Comparison of *Turnip mosaic potyvirus* (TuMV) isolates infecting three crops in Saudi Arabia

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Mohammed A. AL-Saleh
Plant Protection Department, College of Food and Agriculture Sciences, King Saud University, P.O. Box 2460, Riyadh 11451, Saudi Arabia malsaleh@ksu.edu.sa

ABSTRACT

Turnip mosaic virus (TuMV), a member of the potyvirus group, has a wide host range and high degree of biological diversity. During the growing season of summer 2006 and 2007, twenty five samples were collected from symptomatic leaves of lettuce (Lactuca sativa Linn.), garden rocket (Eruca sativa Mill) and radish (Raphanus sativus L). These samples were collected from Al-Hair, Huraymila Al-Oyaynah and Qasseem regions. ELISA test indicated the presence of TuMV in these samples. Seven isolates were selected from these samples which represent both regions and the crops, in order to study the biological and molecular characterization of these isolates.Selected groups of plant species were mechanically inoculated each with one isolate. Systemic mosaics were observed on lettuce, garden rocket and radish, while local chlorotic lesions were expressed on Chenopodium amaranticolor. No symptoms were observed on tomato (Lycopersicum esculentum), Gomphrena globosa, Datura stramonium and Nicotiana tabacum. The viral RNA from these samples was extracted, cDNA was generated, and the coat protein (CP) gene of each was amplified by reverse transcription-polymerase chain reaction assay (RT-PCR). The nucleotide (nt) sequence of the coat protein gene which was determined for each of these isolates ranged between 864- 878 nucleotides. The nt sequences of the coat protein of these Saudi isolates and isolates from other countries were analyzed and a phylogenetic tree was generated using DNAMAN program. The phylogenetic analyses revealed two closely related clusters of the Saudi isolates that were distinct from other isolates reported elsewhere except the radish isolate (TuMV-RaSa-2) that formed one cluster with the South Korian isolate (#AF103789) which was isolated from radish too.

Key words: *TuMV, RT-PCR, CP gene, sequence, comparative analysis.*

INTRODUCTION

TuMV belongs to the genus *Potyvirus*. This is the largest genus of the largest family of plant viruses, the *Potyviridae*, which itself belongs to the picorna-like supergroup of viruses of animals and plants (Barnett *et al*., 1995) and infects cruciferous plants throughout the world. It is the only potyvirus known to infect brassicas (Walsh and Jenner, 2002). It was first reported in crucifers by Gardener and Kendrick (1921) and Schultz (1921) in the USA of America. It is geographically widespread and has been reported in North America, Europe, Africa, Asia, Australia, and New Zealand (Tomlinson, 1970; Feldman and Gracia, 1972; Fujisawa, 1990; Henson and French, 1993; Petrzik and Lehmann, 1996; Omunyin *et al*., 1996; Lehmann *et al*., 1997; Chen *et al*., 2003; Robertson and Ianson, 2005; AL-Saleh *et al*.,

2008; 2009). TuMV has a wide natural host range including crucifers, legumes, ornamentals and weeds (Green and Deng, 1985; Chen *et al.*, 2003). TuMV, like other potyviruses, is transmitted by aphids in the non-persistent manner (Shukla *et al.*, 1994). Eighty nine aphid species were reported to transmit TuMV in a non-persistent manner (Walsh and Jenner, 2002), which adds to the problem imposed by the infection of this virus.

All potyviruses have flexuous filamentous particles 700–750 nm long, each contains a single copy of the genome, which is a single-stranded positive sense RNA molecule (about 10000 nt long). The genomes of potyviruses have a single open reading frame that is translated into a single large polyprotein, which is hydrolysed, after translation, into several proteins by virus-encoded proteinases (Riechmann *et al.*, 1992). Due to variability among TuMV isolates and their broad host range, in addition to the limited information regarding diversity of TuMV in Saudi Arabia, the present work focused on the biological and molecular characterization of *turnip mosaic potyvirus* isolates infecting lettuce, garden rocket and radish plants grown under field conditions in Saudi Arabia.

