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Isolation and purification of *Alfalfa mosaic virus-infecting* potato (*Solanum tuberosum* L.) in Beheira governorate

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ABSTRACT
Alfalfa mosaic virus (AMV) was obtained from naturally infected potato plants, showing bright yellow (Calico) symptoms, grown in Al Nubaria region, El Behaira governorate. The identification was based on the symptoms developed on diagnostic hosts and serological reactions. AMV isolate was transmitted by *Aphis faba* and *Aphis nerii* in non-persistent manner. The purified virus had an ultraviolet absorption spectrum typical of a nucleoprotein and yield of purified virus was 1.8 mg/100g infected leaf tissues. Specific antiserum was prepared and found to have a titer of 1:6400 as determined by indirect ELISA.

***Keywords*:** Virus, Potato, ELISA, Aphis

Introduction

Potato (*Solanum tuberosum* L.) is the world's most important vegetable crop (Abdelkhalek *et al*. 2020). Moreover, potato is a staple food of the world's population. In 2104, four million and eight hundred thousand (4800, 000) tons of tubers were harvested from 439855.8 feddans of potato grown in Egypt according to the FAO (2014). *Solanum tuberosum* (potato) viruses can infect potato naturally and cause a wide range of foliar symptoms ranging from latent infection to plant death, depending on virus species, strain, and potato cultivar (Abdalla *et al*., 2020; Salazar, 1996). AMV is one of the most important and widespread plant viruses and is found to infect 599 species belonging to 245 genera of 68 families (Jaspars and Bos, 1980). In Egypt, AMV are widely spread on potato plants (El-Abhar *et al*., 2018; Abdalla *et al*., 2016; Zaied, 2013). Potato plants exhibiting bright yellow (calico) symptoms were observed in Al Nubaria region, El Behaira governorate.

The present study was directed to isolate and identify the causal agent on the bases of symptomology, reactions of diagnostic hosts, serological reaction, insect transmission, photometrical characters of the purified virus and production of specific antiserum to the isolated virus was also carried out.

Material and Methods

1. Isolation

Leaf samples from Potato plants exhibiting bright yellow calico (Fig.1) were observed in Al Nubaria region, El Behaira governorate. Inoculum was prepared by grinding infected potato leaf tissues in a mortar and pestle with a small amount of 0.1 M phosphate buffer (pH 7) according to previously reported (Abdelkhalek *et al.,* 2018; Hafez *et al.*, 2011). Leaves of plants to be inoculated were kept for 48-72 hrs in darkness then dusted with carborundum (600 mesh) and inoculated with a freshly prepared inoculum using forefinger. Inoculated plants were rinsed with tap water shortly after inoculation and kept in insect proof greenhouse conditions for symptoms development. The isolated virus was maintained in *Nicotiana glutinosa* plants for virus propagation and served as a source of the virus for subsequent studies (Fath-Allah, 1999; Xu and Nie, 2006).

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2. Diagnostic hosts

The following diagnostic hosts; *N. glutinosa, Datura metal, N.repanda, Chenopodium amaranticolor*, *Phaseolus vulgari* and *Gomphrena globosa* known to give characteristic symptoms for AMV as recorded by Abdalla *et al*. (2016) and Paliwal, (1982) were used for tentative identification of the isolate virus. Five seedlings of each tested plant species were mechanically inoculated with AMV isolate and kept under greenhouse conditions. Plants were examined daily for four weeks for symptoms.

3. Serological reaction

3.1. Source of antisera

Antisera to alfalfa mosaic virus (AMV) potato virus Y (PVY), potato leaf roll virus (PFRV) and Potato virus X (PVX) supplied by Bioreba (Switzerland) were used.

3.2. Indirect ELISA

Indirect ELISA was carried out as described by Fegla *et al.* (1997), using antiserum specific to alfalfa mosaic virus. Extracts from infected and healthy plants samples were ground with coating buffer (0.05 M carbonate, pH 9.6) to 1:10. (w\v) antigen was added 100 µl of to the bottom of the well and incubated for 2 hours at 37°C or overnight at 4°C. The plates were rinsed three times, 3 minutes each, by floating wells with PBST. To reduce nonspecific reactions, antiserum requiring cross-adsorption was diluted typically 1: 500 with filtered extract from healthy tissues diluted 1:20 in serum buffer (PBS-Tween 20 containing 2% soluble polyvinylpyrrolidone, 0.2% BSA), and incubated for 45 min. at 37°C. The precipitate, which had formed, was removed by centrifugation for 10 min. at 5000 rpm. 100 µl aliquots from the diluted antisera were added to each well, the plates were incubated at 37°C for 2 hours or at 4°C overnight, then washed as before. Goat anti-rabbit gamma globulin conjugated to alkaline phosphatase was diluted 1:1500 in serum buffer, one hundred µl were added to each well, followed by one hour incubation at 37°C then washed as before. One hundred µl of the enzyme substrate, 1 mg/ml paranitrophenyl phosphate in 10% diethanolamine buffer, pH 9.8 were added to each well and incubated at room temperature (25°C) for about 30 minutes. The enzyme activity was stopped by adding 50 µl of 3 M NaOH. The ELISA In each set of tests, wells laking antigene (coating buffer only) were included as blank. Values measured by sunrise ELISA reader were expressed as absorbency at 405 nm. The absorbance values of at least double that of the healthy control were considered positive.

