cleavage site between NIb and CP was Q/S in all isolates. The DAG motif, conserved in aphid transmitted potyviruses, was present in the majority of isolates (including our newly characterized isolates) at amino acid positions 12-14 from the N-terminal of the CP gene. Altered DAG sequence observed in some isolates (DAG to DAS in NL1, NY15 and US10, to DTG in US3 and to SAG in N17). The 3'-terminal genomic cDNA of four Iranian new isolates were compared to the NCBI GenBank database using Blastn software. Nucleotide sequence comparison of the ORF CP of Ir-D7, Ir-H60, Ir-S31 and Ir-Z1 isolates with 36 different BCMV isolates showed highest homology with US1, US4, US1 and US10 by 100, 94.5, 99.7 and 98.6%, respectively (Table 8). Phylogenetic trees were constructed based on coat protein nucleotide and deduced amino acid of 36 cognate sequences using maximum likelihood method (Fig. 3). In this tree BCMV isolates were not differentiated by original host and location. Ir-S31 and Ir-D7 were more closer to two other Iranian isolates Golestan and Neishabour1 beside CH1 and US7. Nucleotide sequences of isolates Ir-D7, Ir-H60, Ir-S31 and Ir-Z1 have been deposited to NCBI data base under the accession Nos. KU KU051675,

Table 8. Percentage identity of nucleotide (nt) and amino acid (aa) sequences of the coat protein gene of four Iranian BCMV isolates used in this study with different BCMV isolates.

Strain/Isolate	BCMV isolates in this study							
	nt				aa			
	Ir-D7	Ir- H60	Ir- S31	Ir-Z1	Ir-D7	Ir- H60	Ir- S31	Ir-Z1
Ir-D7	100	86.6	**99.7**	96.9	100	86.0	**99.6**	97.0
Ir-H60	86.6	100	86.2	87.9	86.0	100	85.6	87.1
Ir-S31	99.7	86.2	100	96.6	99.6	85.6	100	96.6
Ir-Z1	96.9	87.9	96.6	100	97.0	87.1	96.6	100
1755a	98.3	88.3	97.9	96.6	98.1	87.9	97.7	96.6
AAU	82.4	80.7	82.4	81.7	82.2	80.3	82.2	81.1
CH1	99.0	85.5	98.6	95.9	98.9	84.8	98.5	95.8
D-1	97.9	86.9	97.6	96.2	97.7	86.4	97.3	96.2
GGSSA	82.4	81.0	82.4	81.7	82.2	79.9	82.2	81.1
GGSUS	82.4	81.7	82.4	81.7	81.4	79.9	81.4	80.3
Golestan	99.3	85.9	99.3	96.2	99.2	85.2	99.2	96.2
HB	84.8	83.4	84.8	84.8	84.8	82.6	84.8	84.5
K2	99.3	85.9	99.0	96.2	99.6	85.6	99.2	96.6
L-2	99.7	86.2	99.3	96.6	**100**	86.0	**99.6**	97.0
Lorestan6	85.9	88.3	85.9	86.2	39.4	33.7	39.0	39.0
MB	90.0	84.8	89.7	90.0	90.5	84.1	90.2	90.2
Mexican	83.1	82.1	83.1	82.8	83.3	81.4	83.3	82.6
MS1	98.3	87.6	97.9	97.2	98.5	86.7	98.1	97.0
N17	82.1	81.7	82.1	81.7	82.2	81.1	82.2	81.4
Neishabour1	99.3	85.9	99.0	96.2	99.2	85.2	98.9	96.2
NL1-AY	96.6	85.9	96.2	96.2	96.6	84.8	96.2	95.8
NL1-KM	99.3	87.2	99.0	97.6	99.2	86.7	98.9	97.7
NL1-n	98.3	86.6	97.9	96.6	98.5	86.4	98.1	97.0
NL2	86.9	92.4	86.6	87.6	86.7	92.0	86.4	87.1
NL4	83.4	81.7	83.4	83.1	83.7	81.1	83.7	83.0
NY15	98.6	85.5	98.3	95.5	98.5	84.8	98.1	95.5
PR1	83.4	81.7	83.4	83.1	83.7	81.1	83.7	83.0
PV0915	95.2	88.3	95.2	93.4	94.7	87.9	94.7	93.2
R	88.6	84.8	88.3	89.0	88.6	85.2	88.3	89.4
RU1-OR-B	85.9	88.6	85.9	86.2	85.2	87.5	85.2	85.2
RU1-OR-C	85.5	88.3	85.5	85.9	84.8	87.1	84.8	84.8
Soybean	90.0	85.5	89.7	89.7	90.2	85.2	89.8	90.2
US1	**100**	86.6	**99.7**	96.9	**100**	86.0	**99.6**	97.0
US10 KF	98.6	85.9	98.3	95.5	98.5	85.2	98.1	95.5
US10	98.3	87.9	97.9	**98.6**	98.5	87.1	98.1	**98.5**
US3	95.9	85.2	95.5	95.5	95.8	84.1	95.5	95.1
US4	88.6	**94.5**	88.3	90.0	88.3	**93.9**	87.9	89.4
US5	83.1	80.7	83.1	82.8	83.7	80.3	83.7	83.0
US7	99.7	86.2	99.3	96.6	99.6	85.6	99.2	96.6
Y	89.3	84.1	89.0	89.0	89.4	84.5	89.0	89.4