MATERIALS AND METHODS

Source of virus isolates

Twenty five samples were collected during the growing season of summer 2006 and 2007 from sympotamatic leaves of lettuce (*Lactuca sativa* Linn.), garden rocket (*Eruca sativa* Mill) and radish (*Raphanus sativus* L). These samples were collected from four regions (Al-Hair, Huraymila, Al-Oyaynah and Qasseem). Six garden rocket samples were collected from Al-Hair, and Huraymila areas and six lettuce samples were collected from Al-Oyaynah and Al-Hair areas, 13 radish samples were collected from Al-Hair, Huraymila and Qasseem areas naturally growing and showing systemic mosaic, stunting and chlorosis. Samples were brought to the Plant Virology Laboratory for analysis.

Enzyme linked immuonosorbent assay (ELISA)

ELISA kits for *Radish mosaic virus* (RMV), *Alfalfa mosaic virus* (AMV), *Turnip mosaic virus* (TuMV), and *Cucumber mosaic virus* (CMV) were purchased from Agdia (Agdia Inc., 30380 Country Road, Elkhart, Indiana 46514 USA). Steps of ELISA procedure were applied in the same way explained by the manufacturing company, which were not remarkably different from those indicated in the original procedure by Clark and Adams (1977). Each of four micro titer plates were coated with antibodies of either of RMV, AMV, TuMV, or CMV after being diluted with the coating buffer. Subsequent to incubation and washing, aliquots of 100 ul of each of the samples, which were extracted in the extraction buffer, were added in two wells of each plate. One hundred ul of the proper dilutions of the relevant antibody-alkaline phosphatase conjugate were dispensed in the wells of each plate subsequent to washing 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 hour, the reaction was then stopped using 3 M NaOH, and the plates were read at 405 nm in the minireader.

Inoculation to selected host range

Infected leaf tissues of each TuMV isolate were homogenized in a prechilled mortar and pestle with 0.01 M phosphate buffer, pH 7.2, containing 0.1% sodium sulphite ($Na2SO_3$) using an extraction ratio of 1:4 (w/v). Inoculums were applied on selected

host range and test plants (5 plants/ each plus control) (*Raphanus sativus* L., *Eruca sativa* Mill, *Brassica rapa* L., *B. oleracea* L., *Lactuca sativa* Linn, *Spinacea oleracea, Lycopersicum esculentum* L., *Datura stramonium* L., *Chenopodium ammaranticolor* Cost and Reyn, *Solanum nigrum* L., *Gomphrena globosa, Nicotiana tabaccum* L. and *N. glutinosa* L.) previously dusted with carborundum (600 mesh) using a pad of cheesecloth. Plants were maintained in the greenhouse and observed for symptom development.

