INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
ISSN Print: 1560–8530; ISSN Online: 1814–9596
17–0762/2018/20–6–1353–1358
DOI: 10.17957/IJAB/15.0641
http://www.fspublishers.org

Full Length Article

Biological, Serological and Molecular Characterization of an Isolate of *Red Clover Vein Mosaic Carlavirus* Infecting Alfalfa, other Field Crops and Weeds in Saudi Arabia

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Abstract

Two surveys were performed in the important alfalfa-producing regions in Saudi Arabia during (2012-2014) for detection of viruses suspected of inciting wide spread symptoms in alfalfa using DAS-ELISA and RT-PCR. *Red clover vein mosaic virus* (RCVMV) was among the viruses detected in the five visited regions. It contributed to average infection rates of 1.4% in the first survey (2012-2013) and 18.2% in the second survey (2014). Representative samples from the surveyed regions that tested positive by ELISA, also tested positive by RT-PCR confirming occurrence of this virus. RCVMV was also detected in *Solanum tuberosum, Vicia faba, Sonchus oleraceous* and *Chenopodium* spp. growing adjacent to alfalfa fields for the first time in Saudi Arabia. Sequencing of purified RT-PCR products indicated a close phylogenetic relationship among the Saudi isolates of RCVMV (99-100%) and a distant relationship with isolates deposited in the GenBank. Five of the plant species tested in the host range study were found positive to RCVMV infection and produced mild symptoms. To our knowledge this is the first report of cowpea [*Vigna unguiculata* (L.) Walp.] and black-eyed pea [*V. unguiculata* subspecies *unguiculata* (L.) Walp.] as hosts for RCVMV and can be used for its propagation. © 2018 Friends Science Publishers

Keywords: Alfalfa; DAS-ELISA; RCVMV; RT-PCR; Saudi Arabia; Weeds

Introduction

In the Kingdom of Saudi Arabia (KSA), alfalfa ranks first among all cultivated fodder crops as it is occupying about 126611 hectares out of the total area under fodder crops (195605 hectares) (Anonymous, 2014 2014). Recent surveys have shown wide spread occurrence of viruses of legumes including alfalfa (Guy *et al.*, 2013; Guy, 2014). More than 23 virus species have been found to infect legumes including alfalfa in New Zealand (Fletcher, 1993; Pearson *et al.*, 2006; Denny and Guy, 2009; Guy, 2014; Fletcher *et al.*, 2016). Recently, different striking virus-like symptoms were observed spreading on alfalfa all over Saudi Arabia. In a preliminary study carried out by Al-Shahwan *et al.* (2014), several plant viruses were reported to infect alfalfa crop in the most productive regions in Saudi Arabia. *Red clover vein mosaic virus* (RCVMV) and *Lucerne transient streak virus* (LTSV) were among the detected viruses (AL-Shahwan *et al.*, 2016b; Raza *et al.*, 2017). RCVMV was first reported in red clover in the US in 1937 but was later reported in different legumes in other countries (Varma *et al.*, 1970; Bos *et al.*, 1972; Sherwood, 1997; Larsen and Miklas, 2000; Freeman, 2008; Fletcher *et al.*, 2016). RCVMV belongs to the genus *carlavirus*, family *Betaflexiviridae* (Adams *et al.*, 2004, 2012). Although infection with RCVMV usually symptomless or induces mild symptoms, the virus, was reported to cause economic losses of up to 88% of the grain weight of pea (Khan and Singh, 1997a) and up to 100% in chickpea yield in the US (Larsen and Miklas, 2001). Although its effect on alfalfa is not yet known but the occurrence of its aphid vectors and other susceptible leguminous plant species and weeds in KSA are among the factors that should increase alert for the threat expected from this virus. Since the first report of RCVMV in KSA (Al-Shahwan *et al.*, 2016a), no additional studies have been performed. Therefore, the objectives of this research were (a) To conduct a survey in the five principal alfalfa-producing regions in KSA (Riyadh, Qassim, Hail, Tabuk and Jouf) to determine occurrence of RCVMV and its spread in these regions (b) Biological, serological and molecular characterization of RCVMV isolates in the visited regions along with determination of their phylogenetic relationships among themselves as well as among isolates reported in other countries.

