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Isolation and Identification of the Causal Virus Aucuba Disease in Egypt

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The infected potato plants showed aucuba symptom characteristic to this virus (brilliant and extensive yellow mottle on lower and middle leaves reaches their tips, brown patches and sunken brown areas were frequently observed on the tubers surface) were used as a virus source. Isolation and identification of the virus was performed through studying biological, serological and molecular characters. Electron microscopy helped for illustration of morphology of virus particles as well as alteration in cell organelles. The virus systematically infects most of solanaceae plants, but become diverse to different hosts as it induced local lesions and top necrosis on *Capsicum annuum* L. cvs. California Wonder & Godion as well as *Lycopersicon esculentum* Mill. cvs. Streen B and Casel Rock. *Solanum tuberosum* L. cv. Cara, *Nicotiana glutinosa* L., *Nicotiana benthamiana* gave systemic infection only. On the other hand, systemic symptomless infection had occurred with *N. tabacum* L. cvs. White Burley and Xanthi-nc. Whereas, PAMV didn't infect *Ch. amaranticolor Cost.Reyn.* and *D. metel* L. The virus was transmitted by aphids (*M. persicae*) in a non-persistent manner only when the aphids fed firstly on PVY^N source for 10 min. This indicated a kind of synergistic effect between the two viruses which controlled by role of PVY^N CP and HC-Pro. The ratio of transmission increased by exceeding the acquisition feeding period. The virus isolate showed positive reaction only with polyclonal antibodies specific to *Potato aucuba mosaic virus* (PAMV). Electron microscopy showed spindle-shaped like inclusions as aggregated virus particles slightly flexuous in longitudinal and cross sections of PAMV-infected cells; the virus induced different abnormalities in the structure of chloroplasts and mitochondria. The CP genes of PAMV were detected using RT-PCR with specific primers.

INTRODUCTION

Potato aucuba mosaic Potexvirus (PAMV) has been reported to occur in many regions where potato crop is grown (Todorović *et al.*, 1997). It was recorded to infect potato plants in Egypt (Kabiel, Sannaa 1993). The virus belongs to the genus *Potexvirus* and infects a number of plant species causing a variety of symptoms. The main diseases caused by PAMV are described as potato aucuba mosaic; potato pseudo-net necrosis; potato tuber blotch, whereas it produces yellow spotting or necrosis on potato leaves and necrosis in potato tubers. The effect on yield seems to be small but the tuber necrosis found in some potato varieties with certain strains is important (Beemster and Rozendaal, 1972 and Kassanis and Govier, 1972). PAMV is the only definite potexvirus species known to be transmitted by aphids but requires for its non-persistent transmission, a potyvirus-encoded protein known as the "helper component" (HC) (Govier and Kassanis, 1974 and Manoussopoulos, 2000). According to their frequency of

transmission Kassanis (1961) placed them in four groups: the first group was transmitted with the same frequencies from plants infected with either PVY or PVA whereas those of the second one was transmitted more readily from plants infected with PVY than from those with PVA. Strains of the third group were transmitted only from plants infected with PVY and finally those of the fourth group were transmitted only occasionally from plants infected with either of the helper viruses. Therefore, the present study aimed a) isolation and identification of the causal virus of aucuba disease, b) explain the synergistic mechanism between the two viruses under investigation, i.e., PVY^N and PAMV through insect transmission of the latter by the helper component and aphid transmission factor [Aspartic-Alanine- Glysine (DAG)] tripeptide sequence provided by PVY^N

MATERIALS AND METHODS

Virus isolation

Leaf samples of potato (*Solanum tuberosum*) showing aucuba disease were collected from the potato fields at the Dakahlia Governorates and used as a source for virus isolation. The samples were tested serologicaly using PAbs specific for 9 viruses infecting potato (PVX, PVY, PLRV, PVM, PVA, PVS, AMV, TRV and ToRS) (Agdia, Inc, USA) and with PAbs specific for *Potato aucuba mosaic virus* (PAMV) (provided by Molecular Plant Pathology Dept. Virology Group, The Royal Veterinary and Agricultural University (KVL), Copenhagen-Denmark). The positive samples reacted with PAMV antibodies were used as a source for the virus biologically purified from the single local lesion produced on *Capsicum annuum* cv. California Wonder plants according to the technique described by Khan and Monroe (1963). The local lesions produced by the virus were used as source for mechanical inoculation of healthy *S. tuberosum* cv. Cara.

