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BYMV ON CHICKPEA GERMPLASM IN BULGARIA

S. PETROVA[1], G. PASEV[2] and D. KOSTOVA[2]
[1]*Institute of Plant Genetic Resources, BG – 4202 Sadovo, Bulgaria*
[2] *Maritsa Vegetable Crops Research Institute, BG – 4002 Plovdiv, Bulgaria*

Abstract

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During May and June (2010 and 2011) virus symptoms were observed on significant part of chickpea germplasm grown in native plant nursery of the Institute of Plant Genetic Resources, Sadovo. The symptoms consisted of severe narrowing and twisting of the upper leaflets up to filiform leaves and redness of older leaves. *Bean yellow mosaic virus* (BYMV) was identified in all tested samples using test-plants, ELISA and RT-PCR. Infectious tests with BYMV isolate were carried out in order to evaluate the level of susceptibility of chickpea germplasm. Most of the tested accessions and varieties appeared to be virus susceptible. Few were selected as virus tolerant. Further investigations on progenies are necessary in order to confirm the genetic control of the observed level of resistance.

Key words: Cicer arietinum L., *Potyvirus*, ELISA, RT-PCR, virus susceptibility, virus tolerance

Introduction

Chickpea (*Cicer arietinum* L.) is an old and traditional crop for Bulgaria, utilized as fodder and food since 12 century (Atanassova and Mihov, 2009). Recently an increased interest to this crop is observed due to its versatile use and appreciation as a valuable food. The National Genebank, Sadovo maintains the chickpea collection, which includes mostly introduced accessions and limited number of local germplasm. The health status of the collection is of great importance. During May and June (2010 and 2011) virus like symptoms were observed on significant part of chickpea germplasm grown in native plant nursery. The symptoms consisted of severe narrowing and twisting of the upper leaflets up to filiform leaves and redness of older leaves.

The objective of this study was to identify and characterize the causal agent of the observed disease symptoms on chickpea based on host range, symptomatology, ELISA (Enzyme-Linked Immunosorbent Assay) and RT-PCR – Reverse Transcription Polymerase Chain Reaction.

Materials and Methods

Collection of virus samples

Survey of chickpea germplasm in Genebank field nursery was carried out during the period May to June, 2010 and 2011. Six symptomatic leaf samples were collected and described properly as origin and symptoms. The samples were preserved in cool bag before reaching the laboratory. The long-term preservation of collected samples was at minus 86°C.

Inoculation and host range

The virus was recovered on chickpea plants in insect-proof growth chamber with controlled conditions, at temperature 22°-26°C and 14/10 h day/night. Each plant sample was ground in buffer with 1 $g.kg^{-1} K_2HPO_4$ and 0.1 $g.kg^{-1}$ Na_2SO_3. Carborundum 600 mesh was added as abrasive. For biological characterization of the viral agent the following indicator plants and hosts were used: broad bean (*Vicia faba*), peanut (*Arachis hypogea*), pea (*Pisum sativum*), bean (*Phaseolus vulgaris*), lentil (*Lens esculenta*), white lupin (*Lupinus album*), goosefoot (*Chenopodium amaranticolor)*, tobacco (*Nicotiana tabacum)* cv. Plovdiv. The chickpea and test plants were inoculated mechanically. Symptoms were assessed periodically as local – in the place of inoculation up to 4 to 5 days post inoculation (dpi) and as systemic - on upper leaves, usually 10 to 15 dpi.

Serological test

The samples were analyzed serologically using DAS ELISA (Double Antibody Enzyme-Linked Immunosorbent As-

E-mail: soniapetrova123@abv.bg

say) following the standard procedure of Clark and Adams (1977). The samples were analyzed for the most spread viruses on *Fabaceae – Potyvirus*-group*, Bean yellow mosaic virus* (BYMV), *Bean common mosaic virus* (BCMV), *Clover yellow vein virus* (ClYVV), *Alfalfa mosaic virus* (AMV), *Cucumber mosaic virus* (CMV). The sources of the used polyclonal antisera (As) were as followed: BYMV 0717, ClYVV 0243, *Potyviruses* 0573, AMV 0779 and CMV 0929 from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), Germany; BYMV A287 from Institute of Plant Virology (IPV), Torino, Italy. DSMZ As and antirabbit alkaline phosphatase conjugates were diluted 1:1000, whereas IPV As and conjugates were diluted 1:500. Samples were diluted 1:20. Indirect ELISA was used for *Potyviruses* (Koenig, 1981).

RNA (Ribonucleic Acid) isolation and RT-PCR (Reverse Transcription Polymerase Chain Reaction) analysis

The identified by ELISA BYMV isolate was propagated on chickpea and pea plants for further characterization through RT-PCR analysis and for utilization as infectious inoculum in screening tests.