4. Aphid transmission

Two species of virus free apterous forms of aphids namely, *Aphis faba* and *A. nerii* were starved for one hour in Petri dishes before they were allowed access probes of 3-5 min on infected *N. glutinosa* attached leaves. Aphids were transferred in groups of five to each of 10 healthy *N. glutinosa* plants and allowed an inoculation period of 10 min. before they were killed by spraying by an insecticide. Inoculated plants were kept under greenhouse conditions and symptoms were observed for one month.

5. Purification

Purification of AMV was achieved using a modified method of Clark (1968) and Van Vlotenoting and Jaspers (1972). One hundred grams of systemically infected *N. glutinosa* leaves were collected 21-25 days after inoculation. A Blender was used to grind leaves in 0.1 M K2HPO4, 0.1 M ascorbic acid and 0.02 ethylendiamine tetraacetic acid adjusted to PH 7.1 with KOH (1g / 1.5 ml). The slurry was filtered through muslin and filtrate was mixed 1:1 with chloroform n-butanol for 15 min. The emulsion was broken by centrifugation, 14000 rpm for 20 min at 4C, using (roter). The aqueous layer was removed and brought to 5% (PEG) by the gradual addition of solid Polyethyleneglycol (mol. Wt. 6000) and 0.2 M NaCl was added to aqueous phase after being stirred for 20 min at 4C, the precipitate was collected by centrifugation for 20 min at 14000 rpm and resuspended in resuspention buffer (0.01 M phosphate, PH 7.5). Insoluble material was removed by low speed centrifugation at 6000 rpm for 10 min and the cirus particles were separated by ultracentrifugation (35000 rpm / 2h) in Beckman Optima XE-100 Ultracentrifuge Roter 50.2Tj.

The last pellet was resuspended in 2 ml of the suspending buffer. U.V. absorption spectrum of the purified virus at scanning of wavelength 220-320 nm with 20-nm interval was recorded spectrophotometrically using ultraviolet spectrophotometer. A 260 / 280, A 280 / 260 and A max/min as well as virus concentration were estimated. Virus concentration was calculated by assuming an extinction coefficient E 260nm 0.1% of 5.2 (Noordam, 1973).

6. Antiserum production for AMV

An antiserum against AMV was prepared according to the schedule given by Fath-Allah (1999) and Hamza *et al*. (2018). One white Spain rabbit was immunized with purified virus preparation, First injection in marginal ear veins with purified virus (0.7 mg) diluted with an equal volume of saline solution (0.85 % NaCl) and three intramuscular injections of 0.7 mg virus emulsified with an equal volume of Freund's adjuvant (Difco Lab, U.S.A). Complete adjuvant was used in the second injection and incomplete adjuvant thereafter. Bleedings were made from marginal ear veins one and two weeks after the final injection for immune serum and the blood was left for 2 hr at room temperature to colt, and then stored in refrigerator overnight. The separated antiserum was clarified by centrifugation at 5000g for 30 min, divided in aliquots and kept frozen until needed for different serological tests. Antiserum titer was determined using indirect ELISA as described by Fegla *et al.* (1997). Extracts from infected and healthy *N. gutinosa* plants were diluted with coating buffer to 1:10. Serial dilution of double fold up to 5.12 X 10^4 of antiserum from cross-adsorption with filtered extracts from healthy tissues diluted 1:20 in serum buffer were used (Younes, 2003)

Results

1. Reactions of diagnostic hosts

Diagnostic hosts reacted with symptoms like those produced by AMV. The virus induced Necrotic local lesion after 6 day on *Phaseolus vulgaris* (Fig. 1-B), severe mosaic on *Datura metal* (fig. 1-C), chlorotic local lesion after 6 days of inoculation followed by mosaic and severe distortion and leaf malformation 24 days later on *N. glutinosa* (fig.1-D), and chlorotic local lesions followed by severe mosaic on *Gomphrene globose* 24 days after inoculation (fig. 2-A) and induced apical mild mosaic on *N. repanda* after 18 days of inoculation (fig 2-B).