Total RNA extraction and RT-PCR

Total RNA of each isolate was extracted from infected and uninfected plants using SV–Total RNA Isolation System (Promega, USA). The used oligonucleotide primers designed according to Sanchez *et al* (2003) were as follows, the upstream primer Tu 8705- 8726: 5`- caa gca atc ttt gag gat tat g- 3` and the downstream primer Tu 9690-9669: 5`- tat ttc cca taa gcg aga ata c-3` were used. RT-PCR was performed using the QIAGEN One Step RT-PCR Kit (Qiagen, USA). The reaction was set up according to manufacturer's recommendations. Ten ul of 5x QIAGEN One Step RT-PCR buffer, 2 ul of 10 mM dNTP Mix, 10 ul of 5x Q-Solution, 2 ul 0f 10 pmol of each complementary and homologous primers, 2 ul of QIAGEN One Step RT-PCR enzyme mix, 5-10 Units/ reaction of RNase inhibitor. A total of 5 µl (200 ng) of RNA was added to the One-Step and RNase-free water to 50 ul. The master mix was mixed gently, by pipetting up and down a few seconds. RT-PCR reaction mixture was amplified using the following cycling parameters: hold at 50°C 30 minutes (RT step), hold at 95°C 15 minutes (hot start to PCR), then subjected to 35 cycles of amplification: 30 s at 94 °C for denaturation, 30 s at 54 °C for annealing, and 60 s at 72 °C for extension, followed by a final hold at 72° C for 10 minutes. Aliquots of 5 ul from each of RT-PCR amplified DNA products were mixed with gel loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol, and 30% glycerol). Separation was done on a 1% agarose gel in 1xTBE buffer pH 8.3 (1x = 89 mM Tris , 89 mM borate , and 2 mM EDTA). DNA was stained with ethidium bromide added to the gel at a concentration of 0.5 ug /ml. DNA was visualized on a UV transilluminator and photographed using DNA documentation gel analysis (OptiGo 500, Holland). One Kb DNA Ladder (Promega, USA) was used to determine the size of RT-PCR amplified cDNA products (Sambrook *et al*. 1989).

Sequencing and Data analysis

An amplified DNA fragment of expected size (985 bp) of the coat protein gene of each isolate included 54 bp of the 3`-end of Nib gene and 65 bp of the 3`-UTR. The amplified PCR product was purified using the Wizard PCR clean up kit (Promega, USA). The nucleotide sequence of the isolated gene of TuMV isolates were carried out in two directions with the specific complementary primer in King Faisal Specialist Hospital & Research Center, Molecular Virology & Infectious Diseases Scientist, Biological & Medical Research Department-Research Center, Riyadh, Kingdom of Saudi Arabia using AB 3730xI DNA Analyzer (Hitachi, Japan). Sequence analysis was performed and the homolog tree was constructed and analyzed using DNAMAN trial version 5.2.10 program. The following sequences which were obtained from NCBI database were used for comparison: AF103789, AJ831817, AJ831809, AF103791, AF103786, AF103792, AF434725, AF434726 and AB063251. (Table 1).

Table (1): Accession numbers and isolates of sequences used for comparison.

GenBank accession #	Isolate	Original host	Geographical origin
AF103786	CA7	Chinese cabbage	South Korea
AF103789	RG	radish	South Korea, GeoChang area
AF103791	TU	turnip	South Korea
AF103792	Stock	Stock	South Korea
AJ831809	ZJ2	turnip	China:Hangzhou:Zhejiang
AJ831817	YN2	radish	China:Kunming:Yunnan
AF434725	PRT 1	*Brassica oleracea*	Madeira (Portugal)
AF434726	PV 377	*Alliaria officinalis*	Italy
AB063251	ZYMV-M39	*Cucumis melo*	Japan

Table (2): Reaction of several plant species mechanically inoculated with TuMV- isolates.

Plant Species*	TuMV-Isolates						
	RoSa-1	RoSa-2	RoSa-3	RaSa-1	RaSa-2	LSa-1	LSa-2
Raphanus sativus L.,	SM,d	SM,d	SM, St	St,d	St	St	SM,St
Eruca sativa Mill	SM, St	SM,d	SM,d	SM, St	SM,d	SM	SM
Brassica rapa ,	Ch, E	Ch,E,	Ch,E	Ch,E,St	Ch,E	Ch,E	Ch,E
B. oleracea	-	-	-	-	-	-	-
Lactuca sativa Linn,	M	M	Mo	Mo	M	M	Mo
Spinacea oleracea	-	-	-	-	-	-	-
Lycopersicum esculentum	-	-	-	-	-	-	-
Datura stramonium	-	-	-	-	-	-	-
Chenopodium ammaranticolor	LCL	LCL	LCL	LCL/ LNL	LNL	LCL/ LNL	LCL
Solanum nigrum	-	-	-	-	-	-	-
Gomphrena globosa	-	-	-	-	-	-	-
Nicotiana tabaccum	-	-	-	-	-	-	-
N. glutinosa	-	-	-	-	-	-	-

* Six plants of each species were mechanically inoculated plus one control, and the experiment was repeated twice.
LCL= local chlorotic lesions; LNL= Local necrotic lesion, Sm = systemic mosaic; St = stunting; d =deformation; Ch=Chlorosis, E=epinasty; M= mottling; Mo= mosaic;– = no symptoms.