Identification of the virus biologically

(a) Host range

The infectious saps extracted from the propagative host were used for mechanical inoculation of test plants that belongs to families Amaranthaceae, Chenopodiaceae, Compositeae, Curcubitacae , Fabaceae and Solanaceae. Visual examination for symptom development of the inoculated host plants was carried out at 5 days intervals post inoculation. The symptomless plants were verified by reinoculation to healthy test plants.

(b) Insect transmission:

Aphid transmission of PAMV.

Virus-free green peach aphids (Myzus persicae Sulz) were used in this study as described by Kaiser (1979) for the transmission of virus isolate. The acquisition feeding period on PAMV infected potato plants (S. tuberosum cv.Spunta) was 10 min. followed by transferring to 10 healthy plants (10 aphids/plant) for 10 min as inoculation feeding period. The inoculated plants were sprayed with insecticide (malathion 0.15 %) after feeding to kill the aphids, kept under an insect-proof glasshouse (25-28°C) for symptoms development.

Aphid transmission of PAMV with the aid of the helper virus (PVY).

The experiment was carried out according to Manosopolous (2000 & 2001) with some modifications. Virus-free (*M. persicae*) aphids were starved

for 1-2 h and then fed on the helper virus source (PVY isolated by Abd El-Mohesn, Nashwa, 2003) *D. metel* leaves 20 days post inoculation for 10 min. The viruliferous aphids were divided into two groups, the first group were transferred to indicator plants for PVY, i.e., *N. tabacum* cv. White Burely to confirm the PVY transmission, the second group of aphids were transferred to the PAMV source (*C. annuum* leaves 20 days post inoculation) and fed for different periods (20s, 40s, 1 min, 5 min, 10 min), then were transferred to the indicator plant for PAMV (*C. annuum* 10 aphids/plant) for 1 hr before killed by insecticide. Ten plants were used for each period. The plants were kept under controlled conditions (25-28°C) in an insect-proof greenhouse for symptoms development.

(c) Electron microscopy:-

Ultrathin sections of *S. tuberosum* cv. Cara (21 days post inoculation) were prepared according to Spurr (1969) using 1x1 cm2 of virus-infected leaf samples. They were examined with transmission electron microscope (Philips 301, Specialized Hospital, Ain Shams Univ. Cairo, Egypt).

Serological detection.

The PAMV inoculated potato plants were subjected to serological detection by DAS-ELISA using PAbs specific to PAMV

Detection of virus coat protein gene by reverse transcriptase-polymerase chain reaction (RT-PCR).

Total RNA was extracted from *S. tuberosum* cv. Cara infected with PAMV (21 days post virus inoculation) by SV total RNA isolation system by spin protocol as recommend by the manufacturer instructions of Promega based on Kobs (1998). The cDNA first strand synthesis was primed with the reverse primer (5` TCA GGG GAT TAG GGA A 3`) which are complementary to the sequence derived from PAMV available in GeneBank (accession # NC_003632) for the 3` end of genome between positions 7040 and 7022, as reported by Xu *et al.* (1994) using DNASTAR Lasergene (DNASTAR Inc, MD). The PCR was performed with foreward primer (5` TCA GTC GCA CCT TGG AAC G 3`) in which the 19 residues are the DNA version of the PAMV sequence between 6149 and 6167. The High Performance Liquid Chromatography (HPLC) purified primer pairs were designed by Thermo Hybraid GmbH, Germany. The CP gene synthesis and amplification were obtained using cDNA synthesis kit (Promega corp, Madison, USA). The temperature degrees used for thermocycling programs in PCR shown in Table (1).

Table (1): The temperature degrees used for amplification for PAMV CP gene.

*Thermocycling conditions	Tempe-rature (C)	Period (min)
1-Initial denaturation	-	-
2- Denaturation	94	1
3- Annealing	55	2
4- Extension	72	2
5-Final Extension	72	10

* The cycle numbers repeated 30 times for the target gene.

RESULTS AND DISCUSSION

Identification of the virus biologically.

(a) Host range:

Many researchers determined that PAMV cause serious damage in potato (Vicchi and Garritano, 1999; Gera and Marco, 2001and Loebenstein, 2001). In addition, hosts other than potato including tobacco (*N. tabacum*), tomato (L. *esculantum*), pepper (*C. annuum*). *Ch. amaranticolor* and *Ch. quinoa* were also infected (Juo and Rich, 1961; Harrison, 1971 and Smith, 1972).