Total RNA was extracted from pea plants infected with chickpea BYMV isolate using Plant RNA Gene-Jet extraction kit (Thermo Scientific Inc.) according to supplier's instructions. Total RNAs for positive controls (BYMV and ClYVV) were prepared from infected bean plants. Identification of the chickpea isolate was conducted by two-step RT-PCR. First, cDNA was produced using 0,2 µg of total RNA with First strand cDNA synthesis kit (Thermo Scientific Inc.). Further, amplification was carried out with primers specific to BYMV and ClYVV. For this purpose BYMV primers were designed targeting part of the coat protein gene (BYM-8970 -F, 5'-GTGAATGGACAATGATGAATG-3', BYM-9505-R 5'-TACCCTGTCAGAGTAGAGAG-3') using 17 accessions from NCBI database (KC011006.1, JX173278.1, JX156423.1, NC_003492.1, AB079888.1, U47033.1, AB439731.1, AB439729.1, AB439730.1, FJ492961.1, AM884180.1, AB079886.1, AB079782.1, AB097090.1, D83749.1, X53684.1, X81124.1) and primers targeting part of the coat protein gene of ClYVV (ClYVV-9009F 5'-GAGTGGACAATGATGGATGGAGA-3', CLYVV-9457Rev 5'-CTCCAGCAATGTGATGCATGTT-3') based on 9 accessions (NC_003536.1, EF591473.1, EU860366.1, EU860364.1, S77521.1, AY169801.1, AF203536.1, AF185959.1 и AY169721.1.) from the NCBI database. In addition another two primer pairs were utilized in this study: S1, 5'- GCCTTATGGTGTGGTGCATAG-3'/S2, 5'-CAAGCATGGTGTGCATATCACG-3' for BYMV (Duraisamy et al., 2008) and ClYVV-F, 5'- TTGATGACAGCCAGATG-3′/ClYVV-R, 5'- GAATCGTGCTCCAGCAATG-3′ for ClYVV (Larsen et al., 2008).

PCR reactions were performed in 25 µl volume containing 1x PCR buffer, 1,5 mM MgCl2, 0,4 mM dNTPs, 0,3 µM Primers and 0,2 U BioTaq polymerase (Bioline) and 1 µl cDNA.

PCR conditions: Thermal profile consisted of initial denaturation step at 95°C for 5 min, followed by 35 cycles of 94°C for 30 s annealing step of 50°C (ClYVV-9009F/ CLYVV-9457Rev), 57°C (S1/S2), 61°C (ClYVV-F/ ClYVV-R), 62°C (BYM-8970-F/ BYM-9505-R) for 30 s, 72°C for 1 min and a final extension at 72°C for 10 min.

Analyses of PCR products on agarose gel: 5 µl PCR products were separated on 1 $g.kg^{-1}$ TAE agarose gel for 45 min at 70 V. Fragments were visualized by Serva DNA stain G under UV light.

Preliminary evaluation the level of BYMV susceptibility in chickpea-germplasm

Twenty nine chickpea accessions and varieties were tested in order to investigate the level of their susceptibility resp. resistance towards the identified BYMV isolate. The plants were inoculated with the virus inoculum mechanically at stage of 4 true leaves. Symptoms were assessed periodically as primary and secondary.

Results

Symptoms on test-plants – The collected samples were tested on a number of test plants, presented at Table 1. Most of the tested species reacted as susceptible following inoculation with the viral isolate. Usually no local reaction was observed on inoculated leaves except the diffuse chlorotic spots on *P. vulgaris* plants and small necrotic spots in *L. album,* which appeared after the development of systemic symptoms. The systemic spread of the virus was expressed usually as slight to severe yellow mosaic or mottling. The necrotic reaction on inoculated leaves in *L. album* was observed also as systemic, progressing to top necrosis and death of the plants. The local reaction observed on *Ch. amaranticolor* without systemic invasion of the virus is usually connected with the presence of CMV or *Potyvirus*.

ELISA results – *Bean yellow mosaic virus* (BYMV) was identified in all tested leaves samples by DAS ELISA using polyclonal As of DSMZ and by indirect ELISA with IPV As (Table 2). Positive reaction was observed also with polyclonal As for *Potyviruses*, which supported the previous result. The optical densities (ODs) for positive samples were more than three times over the healthy control. No other viruses were detected in the tested samples.

RT-PCR results - Total RNA extracted from BYMV infected pea and bean plants was analyzed by two-step RT-PCR.