RESULTS AND DISCUSSION

Seven out of 25 samples that were collected from garden rocket, lettuce, and radish plants from Al-Hair, Huraymila, Al-Oyaynah and Qasseem areas gave positive reaction with TuMV using DAS–ELISA. Two of the positive samples were the infected radish grown in Al-Hair (RaSa-1) samples and Al-Oyaynah (RaSa-2) areas, three of them were from the infected garden rocket grown in Al-Hair (RoSa-1), Huraymila (RoSa-2) and Qasseem (RoSa-3) areas, and the other two samples were from the infected lettuce plants at Al-Oyaynah (LSa-1) and Al-Hair (LSa-2) areas. The rest of the samples gave negative reactions to all tested viruses.

When selected plant species inoculated mechanically with each TuMV-isolates then [6-15 days post inoculation (DPI)], mosaic symptoms were observed on the inoculated radish, lettuce, garden rocket, and turnip, whereas local chlorotic lesions were observed on *C. amaranticolor*. No symptoms were observed on the rest of the inoculated plants (Table 2). From that table, it is clear that the inoculated plants categorized into three different phenotypes, systemic, local and no infection.It has been reported that TuMV occurs worldwide and infects a large number of economical crops and wild plants (Zdenka,

1980; Edwardson and Christie, 1991; Stavolone *et al*., 1998 and Hughes *et al*., 2002) and is also being recently reported to continue inducing disease (Robertson and Ianson, 2005; Pallett *et al*., 2008). The virus has many strains which are variable in their pathogenicity, antigenicity and the plant species they infect (Zdenka, 1980; Stavolone *et al*., 1998; Pallett *et al*., 2008 ;).When eight Italian isolates of TuMV were mechanically inoculated into different *Brassica napus* lines, they revealed five pathotypes groups. Also when these isolates were introduced into selected plant species, several pathotypes were observed (Sánchez *et al* 2007).

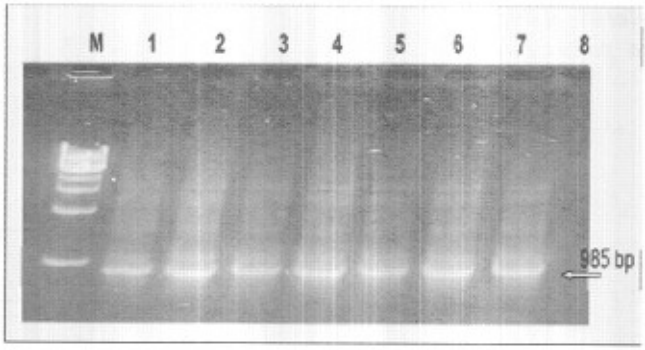

Fig. (1): Agarose gel electrophoresis of RT-PCR amplified TuMV-CP cDNA from infected radish plants were collected from Al-Hair and Al-Oyaynah areas (lanes 1 and 2), garden rocket plants were collected from Al-Haer, Huraymila and Qasseem areas (lanes 3 , 4 and 5), and lettuce plants were collected from Al-Oyaynah and Al-Hair areas (lanes 6 and 7). Lane 8, a healthy tissue control via SV-Total RNA Isolation System kit. M represents 1 Kb DNA ladder (Promega).