The highest values are bold.

KU051676, KU051674 and KU051677, respectively. Maximum and minimum nucleotide sequence homologies within these four isolates were found between Ir- D7 and Ir- S31 by 99.7% and Ir-H60 and Ir-S31 by 86.2% identities, respectively. Evolutionary divergence calculated for CP amino acid ranged from 0.00 to 0.25 (data not shown).

DISCUSSION

BCMV has been previously reported from different bean growing areas of Iran demonstrating its importance and prevalence in pulse crops (Kaiser and Mossahebi, 1974; Shahraeen *et al*., 2005; Naderpour *et al*., 2010; Dizadji and Shahraeen, 2011; Musavi *et al*., 2014). Occurrence of BCMNV NL-3 and NL-5 strains (pathotype VI) and NL-8 (pathotype III) in Iran has been described by Naderpour *et al.* (2010) and Musavi *et al.* (2014) using differential host cultivars and/or strain-specific antibody reactions in biological and serological assays. Nucleotide sequence phylogeny of the 3′-terminal region (373 bp) of Iranian BCMV isolates showed the highest homology and relationship with Mexican isolates as described by Musavi *et al.* (2014), but so far no more studies have been carried out on the pathogenicity groups of BCMV in the country. Thus, the present work was focused on the identification of BCMV isolates from diverse geographical regions of Iran to determine the predominant BCMV pathogroups