2. Serological reaction

AMV antiserum positively reacted with the isolated virus at a dilution of 1:500. No positive reaction was detected with antisera to PLRV, PVY and PVX as determined by indirect ELISA (Table 1).

Table 1: Reaction of infected Potato leaves extracts against antisera to AMV, PVY, PLRV and PVX as determined by indirect ELISA.

Potato leaf extract	Absorbance value at 405			
	AMV	**PLRV**	**PVY**	**PVX**
I	1.840	0.184	0.156	0.220
H	0.410	0.285	0.188	0.266

H= Healthy **I=** Infected

3. Aphid transmission

The virus was transmitted non-persistently by *Aphis fabe* and *A. neri* with average transmission rates of 50% and 20% when five viruliferous aphids were used on each test plant (Table 2).

Table 2: Transmission of AMV by aphid species

Aphis species	Transmission	
	Rate*	**%**
Aphis fabe	5/10	50
A. neri	2/10	20

*Number of infected plants/ no. of tested plants, 5 aphids were used per plant.

4. Photometrical characters and yield of the purified virus

The Potato isolate of AMV was purified by ultracentrifugation and PEG. The U.V. absorption spectrum of the purified virus preparation revealed typical spectrum of nucleoprotein (fig. 3).

The ratios of A260/280 and A280/260 (uncorrected for light scattering) were 1.54 and 0.64 respectively. The yield of the purified virus was about 1.8 mg/100g fresh weight of *N. glutinosa* leaves.