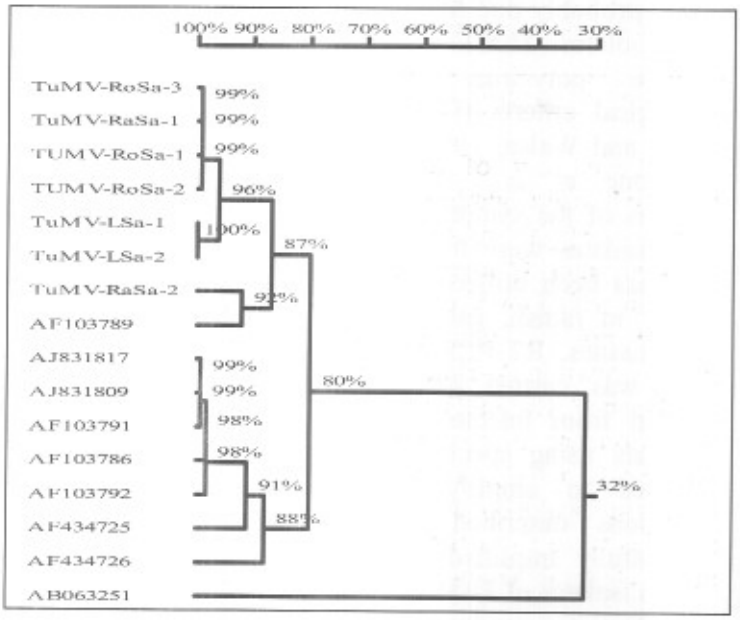

Fig. (2): The phylogenetic homology tree based on multiple sequence alignments of the 7-Saudi Arabian isolates compared to previously sequenced isolates.

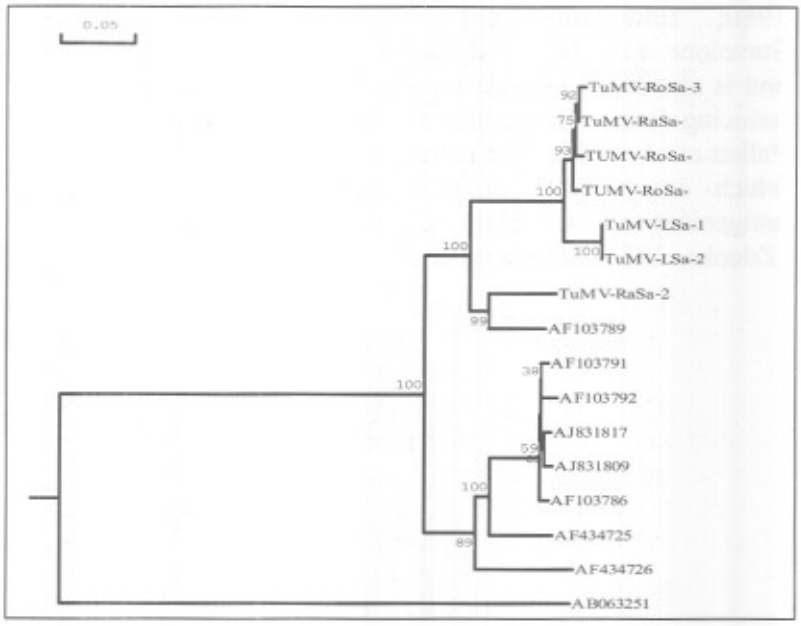

Fig. (3): Phylogentic analysis of TuMV isolates based on the nucleotide sequences of the CP gene. Genetic distances were calculated using bootstrapped parsimony method.