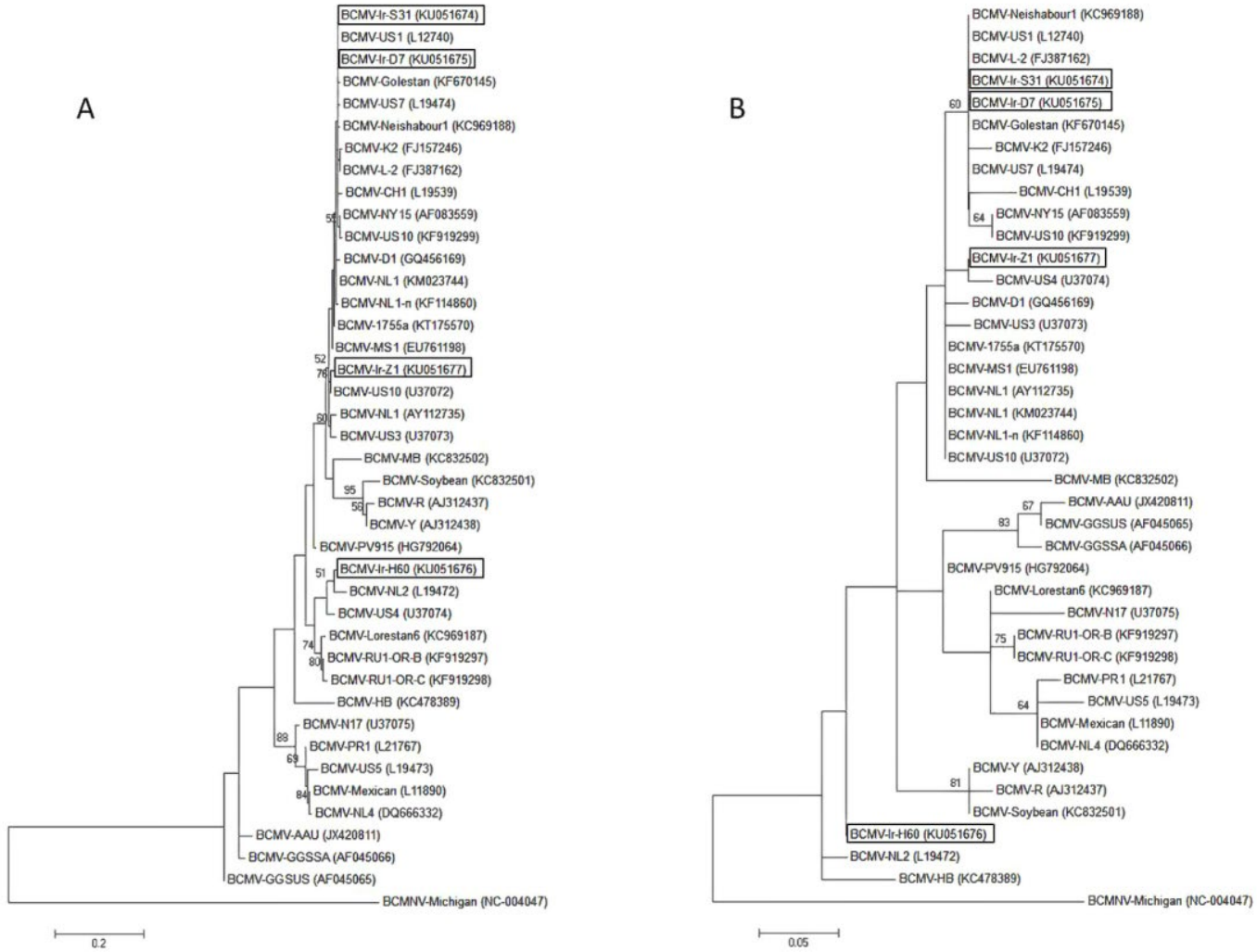

Fig. 3. Phylogenetic tree of the coat protein nucleotide (A) and deduced amino acid (B) sequences of 40 BCMV isolates by maximum likelihood method using MEGA6 with 1000 bootstrap replicates. Bootstrap values (>50%) are shown above the branch points. New Iranian BCMV isolates are shown in box.

and to evaluate phylogenetic relationship among the newly and previously reported BCMV isolates for breeding purposes.

BCMV was detected in symptomatic bean samples of seven provinces of Iran and the infection rate varied between 16.27 to 76.56%, while BCMV-free status of 1100 and 184 symptomatic samples from Alborz and Khuzestan provinces was unexpected. In the absence of random sampling of both symptomatic and asymptomatic plants and unequal number of collected symptomatic samples from each province, it is not possible to determine BCMV prevalence in provinces and infection rate among provinces is not comparable. Inoculation of selected BCMV isolates on test plants induced symptoms which were similar to previous studies (Drijfhout, 1978; Brunt *et al*., 1996), with an exception on *C. amaranticolor* which did not show any symptoms in this study.