Table 1
Host range of narrow leaflets viral isolate

Number	Test plant	Local symptoms	Systemic symptoms
1	Broad bean (*Vicia faba*)	No local reaction	Slight mottling on upper leaves, stunted growth, epinasty on upper leaves
2	Peanut (*Arachis hypogea*)	No local reaction	Slight mosaic on upper leaves, stunted growth
3	Pea (*Pisum sativum*)	No local reaction	Strong yellow mosaic
4	Bean (*Phaseolus vulgaris*)	Diffuse chlorotic spots	Severe light green mosaic; necrosis on main veins
5	Lentil (*Lens esculenta*)	No local reaction	Slight mottling as yellow stripes along the main veins
6	Lupin (*Lupinus album*)	Small necrotic spots following the systemic symptoms	Wilting, top necrosis, death
7	*Chenopodium amaranticolor*	Diffuse chlorotic spots	No systemic reaction
8	*Nicotiana tabacum* cv. Plovdiv.	No local reaction	No systemic reaction

Table 2
DAS and Indirect ELISA results for 5 viruses

Isolates	ELISA OD at 405 nm					
	Polyclonal antisera to					
	BYMV DSMZ	BYMV IPV	ClYVV	AMV	Poty virus	CMV
Sample 1506/10	**0.691***	**0.569**	0.145	0.174	**2.019**	0.127
Sample 0909/10	**0.393**	**0.272**	0.174	0.165	**1.929**	0.136
Sample 0710/10	**0.449**	**0.329**	0.150	0.165	**3.956**	0.124
Sample 1611/11	**0.366**	**0.310**	0.170	0.144	**2.200**	0.122
Sample1602/11	**0.705**	**0.428**	0.143	0.121	**1.559**	0.126
Sample 1006/11	**0.526**	**0.584**	0.154	0.140	**1.345**	0.152
Positive control	0.596	0.476	0.804	0.546	2.404	0.552
Negative (Healthy) control	0.123	0.150	0.154	0.114	0.088	0.121

*Mean value of three repetitions

Amplification reactions performed with primers, designed for identification of BYMV, produced a single band with size of about 500 bp (S1/S2) for chickpea isolate as well as for BYMV-control isolate (Figure 1B). Primers BYM8970F/BYM9505R produced a stronger signal of 535 bp fragment only for chickpea isolate and very slight one for BYMV positive control as well as slight one for ClYVV negative control (Figure 1A). The primer pair BYMV-F/BYMV-R (Larsen et al., 2008) did not amplified the product with the expected size (1113 bp). In this case, multiple fragments appeared on the gel after electrophoresis.

The primer pairs (ClYVV-F/ClYVV-R and ClYVV-9009F/CLYVV-9457Rev), used for detection of ClYVV, revealed single bands of expected size (835bp and 450bp) only for ClYVV-control isolate (Figure 2). No signals were observed with ClYVV primers for BYMV-control isolate and chickpea isolate.

Preliminary evaluation the level of BYMV susceptibility in 29 chickpea cultivars and accessions - Most of the tested accessions did not develop primary symptoms on the place of inoculation with BYMV. The first systemic symptom in susceptible genotypes consisted in wilting of upper branches followed by mottling, yellowing and partial necrosis on the leaflets of compound leaves. The infection progressed rapidly leading to withering of the whole plant. This is so called acute stage of the disease. Later the plant passed in chronicle stage – branches appeared from axillary buds with severe leaf narrowing and deformation as filiform leaves.

In several cultivars and accessions most of the plants, after the development of initial symptoms for viral infection, recovered and later grew normally with well formed branches and leaves without deformations (Balkan, Myles, Progress, BGR 23150, BGR 23151, BGR 23152). As a pre-

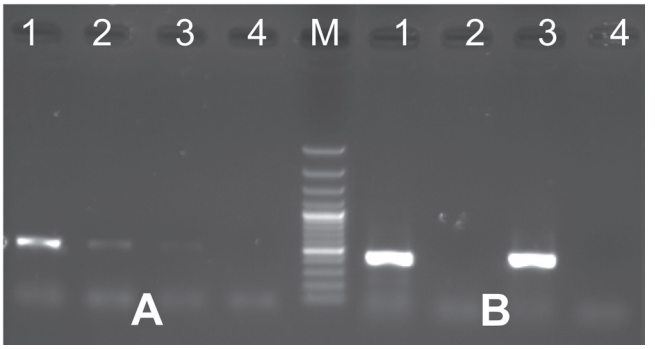

Fig. 1. Agarose gel displaying RT-PCR products amplified by BYM8970F/BYM9505R
(A) and S1/S2 (B) primer pairs for detection of BYMV. M- 100 bp Marker, 1-BYMV chickpea isolate, 2-ClYVV isolate from *P. vulgaris*, 3-BYMV isolate from *P. vulgaris*, 4 –healthy plant

