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ORIGINAL ARTICLE

Detection of new viruses in alfalfa, weeds and cultivated plants growing adjacent to alfalfa fields in Saudi Arabia

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Abstract A total of 1368 symptomatic plant samples showing different virus-like symptoms such as mottling, chlorosis, mosaic, yellow mosaic, vein clearing and stunting were collected from alfalfa, weed and cultivated plant species growing in vicinity of alfalfa fields in five principal regions of alfalfa production in Saudi Arabia. DAS-ELISA test indicated occurrence of 11 different viruses in these samples, 10 of which were detected for the first time in Saudi Arabia. Eighty percent of the alfalfa samples and 97.5% of the weed and cultivated plants samples were found to be infected with one or more of these viruses. Nine weed plant species were found to harbor these viruses namely, *Sonchus oleraceus, Chenopodium* spp., *Hibiscus* spp., *Cichorium intybus*, *Convolvulus arvensis*, *Malva parviflora*, *Rubus fruticosus*, *Hippuris vulgaris*, and *Flaveria trinervia*. These viruses were also detected in seven cultivated crop plants growing adjacent to the alfalfa fields including *Vigna unguiculata*, *Solanum tuberosum*, *Solanum melongena*, *Phaseolus vulgaris*, *Cucurbita maxima*, *Capsicum annuum*, and *Vicia faba*. The newly reported viruses together with their respective percent of detection in alfalfa, and in both weeds and cultivated crop plant species together were as follows: Bean leaf roll virus (BLRV) {12.5 and 4.5%}, Lucerne transient streak virus (LTSV) {2.9 and 3.5%}, Bean yellow mosaic virus (BYMV) {1.4 and 4.5%}, Bean common mosaic virus (BCMV) {1.2 and 4.5%}, Red clover vein mosaic virus (RCVMV) {1.2 and 4%}, White clover mosaic virus (WCIMV) {1.0 and 5%}, Cucumber mosaic virus (CMV) {0.8 and 3%}, Pea streak virus (PeSV) {0.4 and 4.5%} and Tobacco streak virus (TSV) {0.3 and 2.5%}. Alfalfa mosaic virus (AMV), the previously reported virus in alfalfa, had the highest percentage of detection in alfalfa accounting for 58.4% and 62.8% in the weeds and cultivated plants. Peanut stunt virus (PSV) was also detected for the first time in Saudi Arabia with a

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66.7% of infection in 90 alfalfa samples collected from the surveyed regions during the last visit that tested negative to all the previously detected viruses.

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1. Introduction

Alfalfa is considered the most important forage crop in Saudi Arabia that is used for animal feeding. The area under cultivation of alfalfa was 126611 hectares in 2013 producing 2659449 tons (Ministry of Agriculture, 2014). The five most important regions for alfalfa production in Saudi Arabia include Riyadh, Qassim, Tabuk, Jouf and Hail (Fig. 1). More than 90% of the total alfalfa production in the Kingdom was from these regions. In an earlier survey of alfalfa fields in Saudi Arabia, only AMV was detected in several locations (AL-Shahwan, 2002). Several plant viruses besides Alfalfa mosaic virus (AMV) that were reported from alfalfa, such as Bean leaf roll virus (BLRV), Bean common mosaic virus (BCMV), Bean yellow mosaic virus (BYMV), Cucumber mosaic virus (CMV), Lucerne transient streak virus (LTSV), Pea streak virus (PeSV), Red clover vein mosaic virus (RCVMV), Tobacco streak virus (TSV), White clover mosaic virus (WCMV) and Peanut stunt virus (PSV) which were reported to infect this crop and other legumes worldwide (Guy et al., 2013; Jones, 2013; Jones et al., 2012; Massumi et al., 2012; Shah et al., 2006; Latham and Jones, 2001; Guy and Forster, 1996; Alan et al., 1996; Rahman and Peaden, 1993; Hiruki and Hampton, 1990; Stuteville and Erwin, 1990; McLaughlin and Boykin, 1988; Edwardson and Christie, 1986; Forster et al., 1985; Rao and Hiruki, 1985; Hampton, 1983) can negatively affect the production, quality, and durability of alfalfa. If the diseases associated with these viruses are not properly managed they will probably limit alfalfa production. In a previous study, it was reported that some weed and cultivated plant species were found to be infected with AMV besides other viruses that have been reported to infect alfalfa (Al-Shahwan and Abdalla, 1998). Our objective is to generate data that would reveal the situation of the viral disease complex of this crop