BCMV isolates and strains were classified into seven (I-VII) and newly described VIII pathogenic groups on the basis of their interactions with differential bean genotypes (Drijfhout *et al*., 1978; Feng *et al*., 2015). In this work, inoculation of five isolates (selected randomly among infected samples from different geographic origins) on a set of 11 host groups revealed symptoms similar to those previously reported (Drijfhout, 1978; Spence and Walkey, 1995; Kelly, 1997) and surprisingly belonged to five PG-I, II, IV, V and VII which were new reports for Iran. The presence of different BCMV and BCMNV pathotypes in Iran, where susceptible bean genotypes are grown and no significant efforts are made for breeding purposes, is most noticeable. Mixed infection of bean infecting potyviruses can result in interspecific recombination, as a rich source of novel BCMV and BCMNV strains. BCMNV NL-3K, causing earlier and more severe symptoms on bean genotypes, is a stable and natural interspecific recombinant strain derived from BCMV and BCMNV (Larsen *et al*., 2005). BCMV RU1-OR strain is another example of recombination events between US-10 and an unknown potyvirus (Feng *et al*., 2014). Even though survey of mixed infection by other viruses was not included

in this work, continuous cultivation of susceptible bean genotypes would possibly result in the emergence of new variants.

Comparison between 3' terminus of four Ir-S31, Ir-H60, Ir-D7 and Ir-Z1 BCMV isolates genome with related sequences available in GenBank showed highest homology with US1 (L12740), US4 (U37074), US1 (L12740) and US10 (U37072), belonging to BCMV PG- I, IV, I and VII, respectively. This result reflects inconsistency between biological pathogroups and molecular grouping of BCMV isolates based on CP or 3'-terminal region. Although the 3'-terminal region shows the most variation in potyvirus genome, but are not able to distinguish pathogenically strains at molecular level. The evolutionary divergence determined for the CP amino acid sequence (0.00-0.25) indicates that CP sequence is unreliable to distinguish BCMV pathogroups. Lack of correlation between pathogenicity and the sequence of genomic 3'-end has been described before (Sharma *et al*., 2011).

Iran has great potential for cultivation of beans due to appropriate climate, agricultural development and consumer diets that all together encourage breeding programs of beans specifically for resistance to plant pathogens. Only a few attempts have been conducted in Iran so far to breed for traits other than for resistance to plant pathogens. Moreover, 7 out of 8 known pathotypes of BCMV and BCMNV are present in the country according to the results achieved in the present and previous (Naderpour *et al*., 2010; Musavi *et al*., 2014) studies. On the other hand both BCMV and BCMNV are seed transmitted up to 83% (Brunt *et al*., 1996) and are easily distributed via vectors from Aphididae in a more efficient non-persistent manner. Taken all together, breeding the present genotypes for virus resistance is most encouraged. Five bean genotypes, Akhtar, Jules, Derakhshan, Chiti karaj and Red karaj, which are cultivated commonly in Iran, were used to evaluate their reaction against three BCMV isolates collected from different geographical zones. It was found that the evaluated parameters (infection rate and accumulation level of the virus) were significantly affected only by bean genotype but not by BCMV isolate. Based on the results, Chiti karaj and Jules genotypes were characterized as highly sensitive and tolerant genotypes, respectively. Even though, the reaction patterns of all these five genotypes against pathotypes I, II, and IV assigned them to HG-I, but this statement still needs to be more documented. Since BCMV is a widespread seedborne virus, resistant bean cultivars are needed to prevent spread of this seedborne virus. A close relationship between seed color and resistance to BCMV is previously reported explaining that bean cultivars with red seeds are more susceptible whereas cultivars with purple seeds are resistant to BCMV (Steven *et al*., 1986). In accordance with those results, our results showed that genotype Jules, a Great Northern dry bean tolerant to common blight bacterium (*Xanthomonas phaseoli*) (Coyne and Schuster, 1970), with white seeds was more tolerant than genotypes with colored seeds such as Chiti karaj and Red karaj.

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