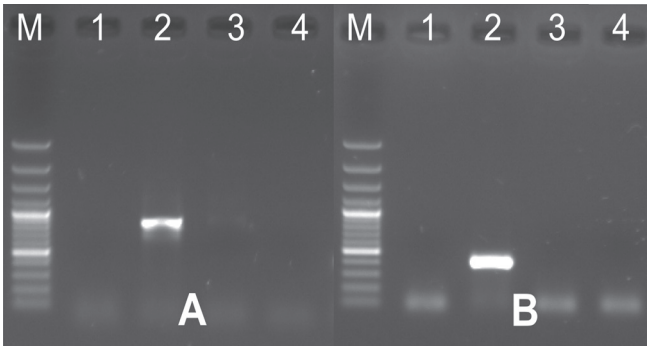

Fig. 2. Agarose gel displaying RT-PCR products amplified by ClYVV-F/ClYVV-R
(A), ClYVV-9009F/CLYVV-9457Rev (B) primer pairs for detection of ClYVV. M- 100 bp Marker, 1 - BYMV chickpea isolate, 2 - ClYVV isolate from *P. vulgaris*, 3 - BYMV isolate from *P. vulgaris*, 4 – healthy plant

liminary study, it could be concluded the existence of level of resistance or tolerance towards BYMV in these materials.

Discussion

BYMV, identified in collected chickpea samples, is generally common and wide spread virus in Bulgaria. Kovachevski (1972), announced it. According to this author, the observed BYMV symptoms on chickpea consisted of chlorotic or yellow leaves, sometimes with vein necrosis, causing their abscission. Other authors observed the described in this study symptom on *Cicer arietinium* L. as filiform leaves also. Kaiser et al. (1988) announced for a potyvirus serologically closed to BYMV, *Blackeye cowpea mosaic virus* (BlCMV) and *Cowpea aphidborne mosaic virus* (CAMV). The isolate was designated as *Chickpea filiform virus* (CFV) due to severe leaflet reduction and the very restricted host range. Yahia et al. (1997) and Singh et al., (2006) identified seed-borne BYMV in the severe narrow leaves of chickpea varieties in Algeria and in sub-tropical zone of Himachal Pradesh province. Kaiser and Danesh (1971) reported BYMV in chickpea in Iran, causing severe stunting, wilting and yield reduction. Similar symptoms consisting of mosaic, stunting, yellowing, wilting, shortening of internodes, and phloem discoloration were observed in chickpea (*Cicer arietinum*) grown in southern Bolivia caused by a Potyvirus named *Chickpea yellow mosaic virus*. (Larsen et al., 2003).

The presence of potyvirus in tested samples was first detected by polyclonal As for *Potyvirus* used in Indirect ELISA. In order to specify and differentiate this potyvirus three As were used in DAS ELISA – BYMV DSMZ, BYMV IPV and ClYVV DSMZ. The positive results with the first two As and the negative with the third As proved the existence of BYMV in chickpea samples. Until recently, ClYVV was considered as a strain of BYMV. Barnett et al. (1987), Uyeda et al. (1991), Tracy et al., (1992), and Sasaya et al. (1998), based on serological and molecular investigations, proved that the both viruses are two distinct species.

Four primer pairs from total five, used in RT-PCR, confirmed the presence of BYMV in tested chickpea samples. Definite results were obtained with S1/S2 primer pair for BYMV and ClYVV-F/ClYVV-R and ClYVV-9009F/CLYVV-9457Rev primer pairs for ClYVV. During the optimization process of amplification reaction for BYM8970F/BYM9505R pair a non-specificity was found regarding the discrimination of BYMV and ClYVV. Lower annealing temperature like 51°C was able to produce signals with equal intensity for both viruses, while raising the temperature up to 61°C produced strong signal only for chickpea isolate and very slight one for BYMV- and ClYVV-control isolates. It could be assumed that the used primer pair was able to differentiate the BYMV-isolates according to the annealing temperature.

The results from screening tests with chickpea accessions showed the high level of susceptibility to BYMV in most of the tested materials. Further investigations are necessary on chickpea materials, selected as BYMV tolerant, in order to confirm the observed level of resistance in the progenies.

Conclusions

BYMV was identified as causal agent of disease symptoms on chickpea germ-plasm. BYMV appeared to be one

of the most spread and dangerous virus on chickpea crop. The importance is increased also by the observed in this study high level of susceptibility in tested lines, cultivars and accessions. Special attention should be paid on virus tolerance observed in several varieties and accessions in respect with breeding for virus resistance.

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