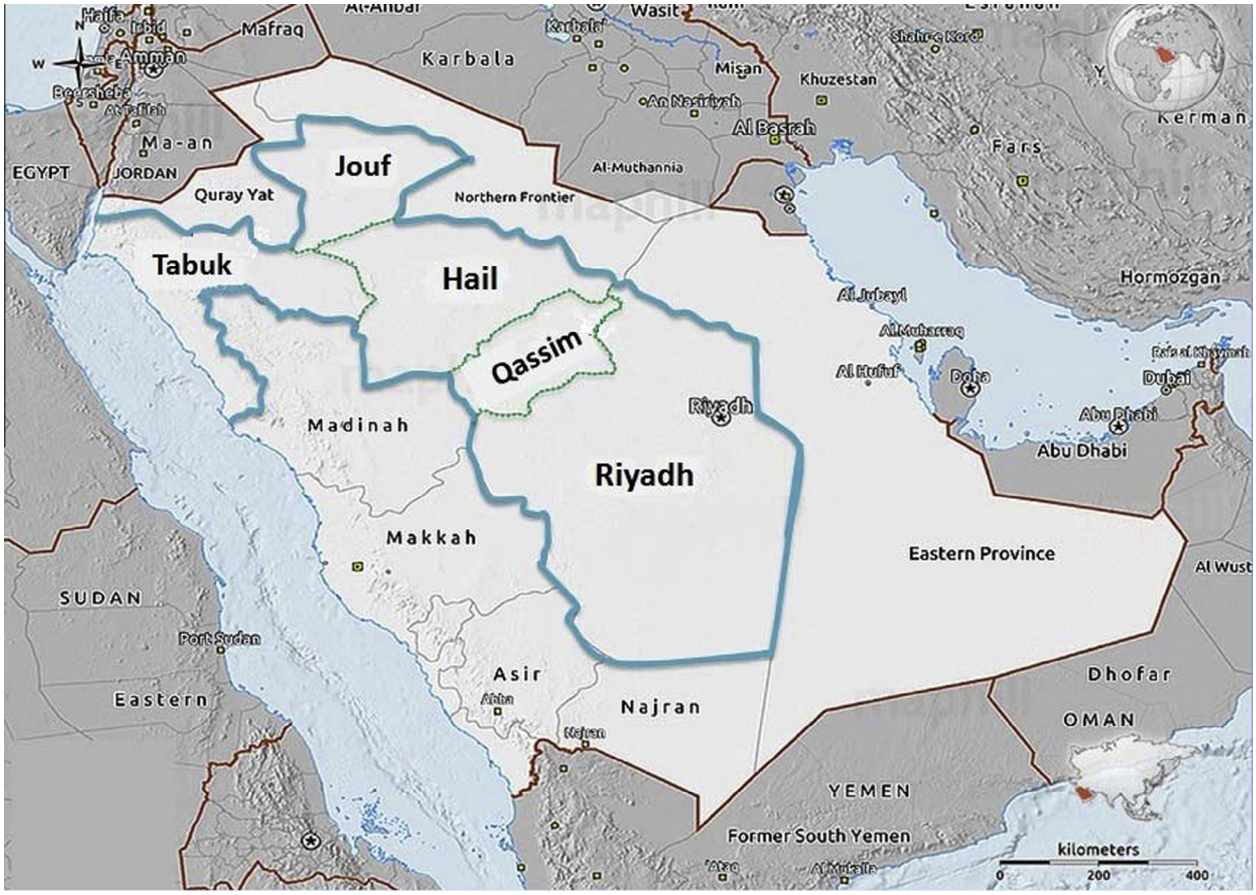

Figure 1 Map of Saudi Arabia showing the surveyed regions (Riyadh, Qassim, Hail, Jouf, and Tabuk) during the two year survey (2012–2013).