These results are in harmony with others when using isolates mechanically inoculated to *C. amaranticolor*, garden rocket, and *Datura stramonium*. However, other plant species, such as *Gomphrena globosa*, tomato and lettuce, had variable reaction (Sachez *et al* 2007) probably due to the biodiversity of the virus isolates. TuMV is considered a highly variable potyvirus by biological and serological criteria (Green and Deng 1985; Jenner and Walsh, 1996; Jenner *et al.*, 1999; Stavolone *et al.*, 1998). The biological analyses of the symptomology suggested that these isolates were biologically distinct. RT-PCR has been utilized successfully to detect TuMV in radish, garden rocket and lettuce plant tissues. RT-PCR amplification of viral RNA was carried out on the total RNA isolated from infected and uninfected plant materials using specific primers for TuMV designed to amplify 985 bp. The PCR conditions described in this investigation successfully amplified CP gene of the Saudi Arabia isolates of TuMV. Obtained data in Fig (1) illustrate product of the agarose gel electrophoresis of RT-PCR amplified TuMV-CP cDNA from infected radish plants that collected from Al-Hair and Al-Oyaynah areas (lanes 1 and 2), garden rocket plants that brought from Al-Hair, Huraymila and Qasseem areas (lanes 3 , 4 and 5), and lettuce plants from Al-Oyaynah and Al-Hair areas (lanes 6 and 7). No RT-PCR product was observed from healthy tissues (Fig.1, lane 8). Lane M, represents 1 Kb DNA ladder (Promega). PCR is extremely sensitive technique that widely used as a diagnostic tool for infection of plant viruses' belonging to several groups (Langeveld *et al.*, 1991; Robertson *et al.*, 1991; Henson and French, 1993; Rojas *et al.*, 1993). RT-PCR which has been used to detect plant viruses (Singh *et al.*, 1995) has also been applied to enhance detection sensitivity of potyviruses such as *Plum pox virus* (Wetzel *et al.*, 1991), *Zucchini yellow mosaic virus* (ZYMV) (Thomson *et al.*, 1995), and *Soybean mosaic virus* (SbMV) (Omunyin *et al.*, 1996). With these viruses, the PCR procedure utilizes primers designed to amplify a variable region of the potyvirus

genome to distinguish between different viruses within the group. Lehmann *et al* (1997) compared the CP gene of 12 TuMV-isolates and identified three groups of these isolates. Group 1, mostly infects *Brassica*, from Europe and North America and Group 2, from Asia infecting *Raphanus*, while the third group only represent one isolate from Greece that did not fall into the two groups. In another study, Chen *et al* (2002) reported that the variation among the Ten TuMV-isolates infecting different crops in China has tight relation to their host that being infected. *Brassica* isolates were placed in group 1, while *Raphanus* isolates were placed into group 2. There was an evidence of recombination in one of these isolates.

The CP coding sequence of the Saudi Arabia isolates of TuMV from infected radish, garden rocket and lettuce plants was found to be 862-878 nucleotides in length. A multiple alignment was done along with previously obtained sequences by GenBank sequence database. Analysis of the genetic distance of 16 TuMV sequences covering the complete CP gene was performed to build up a reference homology tree (Fig. 2) and phylogenetic tree (Fig. 3). The dendogram indicated that these sequences fell into 7 clusters readily distinguishable according to their relative genetic distances. One cluster included 4 sequences of isolates from hosts in *Brasica* genus isolated from garden rocket and radish plants (RoSa-3, RaSa-1, RoSa-1 and RoSa-2). The second cluster included two sequences of isolates of lettuce plant (LSa1 and LSa-2). The third cluster included two sequences isolated from radish (TuMV-RaSa-2 and AF103789). The fourth cluster included five sequences of isolates mostly originating from hosts in the *Brassica* genus (AF103791, AF103792, AJ831817, AJ831809 and AF103786). One cluster sequence of the isolate of *Brassica oleracea* (AF434725). One cluster sequence of the isolate of *Alliaria officinalis* (AF434726) and the seventh cluster sequence of the isolate *Zucchini yellow mosaic virus* (ZYMV).

Based on the results obtained from Figs. 2 & 3, the TuMV-isolates obtained from the three crops collected from the four areas in the central region of Saudi Arabia, formed two closely related clusters. Only one isolate, collected from radish at Al-Oyaynah area, formed a distinct cluster with the Korian isolate (AF103789) which was isolated from radish also. Moreover, it can be concluded from the dendogram that the Saudi isolates that infect *Brassica* were grouped in one cluster which was also indicated when our isolates were compared with other TuMV-isolates reported elsewhere infecting *Brassica* crops, and clustered closely. However, the two Saudi isolates LSa1 and LSa-2 that were collected from lettuce were placed in a different cluster.