Materials and Methods

Sample Collection and ELISA Test

Two surveys were performed in the five major regions for

To cite this paper: AL-Shahwan, I.M., T. Farooq, O.A. Abdalla, M.A. AL-Saleh and M.A. Amer, 2018. Biological, serological and molecular characterization of an isolate of *Red Clover Vein Mosaic Carlavirus* infecting alfalfa, other field crops and weeds in Saudi Arabia. *Int. J. Agric. Biol.*, 20: 1353–1358

alfalfa production in Saudi Arabia, namely, Riyadh, Qassim, Hail, Tabuk and Jouf (Fig. 1) during 2012-2014 for detection of viruses infecting alfalfa. A total of 1474 samples were collected from alfalfa plants showing virus symptoms. The samples were tested for occurrence of RCVMV by DAS-ELISA using kits provided by AC-Diagnostic Inc. (USA). Absorbance values were recorded at 405 nm. The three most alfalfa producing regions (Riyadh, Qassim, Hail) were visited in the second survey and 308 alfalfa samples were collected and tested using the same technique for comparison.

Host Range Test

The tested plants in the host range study included *Medicago sativa* (Hasawi cultivar), *Chenopodium amaranticolor, C. quinoa, Gomphrena globosa, Pisums ativum, Trifolium alexandrinum,* two cultivars of *Vigna unguiculata, Phaseolus spp., Vicia faba, V. sativa, Solanum nigrum, S. melongena, Nicotiana tabaccum, N. glutinosa, N. occidentalis, and N. Benthamiana* which were mechanically inoculated as described by Hill, 1984, using a pure RCVMV isolate identified by Al-Shahwan *et al.* (2014).

RT-PCR, Sequencing and Sequence Analysis

A set of primer pairs was designed for identification and amplification of RCVMV (accession number NC_012210.1) based on the coat protein gene of the virus (RCVMVCP-F & R), in order to confirm the presence of RCVMV in the ELISA-positive samples. The forward primer was RCVMVCP-F 5'-ATAGAGATGTCAGAAACCGC-3', and reverse primer pair was RCVMVCP-R 3'-TAGGTATTAAGCCACGCAAA- 5', respectively. RT-PCR as a sensitive and specific method for nucleic acid based detection of plant viruses (Uehara-Ichiki *et al*., 2013) was used to confirm the ELISA results. The expected size of the amplified bands was 932 bp. Both primers were used following similar RT-PCR cycles with minor changes in the annealing temperature.

Six alfalfa samples, two from each of Qassim and Hail and one from each of Riyadh and Tabuk that showed high absorbance values in ELISA were designated (7Q, 21Q, 22H, 28H, GH3, & 41R) respectively, and selected for RT-PCR analysis. Total RNA was extracted using The SV total RNA Isolation System from Promega Corporation (2800 Woods Hollow Road, Madison, WI 53711-5399 USA, www.promega.com). The MyTaq^{TM}One-Step RT-PCR kit from Bioline Ltd, United Kingdom, www.bioline.com) was used for the RT-PCR assay. The Advance-Primus 96 machine (PEQ LAB 3411 Silverside Road, Weldin Building, Wilmington, DE 19810, USA) was used for PCR amplification.

The reverse transcription started with a cycle of 30 min at 45°C. The amplification program started with an initial denaturation step at 95°C for 1 min, followed by 35 cycles at 95°C for 30 sec, 50.6°C for 30 sec, 72°C for 1 min, and a final extension at 72°C for 5 min.

The electrophoresis method described by Sambrook and Russel (2001) was followed to visualize the RT-PCR products on 1% agarose gel with simultaneous run of Gene Direx 1Kb DNA marker. Positive results were documented using Gel Documentation System (syngene Bio Imaging-Ingenius: Beacon House Nuffield Road, Cambridge, United Kingdom). The specific 932 bp bands visualized on the 1% agarose gel were purified using the AxyPrep DNA Gel Extraction Kit from Axygen Biosciences (33210 Central Avenue, Union City, CA 94587, USA). To increase DNA concentration, re-PCR was conducted using the purified RT-PCR product as template. KAPA2G Ready Mix Kit (KAPA Biosystems, USA) was used for PCR amplification.

The PCR conditions were 2 min cycle at 95^{o}C for initial denaturation, 35 cycles at 95^{o}C for 30 sec for denaturation, 35 cycles at 50.6^{o}C for 30 sec for annealing, 35 cycles at 72^{o}C for 1 min for elongation and a 5 min cycle at 72^{o}C for final elongation. Re-PCR products along with specific primers were sent to the Advanced Genetic Technology Center, College of Agriculture Sciences, Plant Sciences Department, University of Kentucky, USA for bidirectional sequencing of PCR products. BLASTN and BLASTX programs were used to search the homologous DNA and protein sequences respectively. CODONCODE software trial version 6.0.2 (http://www.codoncode.com) was used to align and clean the obtained sequences. These sequences were compared with each other and with published coat protein sequences available at the national center for biotechnology information (NCBI).