Figure 2 Virus like symptoms on alfalfa (A, B and C), weed (D: *Sonchus oleraceus*), and cultivated plant species (E: potato and F: cowpea).

and the relative importance of the components of this disease complex as an important step toward investigations essential for adopting the proper methods of their management.

2. Materials and methods

Four field trips were made to each of the five principal alfalfa–producing regions in Saudi Arabia, namely, Riyadh, Qassim, Tabuk, Jouf and Hail in four successive growing seasons (i.e.) a total of twenty field trips were made to these regions within the duration of the two year survey. One trip was made to each of these five regions during the first six months of the year while a second trip was made to each of the same regions during the last six months of the year during 2012 and 2013. A total of 1368 samples were collected from alfalfa, weeds and cultivated plants growing adjacent to alfalfa fields and showing virus like symptoms (Fig. 2). Virus Detection in the collected alfalfa, weed and cultivated plants samples was carried out using DAS-ELISA as demonstrated by Clark and Adams (1977). ELISA kits for BLRV, BYMV, BCMV, CMV, LTSV, PeSV, RCVMV, TSV, WCMV, PSV, and AMV were purchased from Agdia (Agdia Inc., 30380 Country Road, Elkhart, Indiana 46514 USA), or from AC-Diagnostics (1131 W Cato Springs Road, Fayetteville, AR 72701, USA).

The microtiter plates were coated with antibodies for each of the above-mentioned viruses after being diluted with the coating buffer. Subsequent to incubation and washing, aliquots of 100 µl of each of the samples which were extracted in the extraction buffer, were added in two wells of each plate. 100 µl of the dilutions of the relevant antibody-alkaline phosphatase conjugate, recommended by the kit provider, was dispensed in the wells of each plate following washing of the plates from samples sap. *P*-Nitrophenyl phosphate solution was then added in the wells of each plate after washing from the conjugate solution. The plates were incubated for 1 h before the reaction was stopped using 3 M NaOH, and the absorbance at 405 nm was recorded using BioTek Instruments, ELx 808 reader, USA.

3. Results and discussion

ELISA test of the 1368 plant samples showing different virus symptoms (Fig. 2) indicated occurrence of ten viruses, for the first time in alfalfa in Saudi Arabia. These were BLRV, LTSV, BYMV, BCMV, RCVMV, WCIMV, CMV, PeSV, TSV and PSV. The frequencies of detection of these viruses in alfalfa plants were 12.5%, 2.9%, 1.4%, 1.2%, 1.2, 1.0,%, 0.8%, 0.4%, 0.3% respectively (Table 1). These ten viruses were also detected in weed and cultivated plant species and their respective frequencies of detection were 4.5%, 3.5%, 4.5%, 4.5%, 4%, 5%, 3%, 4.5% and 2.5% (Table 2). AMV which was reported earlier in alfalfa in Saudi Arabia was found to have the highest percentage of detection in the collected alfalfa samples (58.4%), weed and cultivated plants samples (62.8) as shown in Tables 1 and 2, respectively. The frequency of detection of PSV which was detected in the last field trip of the survey was 66.7% in 90 tested alfalfa samples that were negative to all other ten viruses including AMV.

The number of the newly detected viruses in alfalfa varied from region to region and from location to location within the same region. The number of viruses detected in alfalfa per region was greater in the four regions, north of Riyadh, which include Jouf, Tabuk, Hail, and Qassim where at least seven viruses were detected per region compared to the

Table 1 Viruses detected in alfalfa samples in the major producing regions in the Kingdom of Saudi Arabia during two year survey (2012–2013).