It was found that the collected potato samples exhibit a brilliant and extensive yellow mottle on lower and middle leaves reaches to their tips, then spreads all over the plants. Brown patches and sunken brown areas frequently observed on the tubers surface and pith as necrosis symptoms developed on infected plants (Table 2 & Fig. 1). The virus was found to infect hosts other than potato plants (Table 2 & Fig. 1), and the results as follows:-

Hosts reacted with local and systemic symptoms include: *C. annuum* L. cvs. California Wonder and Godion, *C. frutescence* L., *Lycopersicon esculentum Mil*L. cvs. Streen B and Casel Rock. These results are in agreement with Beemster and Rozendaal (1972); Kassanis and Govier (1972); Smith (1972) and Todorović *et al* (1997). But *C. annuum* is unsuitable as a differential host for PAMV. Since the same symptoms was induced by *Potato X Potexvirus*. *C. frutescence*, however, was a suitable for PAMV differentiation since none of the PVX isolates induced top necrosis (Kratchanova and Ivanoova, 1979).

Hosts reacted with systemic symptoms varied from aucuba symptoms to mild mottle and mosaic include: *S. tuberosum* L. cv. Cara, *N. glutinosa* L., *N. occidentalis*, *N. benthamiana*, *N. depneyi Domin.*, *Ph. floridana Rydb.* and *D. stramonium* L.. These results are in agreement with several researchers (Beemster and Rozendaal, 1972; Kassanis and Govier, 1972; Smith, 1972; Beemster, 1981 and Manosoupoloous, 2000 & 2001).

Hosts reacted with symptomless infection include: *N. tabacum* L. cvs. White Burley, Xanthi-nc, *Petunia hybrid vilm.* Similar results were reported by Smith (1972) and Beemster (1981).

On the other hand, some hosts are not susceptible to PAMV include: *Gomphrena globosa* L., *Ch. album* L., *Chenopodium amaranticolor Cost&Reyn.*, *Ch. bushmanium*, *Ch. quinoa* wild., *Zinnia* sp, *Cucumis sativus* L. cv. Ashley and *Phaseolus vulgaris* L. cv. Giza 3, *D. metel* L., *Nicandra physaloides* L., *N. rustica* L., *N. tabacum* L. cv. Turkish. Juo and Rich (1961) and de Bokx (1975) found that inoculation of PAMV on *Ch. amaranticolor*, *Ch. quinoa*, *G. globosa*, *Ph. vulgaris cv.* Bruine Stam gave local lesions. It was suggested that the difference might be attributed to genetic variation in PAMV strains.

(b) Insect transmissions:-

Aphid transmission of PAMV.

The experiment for testing transmissibility of PAMV isolate by aphid (*M. persicae*) showed that the isolate was non-aphid transmissible, thus the inoculated potato plants didn't show any symptoms.

This result for testing PAMV transmissibility agree with that obtained by Clinch *et al* (1936) and Kassanis (1961), who reported that PAMV can be transmitted by aphids from plants coinfectd with either of two potyviruses, PVA and PVY, but

not from plants containing PAMV alone.

Aphid transmission of PAMV with the aid of the helper virus PVY.

In this experminet, it was found that PVY^N was easily transmitted by *M. persicae*. In addition, aphids that firstly probed on PVY^N infected *D. metel* leaves, and then on PAMV source could transmit the virus to *C. annuum* plants.

It was also observed that the percentage of insect transmission of PAMV increased by increasing the aphid acquisitions feeding period (Table 3).