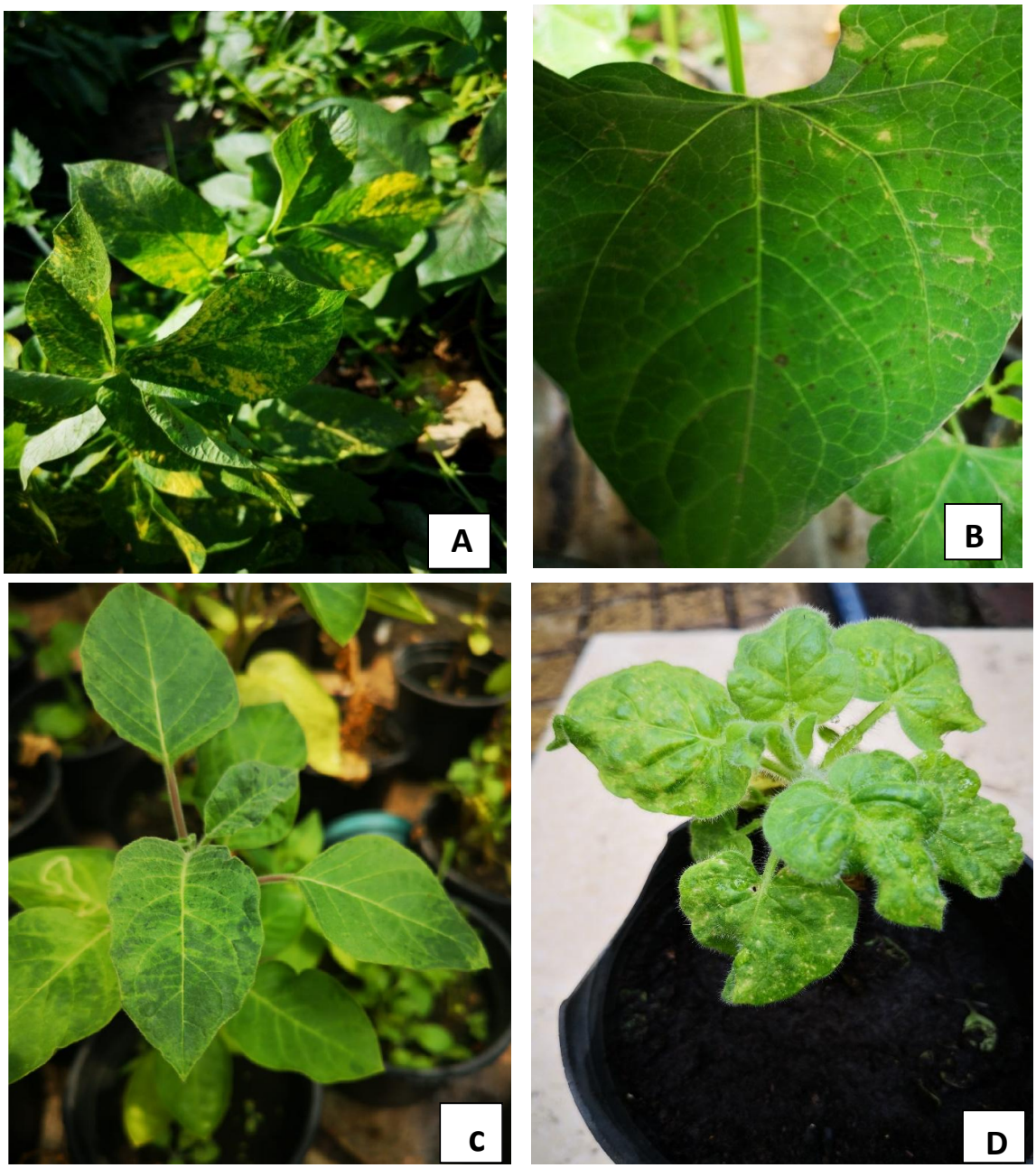

Fig. 1: Symptoms of AMV: A-Bright yellowing (Calico) natural infected *Solanum tuberosum* B-Necrotic local lesion induced on *Ph. vulgaris* C- severe mosaic on *Datura metal* D-chlorotic local lesion, mosaic and severe distortion and leaf malformation

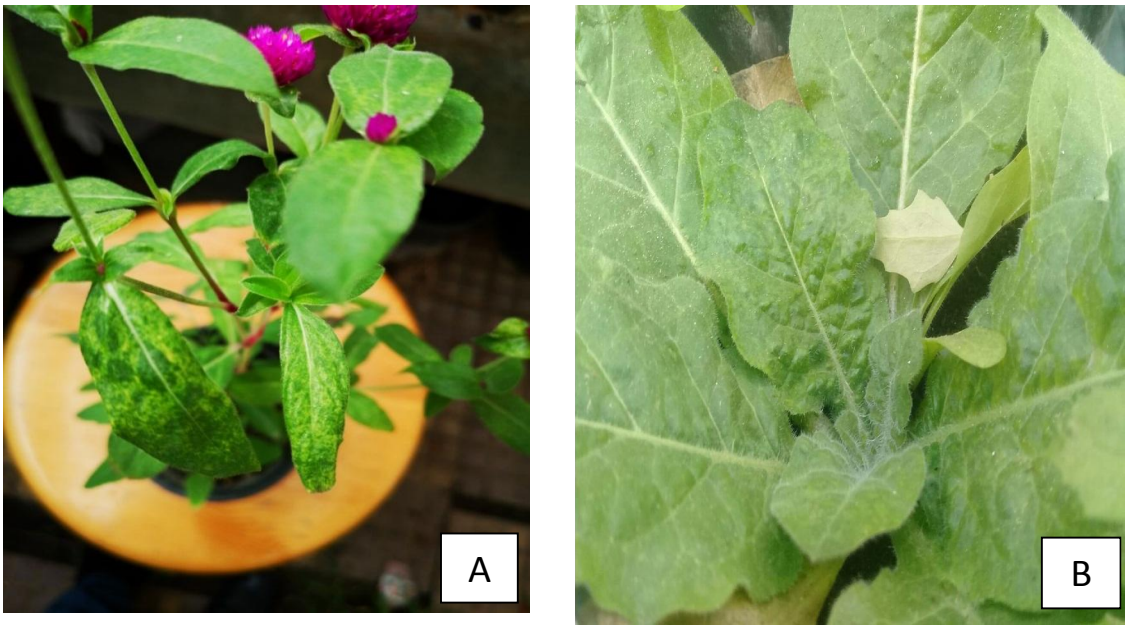

Fig. 2: Symptoms of AMV: a- chlorotic local lesions followed severe mosaic on *Gomphrene globose* and b- mild mosaic on *Nicotiana repanda*