Region & location	No. of samples	Viruses									
		AMV	BCMV	BLRV	BYMV	CMV	LTSV	PeSV	RCVMV	TSV	WClMV
Jouf	169	119	9	44	2	2	9	0	2	0	1
Tabuk	178	82	0	12	4	6	10	1	1	1	1
Hail	164	106	0	28	6	1	8	3	5	0	3
Qassim	189	107	5	22	2	0	2	1	2	0	2
Riyadh region											
Wadi Aldawasir	129	54	0	3	0	0	0	0	1	2	0
Shaqra	40	33	0	10	0	0	0	0	1	0	0
Sagir	87	52	0	10	2	0	1	0	1	1	3
Harad	13	7	0	0	0	0	0	0	0	0	0
Zulfi	56	38	0	5	0	0	0	0	0	0	0
Alkharj	51	33	0	1	0	0	2	0	0	0	1
Hota	40	26	0	1	0	0	2	0	0	0	1
Aflaj	50	24	0	10	0	0	0	0	1	0	0
Total	1166	681	14	146	16	9	34	5	14	4	12
Percentage %		58.4	1.2	12.5	1.4	0.8	2.9	0.4	1.2	0.3	1

Table 2 Viruses that were detected in weeds and cultivated plants growing in the vicinity of alfalfa fields in the visited regions during two year survey (2012–2013).

Host	No. of samples	Viruses									
		AMV	BCMV	BLRV	BYMV	CMV	LTSV	PeSV	RCVMV	TSV	WCMV
Sonchus oleraceus	113	96	3	4	5	1	6	7	6	2	6
Vigna unguculata	8	5	1	0	0	3	0	0	0	0	0
Hippuris vulgaris	7	2	1	0	0	0	0	0	0	2	0
Chenopodium spp.	17	8	1	1	0	0	0	1	1	0	1
Rubus fruticosus	5	1	0	1	0	0	0	0	0	0	0
Hibiscus spp.	20	7	0	1	0	1	1	0	0	0	0
Solanum tuberosum	11	2	2	1	0	0	0	0	1	0	0
Vicia faba	1	0	1	1	1	0	0	0	1	1	0
Cichorium intybus	4	2	0	0	0	0	0	0	0	0	0
Phaseolia vulgaris	2	0	0	0	0	0	0	0	0	0	0
Capsicum annuum	1	0	0	0	1	0	0	0	0	0	0
Flaveria trinervia	1	0	0	0	0	0	0	0	0	0	0
Solanum melongena	2	2	0	0	0	0	0	0	0	0	1
Malva parviflora	4	1	0	0	0	0	0	0	0	0	0
Convolvulus arvensis	4	1	0	0	0	0	0	0	0	0	0
Cucurbita maxima	2	0	0	0	0	1	0	1	1	0	0
Total	202	127	9	9	7	6	7	9	10	5	8
Percentage %		62.8	4.5	4.5	3.5	3	3.5	4.5	5	2.5	4

locations of Riyadh region where relatively fewer viruses were detected per location. Also the percent of infection with these viruses was greater in the northern regions compared to the eight locations of Riyadh region. Most of alfalfa samples were found to be multiply infected with two or more viruses (Table 1). In an earlier study Alshahwan reported only AMV from different regions and locations in Saudi Arabia (AL-Shahwan, 2002) which may suggest appearance of ten new viruses in alfalfa in at least Riyadh region within one decade.

The viruses infecting alfalfa were also found in nine weed plant species growing adjacent to alfalfa fields including *S. oleraeus*, *Chenopodium* spp., *Convolvulus arvensis*, *Malva parviflora*, *Hibiscus* spp., *Rubus fruticosus*, *Hippuris vulgaris*, *Cichorum intybus*, *Flaveria trinervi* (Table 2). These viruses were also found in seven cultivated plant species growing in the same area. These were *Vigna unguiculata*, *Solanum tuberosum*, *Solanum melongena*, *Phaseolus vulgaris*, *Cucurbita maxima*, *Capsicum annuum*, and *Vicia faba* (Table 2). It was reported earlier by Alshahwan and Abdalla that some weed and cultivated plants harbor AMV in addition to other viruses that may affect alfalfa (Al-Shahwan and Abdalla, 1998).