Homology Tree and Identity Percentages for RCVMV Isolates

The software DNAMAN trial version 7.0 (Lynnon Biosoft., San Ramon, CA 94583, USA, http://www.lynnon.com) was used to Check the identity percentage between the obtained sequences. Three sequences of RCVMV isolates retrieved from GenBank database, two of which from Washington and one from New Zealand (Accession numbers: NC_012210.1 & FJ685618.1, KR108251.1) respectively, were aligned with sequences of 10 Saudi isolates of RCVMV sequenced at USA to check the percent of similarity among them.

Results

Sample Collection and ELISA Test

Different virus symptoms such as mottling, chlorosis and mosaic were observed on alfalfa plants from which samples were collected. RCVMV was detected in 14 out of the 1166 alfalfa samples collected from the five major alfalfa-producing regions during the first survey. Its percent of detection varied between 0.6–3.0% among the surveyed regions with an average of 1.2% (Table 1).

Table 1: Number of samples and the percentages of infection with *Red clover vein mosaic virus* (RCVMV) in the major alfalfa-producing regions during the surveys

Region	Number of collected Samples		Number of infected samples		Infection Percentages	
	2012-13	2014	2012-13	2014	2012-13	2014
Riyadh	466	185	4	24	0.9	12.9
Qassim	189	61	2	19	1.1	31.1
Hail	164	62	5	13	3.0	20.9
Jouf	169	-	2	-	1.2	-
Tabuk	178	-	1	-	0.6	-
Total	1166	308	14	56	1.2%	18.2%

- Region was not visited during 2014 survey

Fig. 1: Map of Saudi Arabia showing the five surveyed regions (Riyadh, Qassim, Hail, Jouf, and Tabuk)

In the 2014 survey, 56 out of the 308 alfalfa samples collected from the three most important alfalfa-producing regions visited (Riyadh, Qassim, Hail), were positive to RCVMV, accounting for 18.2% compared to 1.2% in these three same regions from which 819 samples were collected during 2012/13. This shows that the percent of virus detection was higher in the three regions surveyed during 2014 compared to 2012–2013 indicating increase in the infection rate of this virus in these regions. RCVMV was also detected by ELISA in samples collected from symptomatic *S. tuberosum* and *V. faba* plants growing adjacent to alfalfa fields in Hail and Jouf respectively. The virus was also detected in weeds such as *S. oleraceous* in Jouf and Hail and in *Chenopodium* spp. in Jouf which were also found growing within and adjacent to alfalfa fields.

Host Range of RCVMV

Five out of the tested 17 plant species used in the host range experiment showed symptoms four weeks post inoculation with RCVMV. These were alfalfa, which showed mottling for a very brief period of time and disappeared later on, cowpea and black eye pea which showed leaf malformation, small leaves and mottling, *N. occidentalis* and *G. globosa* which showed mosaic symptoms (Fig. 2).

The symptoms that appeared on the inoculated plants were confirmed to be due to RCVMV through back inoculation and RT-PCR using specific primers of the virus. All other tested plants didn't show any symptoms. Response of the plant species tested in the host range study within four weeks after inoculation with RCVMV are shown in Table 2.

RT-PCR, Sequencing and Sequence Analysis

RT-PCR products of the six selected representative samples from the surveyed regions, indicated formation of bands of equivalent sizes of 932 bp on ethidium bromide stained 1% agarose gel (Fig. 3) confirming the presence of RCVMV in these samples. No amplification was detected in samples collected from plants that did not show symptoms.

When sequences of ten of the Saudi isolates of RCVMV that were sequences at USA were aligned with sequences of three isolates of RCVMV, two of which were reported from Washington and one from New Zealand (NC-012210.1, FJ-685618.1 & KR-108251.1 respectively), high percent of similarity was observed among the Saudi isolates of RCVMV (99–100%), however low percentage of similarity (35%) was observed among the Saudi isolates and those from Washington and New Zealand (Fig. 4). When these sequences were blasted against NCBI GenBank database using blastx software, they hit mainly proteins like DNA/RNA polyprotein and *gypsy*/Ty3 protein (accession numbers XP 007049837.1 & XP 007028165.1 respectively).