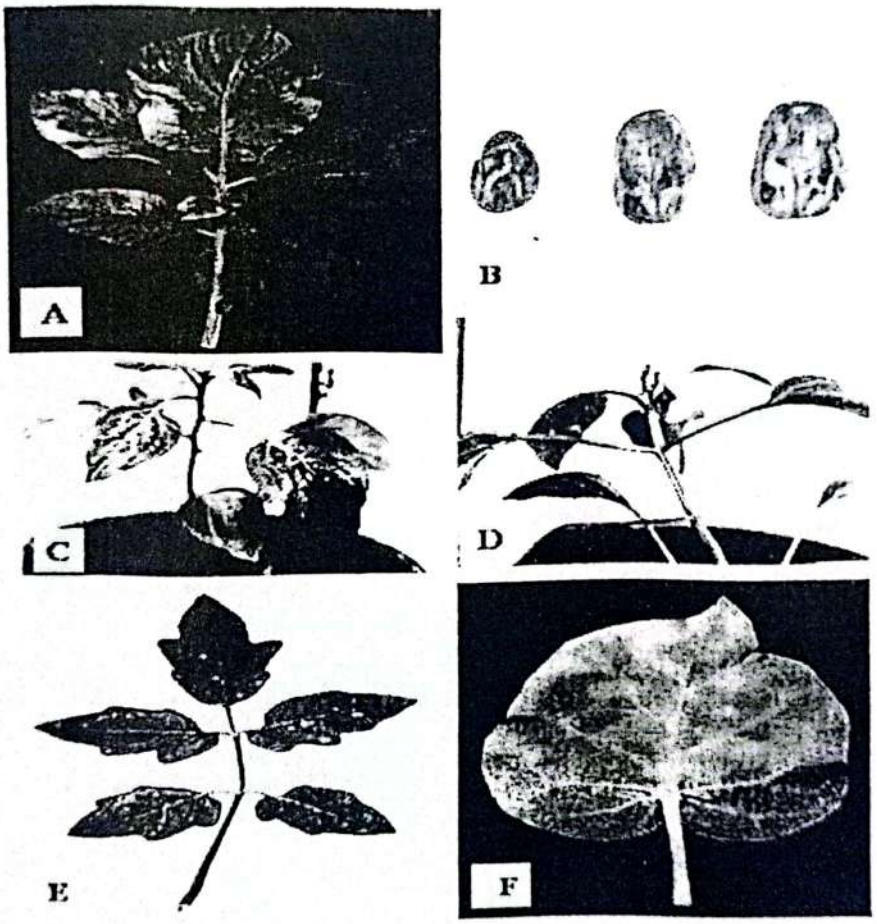

Fig (1): Typical symptoms of PAMV on potato plants resulting from naturally virus infection (A) symptoms on foliage occurring as a transient diffuse yellowish mottle of the leaf and (B) symptom on tubers, tuber necrosis develops as necrosis on the surface (brown patches and sunken brown areas). (C) Brown irregular local lesions on the inoculated leaves of *C. annuum* cv. Godion developed after 12 days from inoculation with PAMV. (D) Systemic leaf necrosis in pepper after 24 days appeared as severe necrosis on the top of the leaves followed by plant death. (E) PAMV produces small yellow spots on inoculated leaves of L. *esculentum* cv. Streen B after 15 days from inoculation; followed by systemic infection (the same symptoms) on the young leaves with malformation after 26 days. (F) *N. glutinosa* develops systemic infection of PAMV as yellow blotches and mosaic with vein banding after 24 days from inoculation.

Table 2. Reaction of different hosts for PAMV using mechanical inoculation.

Host	Cultivar	Symptoms
Amaranthaceae		
Gompherna globosa L.		-
Chenopodiaceae		
Chenopodium album L.		-
Ch. amaranticolor Cost.Reyn.		-
Ch. bushmanium		-
Ch. quina wild.		-
Compositeae		
Zinnia elegans		-
Curcubitaceae		
Cucumis sativus L.	Ashley	-
Fabaceae		
Vicia faba L.	Giza 2	-
Ph. Vulgaris L.	Giza 3	-
Solanaceae		
Capsicum annuum L.	California Wonder	NLL+SN
	Godion	NLL+SN
Capsicum frutescens L.		NLL+SN
Datura metel L.		-
Datura staramonium L.		M
Lycopersicon esculentum Mill.	Streen B	CLL
	Casel Rock	CLL
	Pett 86	-
Nicandra phycaloides L.		-
N. benthamiana		M+S
N. debneyi Domin		M
N. glutinosa L.		M+VB
N. occidantalis		M
N. rustica L.		-
Nicotiana tabacum L. cvs.	White Burley	SSL
	Samsun	M
	Turkish	-
	Xanthi -nc	SSL
Petunia hybrid Vilm.		SSL
Physalis floridana Rydb.		Mo
Solanum tuberosem L.	Cara	A

M=Mosaic; A=Aucuba; CLL =Chlorotic local lesion; NLL =Necrotic local lesion; SM =Severe mosaic; SN = Systemic necrosis; VN= Veinal necrosis; VB =Vein banding; Mo =Mottle; S =Stunting; SSL=Systemic symptoless gave CLL upon testing by reinoculation onto tomato plants; - =No reaction.