It is interesting that all the viruses detected in alfalfa were also detected in *S. oleraceus* the most frequently encountered weed growing close to or within alfalfa fields from which most of the weed samples were collected (Table 2). From the same

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Table 3 Viruses that were detected in weeds and cultivated crops growing in vicinity of alfalfa fields that were collected during January–June of 2012 and 2013.

Region & location	Host	No. of samples	Viruses									
			AMV	BCMV	BLRV	BYMV	CMV	LTSV	PeSV	RCVMV	TSV	WCMV
Jouf	*Sonchus oleraceus*	11	8	2	2	3	1	3	2	1	0	3
	Vigna unguculata	2	2	1	0	0	1	0	0	0	0	0
	Hippuris vulgaris	1	0	1	0	0	0	0	0	0	1	0
	Chenopodium spp.	2	0	1	0	0	0	0	1	1	0	0
Tabuk	*Sonchus oleraceus*	13	9	0	0	0	0	0	1	0	0	0
	Hippuris vulgaris	2	0	0	0	0	0	0	0	0	1	0
	Rubus fruticosus	2	0	0	0	0	0	0	0	0	0	0
	Hibiscus spp.	1	0	0	0	0	0	0	0	0	0	0
Hail	*Solanum tuberosum*	5	0	1	0	0	0	0	0	1	0	0
	Sonchus oleraceus	15	13	0	2	2	0	2	4	5	2	3
Qassim	*Sonchus oleraceus*	10	10	0	0	0	0	0	0	0	0	0
	Phaseolia vulgaris	2	0	0	0	0	0	0	0	0	0	0
Riyadh region												
WadiAldawasir	*Sonchus oleraceus*	5	5	0	0	0	0	0	0	0	0	0
	Cichorium intybus	1	0	0	0	0	0	0	0	0	0	0
	Hibiscus spp.	2	1	0	1	0	0	0	0	0	0	0
Shagra	*Sonchus oleraceus*	5	5	0	0	0	0	0	0	0	0	0
	Cichorium intybus	1	1	0	0	0	0	0	0	0	0	0
	Hippuris vulgaris	2	0	0	0	0	0	0	0	0	0	0
	Capsicum annuum	1	1	0	0	1	0	0	0	0	0	1
Haradh	*Sonchus oleraceus*	1	1	0	0	0	0	0	0	0	0	0
	Malva parviflora	1	0	0	0	0	0	0	0	0	0	0
Azulfi	*Sonchus oleraceus*	2	2	0	0	0	0	1	0	0	0	0
Alkharj	*Sonchus oleraceus*	8	6	0	0	0	0	0	0	0	0	0
	Chenopodium spp.	3	1	0	0	0	0	0	0	0	0	1
	Cichorium intybus	1	0	0	0	0	0	0	0	0	0	0
	Hibiscus spp.	1	1	0	0	0	0	0	0	0	0	0
	Solanum spp	1	1	0	0	0	0	0	0	0	0	1
	Malva parviflora	1	1	0	0	0	0	0	0	0	0	0
	Hibiscus vulgaris	2	0	0	0	0	0	0	0	0	0	0
Alhotah	*Sonchus oleraceus*	7	7	0	0	0	0	0	0	0	0	0
	Convolvulus arvensis	1	1	0	0	0	0	0	0	0	0	0
	Malva parviflora	2	0	0	0	0	0	0	0	0	0	0
	Chenopodium spp.	1	1	0	0	0	0	0	0	0	0	0
	Hibiscus spp.	2	2	0	0	0	0	0	0	0	0	0
Aflaj	*Sonchus oleraceus*	2	2	0	0	0	0	0	0	0	0	0
	Hippuris vulgaris	1	1	0	0	0	0	0	0	0	0	0
	Chenopodium spp.	2	2	0	0	0	0	0	0	0	0	0
	Convolvulus arvensis	3	0	0	0	0	0	0	0	0	0	0
	Hibiscus spp.	3	2	0	0	0	0	0	0	0	0	0
Total		128	85	6	5	6	2	6	8	8	4	8