This report gave an idea on the genetic diversity of TuMV isolates infecting three economic crops in four areas of the central region of Saudi Arabia. Due to the small numbers of TuMV isolates that being collected in this study in the two growing seasons 2006-2007 and also, these samples collected from a limited area (the central region of Saudi Arabia), future studies are needed to address other economic crops and/or weeds in other regions that can be infected by this virus. That will give a better idea on the genetic diversity of this destructive virus in the whole country.

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الملخص العربي

مقارنة لعزلات فيروس موزيك اللفت التي تصيب ثلاث محاصيل في المملكة العربية السعودية

محمد على الصالح

قسم وقاية النبات- كلية علوم الأغذية والزراعة – ص ب. 2460 الرياض- 11451 – جامعة الملك سعود- المملكة العربية السعودية

يعتبر فيروس موزاييك اللفت (*Turnip mosaic potyvirus*) من أهم فيروسات مجموعة البطاطس (Potyvirus group) والذى له مدى عائلي واسع ودرجة عاليه من الاختلافات البيولوجية . خلال الموسم الزراعي لصيف 2006،2007 م تم جمع خمسة وعشرين عينة نباتية تظهر عليها أعراض شبيهه بأعراض الأمراض الفيروسية من أوراق نباتات الخس والجرجير والفجل من المزارع المنتشرة فى كل من الحائر وحريملاء والعيينة بمنطقة الرياض وكذلك من منطقة القصيم. ثبت وجود فيروس موزاييك اللفت فى هذه العينات المختبرة باستخدام طريقة اختبار الأليزا ELISA . تم اختيار سبع عزلات من العينات النباتية التي تم جمعها ممثلة للمناطق والمحاصيل قيد الدراسة وذلك لدراسة خواصها البيولوجية والجزيئية ومقارنة هذه العزلات ببعضها البعض وكذلك مقارنتها مع العزلات التي تم دراستها عالميا لهذا الفيروس. نتج عن العدوى الميكانيكيه لكل عزلة من هذه العزلات السبع لبعض الأنواع النباتية ظهور الأعراض الجهازية على نباتات الخس والجرجير والفجل. أما الأعراض الموضعية المتمثله بالبقع الشاحبه فقد ظهرت على نباتات الزربيح فقط. لم تظهر أي أعراض على نباتات الطماطم والداتوره وعنب الثعلب والمخلده (الجومفرينا) ونباتات التبغ وذلك بعد 21 يوم من العدوى الصناعية. تم استخلاص الحامض النووي لهذه العزلات والحصول على الحامض النووي المكمل وإكثار جين الغلاف البروتينى لكل عزلة من هذه العزلات بواسطة تفاعل البلمرة المتسلسل العكسي النسخ. وتم دراسة تحديد التتابع النيوكليتيدى لكل عزله من هذه العزلات فى منطقة جين الغلاف البروتينى والذي تراوح بين 864 إلى 878 نيوكليتيدة. وقد تم تحليل نتائج التتابع النيوكليتيدى لهذه العزلات والحصول على شجرة التقارب بين العزلات باستخدام برنامج DNAMAN . أوضحت نتائج هذه التحاليل انه بمقارنة التتابع النيوكليتيدى للعزلات السعودية مع العزلات الأخرى وجد أن العزلات السعودية قد شكلت مجموعتين متقاربتين فيما بينهما، بينما كانت هذه العزلات متباعده عن العزلات الأخرى المسجلة عالميا، عدا عزلة الفجل السعودية رقم 2 والتي شكلت مجموعة ثالثة مع عزلة كوريا الجنوبية من نباتات فجل مصابه .