Table (3): Aphid transmission for PAMV.

No. Aphids/plant	Acquisition feeding period	Proportion of plants infected	
		No.	%
10	20 s	0	0
10	40 s	1	10
10	1min	1	10
10	5min	4	40
10	10min	6	60

These results confirmed that PAMV could be transmitted in the field by aphids, which fed firstly on PVY source or mixed infection by two viruses. This is the main procedure for PAMV spread in potato crop in addition to spreading by contact between plants. These data are confirmed by Kassanis and Govier (1971a, b) who showed that aphids are capable for PAMV transmission only after provides with PVY source. On the contrary, the aphids couldn't transmit PAMV when fed firstly on the virus-infected plants, then on PVY source. Govier and Woods (1971) deduced that supporter virus change the circumstance for aphid that helped in acquiring PAMV.

Concerning factors responsible for PAMV movement *via* aphids by (the specific adsorption hypothesis), Pirone (1969) explain the correlation between carrier and virus through non-persistant transmission. Baulcombe *et al.* (1993) concluded that potyvirus-assisted aphid transmission of PAMV, through DAG tripeptide sequence in both viruses aside N-terminus in CP gene of PVY strain controlled aphid transmission companied with HC-Pro gene. Differences among the PAMV CPs seem likely to be the most effective factor on the extent to which the different PAMV strains were transmitted.

The mechanism of transmission of PAMV only when PVY present is account for the HC-Pro has to be acquired by the aphid's styles either during or before virus acquired. There is no insect (aphid) transmission if HC-protein is acquired after PAMV.

The HC-Pro properties are reviewed by Raccah *et al.* (2001) as the biologically active form of helper component appears to be a dimmer with molecular weight ranging between 100 and 160 KDa. Potyviral HC-Pro binds non-specifically to single-stranded nucleic acids, with a preference for RNA (Urcuqui-Inchima *et al.*, 2000). The protein contains two nucleic acid-binding regions.

In conclusion, PAMV transmission by *M. persicae Sulz.* confirmed that, the isolate was aphid transmissiible because it has the DAG (Aspartic-Alanine-Glysine) tripeptide sequence in its CP protein while the PAMV isolates which couldn't transmit, the DAS (Aspartic-Alanine-Serene) tripeptide sequence in their CP protein was found instead of DAG.

(c) Electron microscopy:-

Potexvirus group usually occur in high concentration in systemically infected hosts and the virus particles seem to induce remarkably uniform cytopathic effects and accumulate in the cytoplasm, forming massed aggregates that may exhibit conspicuous banding specially in meristematic dome cells (Appiano and Pennazio, 1972 and Pennazio and Appiano, 1975).

Data herein indicate that the ultrathin sections of potato leaf cells infected with PAMV show several patterns of filamentous particles arranged in aggregates. The PAMV particles appeared as fibrous mass within the cytoplasm and are not arranged in parallel but at random and appear strongly curved occoupied large area of the cell lumen (Fig. 2a) and occur as dense bands (Figs. 2b). It is consider that dense bands correspond to bundles of virus particles in which individual particles aggregates compactly in an end-to-end and side-by-side manner. Similar structure was described by Kassanis and Govier (1972).

The infection with PAMV induces structural modifications of intracellular organelles, the chloroplast ultrastructure showed full destruction, the lamella membrane system and chlorophyll were distrubed or inhibited. This may explain the chlorotic symptoms produce in potato leaves cv. Cara. In addition, some vesicles or vacuoles were observed within the chloroplast stroma (Fig. 2 c, d). These data are in agreement with those described for a virus belong to the same group i.e., PVX (Doraiswamy and Lesemann, 1974; Palilova *et al.*, 1978; Ambrosov *et al.*, 1981; Mayee and Sarkar, 1982 and Lupan, 1993).