table, it can be seen that other important weed plant species growing in close proximity to alfalfa fields were found to be multiply infected with two or more of these viruses. The results also indicated that the prevalence of these viruses depends on the geographical location and the weed. For instance, the number of viruses detected in *S. oleraceus* in Jouf during the July–December visits was much greater than the number of viruses detected in the same weed anywhere in the other regions and locations during the same season and that also the number of viruses detected in the same weed in the same region (Jouf) dropped from 7 to 9 viruses during the July–December visits to a single virus during January–June visits (Tables 3 and 4). Some of the viruses that infect alfalfa were found infecting other cultivated plant species growing adjacent to alfalfa fields. BCMV was detected in *V. unguiculata,* CMV was detected in *C. maxima,* AMV, BYMV and WCMV were detected in *C. annuum*, AMV was detected in *V. unguiculata*, *S. tuberosum*, and *S. melongena* in one or more of the surveyed regions (Tables 2–4).

The results of detection of PSV for the first time in 60 out of 90 alfalfa samples that tested negative to all other ten viruses reported herein were two folds, firstly this is the first detection ever of this virus in Saudi Arabia and secondly these results also help to explain the negative results obtained in 20% of

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Table 4 Viruses that were detected in weeds and cultivated crops growing in vicinity of alfalfa fields that were collected during July–December of 2012 and 2013.

Region & location	Host	No. of samples	Viruses									
			AMV	BCMV	BLRV	BYMV	CMV	LTSV	PeSV	RCVMV	TSV	WCMV
Jouf	*Sonchus oleraceus*	4	4	0	0	0	0	0	0	0	0	0
	Vigna unguculata	2	1	0	0	0	2	0	0	0	0	0
	Faba bean	1	0	1	1	1	0	0	0	1	1	0
	Cucurbita maxima	1	0	0	0	0	1	0	1	1	0	0
	Chenopodium spp.	2	1	0	0	0	0	0	0	0	0	0
Tabuk	*Sonchus oleraceus*	2	2	0	0	0	0	0	0	0	0	0
	Hibiscus spp.	4	0	0	0	0	1	0	0	0	0	0
Hail	*Solanum tuberosum*	4	1	1	0	0	0	0	0	0	0	0
	Sonchus oleraceus	4	1	1	0	0	0	0	0	0	0	0
	Hibiscus spp.	1	0	0	0	0	0	0	0	0	0	0
Qassim	*Sonchus oleraceus*	1	0	0	0	0	0	0	0	0	0	0
	Cucurbita maxima	1	0	0	0	0	0	0	0	0	0	0
	Chenopodium spp.	1	0	0	0	0	0	0	0	0	0	0
Riyadh region												
WadiAldawasir	*Sonchus oleraceus*	13	11	0	0	0	0	0	0	0	0	0
	Vigna unguculata	1	1	0	0	0	0	0	0	0	0	0
	Hibiscus spp.	3	1	0	0	0	0	0	0	0	0	0
	Solanum tuberosum	2	1	0	1	0	0	0	0	0	0	0
	Chenopodium spp.	1	0	0	1	0	0	0	0	0	0	0
Shagra	*Solanum melongena*	1	1	0	0	0	0	0	0	0	0	0
Sagir	*Flaveria trinervia*	1	0	0	0	0	0	0	0	0	0	0
Haradh	*Sonchus oleraceus*	2	2	0	0	0	0	0	0	0	0	0
	Cichorium intybus	1	1	0	0	0	0	0	0	0	0	0
	Chenopodium spp.	1	0	0	0	0	0	0	0	0	0	0
Azulfi	*Sonchus oleraceus*	2	2	0	0	0	0	0	0	0	0	0
	Vigna unguculata	1	0	0	0	0	0	0	0	0	0	0
Alkharj	*Hibiscus vulgaris*	1	0	0	0	0	0	1	0	0	0	0
Alhotah	*Vigna unguculata*	2	1	0	0	0	0	0	0	0	0	0
	Chenopodium spp.	2	1	0	0	0	0	0	0	0	0	0
	Rubus fruticosus	2	0	0	1	0	0	0	0	0	0	0
Aflaj	*Sonchus oleraceus*	6	6	0	0	0	0	0	0	0	0	0
	Hippuris vulgaris	1	1	0	0	0	0	0	0	0	0	0
	Chenopodium spp.	2	2	0	0	0	0	0	0	0	0	0
	Rubus fruticosus	1	1	0	0	0	0	0	0	0	0	0
Total		74	42	3	4	1	4	1	1	2	1	0