Discussion

Appearance of peculiar wide spread symptoms on alfalfa in different regions in Saudi Arabia encouraged conduction of this study. Detection of RCVMV in some symptomatic samples and lack of its detection in others suggests occurrence of other viruses infecting that crop besides RCVMV. This was confirmed in a separate recent study (Al-Shahwan *et al.*, 2016a). The two conducted surveys did not only indicate occurrence of RCVMV in Saudi Arabia but they also indicated the gradual increase in the rate of infection of alfalfa with this virus, starting with 1.4% during 2012/13 and reaching 18.2% in 2014 for Riyadh, Qassim and Hail which were the three regions visited in the two surveys. The increase in the infection rate of RCVMV during 2014 compared to 2012–2013 could probably be attributed to factors such as the activity of the aphid vectors that were reported to transmit this virus in a non-

Table 2: Response of the plant species used in the host range test four weeks after mechanical inoculation with the RCVMV isolate under greenhouse conditions and their results by DAS-ELISA and RT-PCR

Plant Species	Symptoms*	Results for RCVMV with DAS-ELISA and RT-PCR
Medicago sativa var Hassawi	Mt	+
Trifolium alexandrium	-	-
Solanum melongena	-	-
Solanum nigrum	-	-
Chenopodium amaranticolor	-	-
Chenopodium quinoa	-	-
Gomphrena globosa	Mo	+
Nicotiana tabacum	-	-
Nicotiana benthamiana	-	-
Nicotiana occidentalis	Mo	+
Nicotiana glutinosa	-	-
Datura stramonium	-	-
Vigna unguiculata (Cowpea)	LM	+
Vigna unguiculata (Black Eye Pea)	Mt	+
Phaseolus spp.	-	-
Pisumsativum	-	-
Vicia sativa	-	-

*: At least four plants of each species were mechanically inoculated
**: Mo = Mosaic
SL = Small Leaves
Mt = Mottle
LM = Leaf Malformation
- = No Reaction

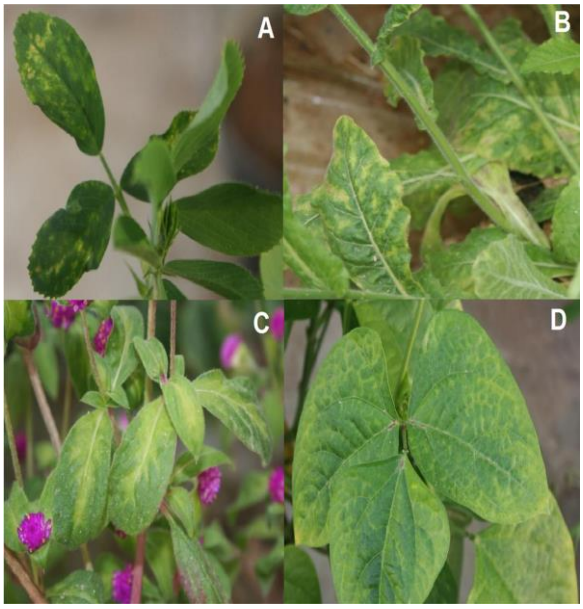

Fig. 2: Symptoms of RCVMV on some of the plant species that were mechanically inoculated with virus in the host range test under greenhouse conditions. A) Mottling on alfalfa. B) Mosaic symptoms on *N. occidentalis*. C) Mosaic on *G. globosa* leaves and D) Mottling on black eye pea leaves

persistent manner (Graves and Hagedorn, 1956; Freeman, 2007) and also to transmission of this virus through seeds (Freeman, 2007). Other field crops and weeds that were also

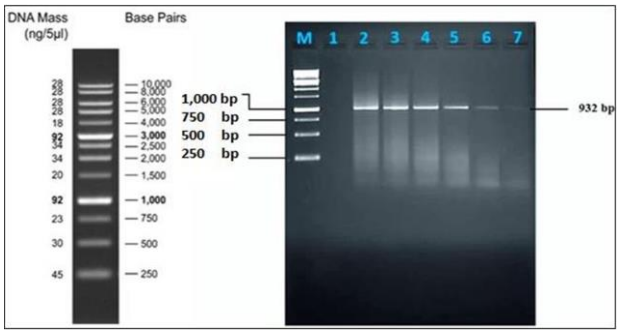

Fig. 3: Electrophoresis in 1% agarose Gel showing products of RT-PCR amplified from six alfalfa samples using specific primer pair designed to amplify 932 bp fragment of coat protein gene of RCVMV. Representation of each well is written above the well. 1 = Negative control. 2, 3, 4, 5, 6, & 7 represent GH3, 41R, 7Q, 21Q, 22H, & 28H respectively. Lane M represents Gene Direx 1 Kb DNA marker whose picture is pasted on the left side