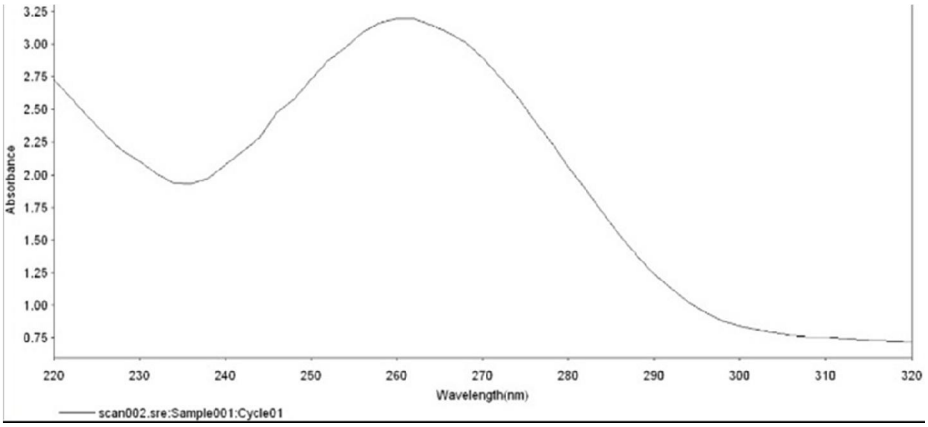

Fig. 3: Ultraviolet absorption spectrum of AMV purified from *N. glutinosa*.

Production of AMV antiserum

An antiserum against AMV was produced. Antiserum titer was determined by indirect ELISA. Positive ELISA values were obtained up to dilutions of 1:6400 and not with 1:12800 (Table 3)

Table 3: Indirect ELISA absorbance values (E 405 nm) of extract of AMV infected *N. glutinosa* plants in various dilutions of AMV antiserum*

Antiserum dilution	First week		Second week	
	Infected	Healthy	Infected	Healthy
1:4X10^2	3.297	1.184	2.258	1.012
1:8x10^2	1.987	0.817	2.414	0.713
1:1.6X10^3	1.295	0.605	1.579	0.563
1:3.2X10^3	0.701	0.256	0.645	0.534
1:6.4X10^3	**0.527**	**0.247**	**0.524**	**0.475**
1:1.28X10^4	0.397	0.261	0.424	0.449
1:2.56X10^4	0.318	0.203	0.330	0.190
1:5.12X10^4	0.327	0.195	0.327	0.191

*The experiment was repeated twice and indirect absorbance values at 405 nm are average of two replicates each.
*Absorbance value of at least double that of the healthy control were considered positive.

Discussion

AMV is one of the most significant infections worldwide and has a wide host range (Parrella *et al*., 2011; Jaspars and Bos, 1980). AMV showed up on potato plants in a few area causing serious loses (El-Helaly *et al*., 2012). AMV isolate infecting potato crop in Al Nubaria area, El Behaira governorate was isolated and identified. Based on symptoms produced on diagnostic hosts and serological reactions with antisera to AMV, PLRV, PVY, and PVX, utilizing indirect ELISA. The infection separated from normally tainted potato plants, developed in, with bright yellowing (calico) was identified as AMV in many countries, Isolate could be similar to those described by Hajimorad, (1991); Fatallah, (1999); Fegla and Younes, (1999), Xu and Nie, (2006), El-Helaly *et al*., (2012), Abdalla *et al*., (2016) and El-Abhar *et al*., (2018).

In this investigation, AMV was isolated from naturally infected potato plants collected from, Al Nubaria region, El Behaira governorate, showing bright yellow (calico) symptoms. These results match also isolated this virus from potato (El-Helaly *et al*., 2012; Zaeid, 2013). AMV can be readily detected in *Nicotiana glutinosa* showing no symptoms and the virus cannot be determined with certainty from the symptoms appearing on any type of host plant (Hajimorad, 1990), as more than once plants were tested that had been infected with no symptoms and gave high virus concentrations when tested by ELISA. In this work, symptoms produced on the artificially inoculated plants by the virus isolate (nine plant species and cultivars belonging to four families, Amaranthaceae, Chenopodiaceae Solanaceae, Leguminosae) varied in severity in the selective host plants studied from Necrotic local lesion, chlorotic local lesion, mosaic, leaf malformation Isolation gave typical symptoms where it induced with the *Phaseolus vulgaris* and *Ch. amaranticolor* necrotic local lesion, while it did not give with Datura metal. Put induced chlorotic local lesion followed by leaf deformation on *N. glutinosa*; mosaic on *N. repanda* and *G. globosa*. These results were in parallel with those reported by Jaspers and Bos (1980); Sutic *et al*. (1999) and Hajimorad (1990). AMV isolate was transmitted non-persistently by *A. fabe and A. nerii*, these results are in harmony with the finding of Zaeid (2013). AMV was purified. Some properties of virus particules were determined and injection rabbit with purified virus prepared specific antiserum. Results show that the absorption spectrum of the purified is typical for nucleoprotein. The yield of the virus as well as its spectrophotometrical data such as A260/280, A280/260 fall in the range reported for AMV. The titer of antiserum obtained for AMV was 1: 3200 (Avgelis and Katis, 1989; Malapi-Nelson, 2008; Hajimorad and Francki, 1988 and Fath-Allah,1999; Zhao *et al.,* 2020).

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