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the alfalfa samples collected in this study. The occurrence of these viruses in alfalfa, weeds and cultivated plant species as well, has a great implication on the epidemiology of the diseases caused by these viruses on alfalfa since we also encountered same aphid species in the surveyed alfalfa fields, weeds and cultivated crops which were probably vectors for most of the detected viruses and enhance their transmission between weeds, other cultivated plants, and alfalfa. Previous investigations on viruses associated with alfalfa indicated infections of this crop with viruses similar to what was reported herein. In a recent survey alfalfa was reported to be infected with several of these viruses (Massumi et al., 2012; Peck et al., 2012; Jones, 2012, 2014; Shah et al., 2006; Rahman and Peaden, 1993). Van Leur and Kumari, 2011, detected only two viruses in their Lucerne survey in New South Wales. Interestingly, similar to our finding, AMV was found to be the most widely spreading in Lucerne in that study followed by BLRV, the second important virus in our survey. Shah et al. (2006) reported four viruses, AMV, BYMV/ clover yellow vein virus (CLYVV) and CMV from alfalfa in New York with the following disease incidences 41.96, 6.56 and 6.69, respectively. Similar to what we have got, their results showed mix- virus infections and also that AMV had the highest disease incidence. Massumi et al. (2012), detected four viruses from alfalfa in Iran namely, AMV, BLRV, PSV and BYMV with disease incidences of 23.3, 12, 0.7 and 0.28%, respectively, however, CMV and BCMV were not detected. Rahman and Peaden (1993), detected AMV, BLRV, PeSV and TSV in a survey carried out in Washington, Oregon, Idaho, California, and British Colombia with their importance in the same sequence similar to what we found in Saudi Arabia.

It is clear from the previous reports that the problematic viruses in alfalfa are the same in all alfalfa –producing countries worldwide with AMV being the most prevalent. Also the number of viruses detected in the previous reports ranged between 2 and 4 viruses, however, 11 viruses were detected in alfalfa in Saudi Arabia, 10 of which were reported for the first time in this study. Diseases caused by these viruses probably constitute a potential threat to alfalfa production in Saudi Arabia as some of them are mechanically transmitted, some are transmitted with vectors and some are transmitted with seeds and some are multiply transmitted with more than one of these means, in addition to the fact that some have reservoir weed and other cultivated plants that harbor them during the off-season periods.

Previous investigations also indicated that the viruses infecting alfalfa were also reported in wild reservoir plants such as *Datura* which was found to harbor AMV and CMV (Juan et al., 2006), *Chenopodium* spp. in which AMV, CMV and BYMV were detected, *Solanum nigrum* in which AMV and CMV were reported, and other weeds in which other viruses were also detected (Zitter, 2001; Barnett and Zeyong, 1982).

In conclusion, it is clear from this investigation that most of the previous studies with alfalfa viral diseases were aiming at detecting only AMV in this crop. (AL-Saleh and Amer, 2013; Al-Shahwan et al., 2010; Al-Abrahim, 2004; AL-Shahwan, 2002; Cook et al., 1984). This crop is in fact found infected with 11 viruses as shown from this study. Ten of these viruses were reported for the first time in alfalfa in Saudi Arabia. The fact that these viruses belong to different virus groups, have different means of transmission, have wild reservoir weed plants at least for several of them, and infect several cultivated crops, in addition to the occurrence of insect vectors for some of them in the field such as aphids, thrips and leafhoppers does not only draw the attention to the diseases caused by these viruses but also to the threat of the potential epidemics that are probably caused by these pathogenic agents as all the pre-requisites of their occurrence are documented if proper management of these diseases was not taken in consideration. In conclusion, future studies will include characterization of the new viruses especially those associated with great percentages of infection.

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