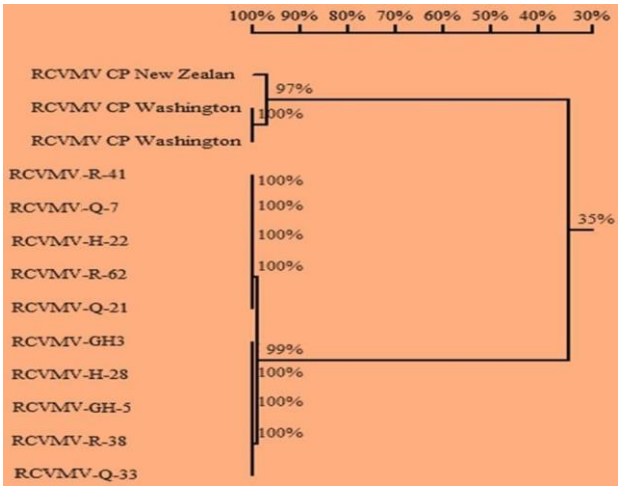

Fig. 4: The homology tree constructed based on multiple sequence alignment of the coat protein gene of Saudi isolates and three other isolates of RCVMV obtained from GenBank

found infected with RCVMV such as *V. faba*, *S. tuberosum*, *Sonchus oleraceous* and *Chenopodium* spp. were probably not only enhancing persistence of this virus but may also be playing a role in its epidemiology.

Although the actual effect of this virus on alfalfa has not been studied thoroughly yet it could probably pose threat for that crop since significant growth reduction and yield losses were reported on its infection to other crops such as chickpea in the United States (Larsen and Miklas, 2001) and pea in India (Khan and Singh, 1997a, b) on which significant losses were reported. This is in spite of the mild symptoms or symptomless infection induced with this virus as observed in the host range experiment conducted in this study which sometimes does

not show except in the cases of mixed infection with other viruses (Alconero *et al.*, 1986; Sherwood, 1997; Fletcher *et al.*, 2016).

The two *V. unguiculata* cultivars, cowpea and black eye pea among the five plant species that were infected with RCVMV, were reported for the first time as hosts for this virus and can be used as propagative hosts for it. Also the host range experiment results indicated differences between the Saudi isolate of this virus and a New Zealand isolate (Fletcher *et al.*, 2016) that both isolates shared infection of only one plant species (*N. occidentalis*), which even showed different symptoms for each isolate, out of 12 plant species inoculated with each of these isolates, separately. Interestingly, four out of the other 11 species mentioned in table 2, were infected with the Saudi isolate but not infected with the New Zealand isolate, two were vice versa and five plant species were neither infected with any of the two isolates. Since sparing and sometimes latent symptoms accompanied infection of the host range plants of this virus, these plants were also tested with PCR to confirm their observed responses following their inoculation with this virus. The 932 bp PCR product observed on the 1% agarose gel stained with ethidium bromide confirmed infection of the field plants collected during the survey and the symptomatic host range plants that were inoculated with the Saudi isolates of RCVMV. Despite the close phylogenetic relationship observed between Saudi isolates of RCVMV they were found to have distant relationships with the Washington and New Zealand isolates. The biological and molecular discrepancies observed in the host range and in the distant phylogenetic relationships between the Saudi isolate and the New Zealand isolate which is closely related to other isolates of RCVMV such as Washington isolate may suggest that the RCVMV isolates detected in Saudi Arabia are probably of unique characteristics. Such deviating RCVMV isolates have also been reported earlier (Bos *et al.*, 1972). Conclusive evidence for these speculations would probably require further investigations.

Conclusion

Red clover vein mosaic virus (RCVMV) was detected for the first time in alfalfa in Saudi Arabia by ELISA and RT-PCR. Its rate of infection was found to be appreciably increasing between the two surveys. Besides alfalfa, the virus was also found for the first time to naturally infect *S. tuberosum, V. faba, S. oleraceous* and *Chenopodium* spp. growing adjacent to alfalfa fields. Cowpea, [*Vigna unguiculata* (L.) Walp.] and black-eyed pea, [*V. unguiculata* subspecies *unguiculata* (L.) Walp.] are reported for the first time in this study as hosts for RCVMV. The phylogenetic relationships among Saudi isolates of RCVMV were found to be closer than their relationships with isolates deposited in the GenBank based on their nucleotide sequences analysis.

Acknowledgements

The authors would like to express their thanks and gratitude for The Agriculture Research Center at The College of Food and Agriculture Science, King Saud University for financing this research.

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(Received 13 July 2017; Accepted 13 November 2017)