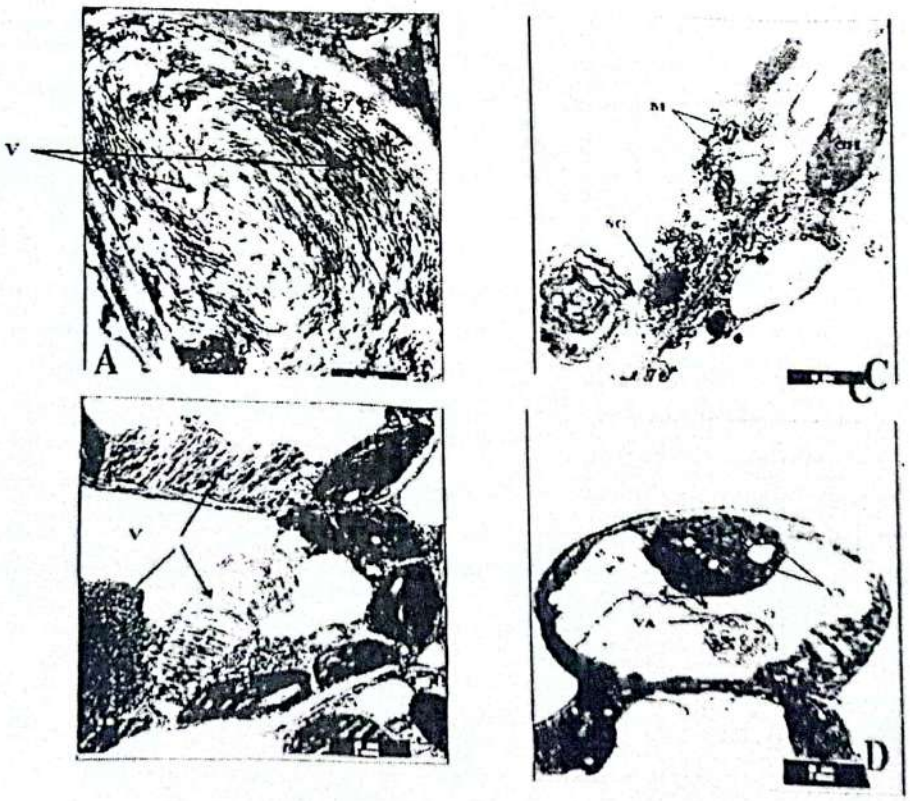

Fig. (2): Ultrathin sections of leaf cells of PAMV-infected *S. tuberosem* cv. Cara plants (21 days post inoculation) showing spindle-shaped like inclusions (A) aggregated particles slightly flexuous in cross section (magnification: 28,000-X), (B) Bundles of virus aggregates fill the spindle shaped structure (magnification: 8,000-X), while inoculated plants showing numerous vesicles (VS) and vacuoles (VA) within the cytoplasm and chloroplast (CH) and spherosomoes crystal (SC), in addition to mitochondria (M) malformation (C, D) (magnification: 13,000-X & 8,000-X, respectively).

ELISA detection.

Potato plants infected with PAMV reacted positively with polyclonal antibodies (PAbs) specific to PAMV and didn't react with any PAbs specific for other tested potato viruses (PVX, PVY, PLRV, PVM, PVA, PVS, AMV, TRV and ToRSV). Data agree with that mentioned before by Kassanis and Govier (1972) who found no serological relationship to PAMV with other potato viruses, but PAMV resembles viruses of the *Potato virus X* group morphologically. Artyukova and Krylov (1983) indicate that amino acids composition of the PAMV protein was similar to that of *Potato virus X*, differing only in theronine, leucine, histidine, tyrosine and methionine, these resemble those of other potexvirus.

RT-PCR detection.

The CP genes of PAMV were isolated using RT-PCR with specific primers and consist of 892 nt (Fig. 3).

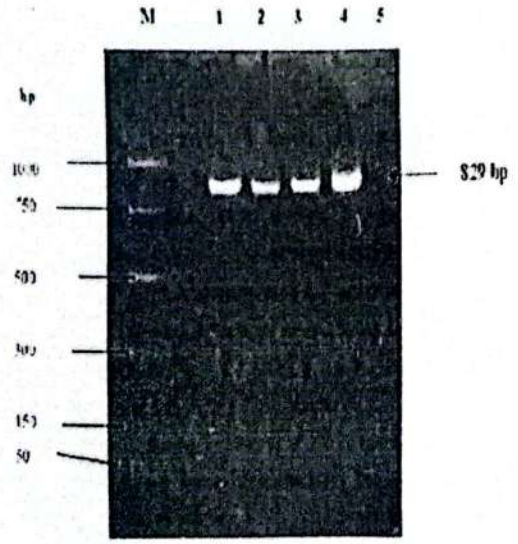

Fig. (3): Detection of CP genes of PAMV (b) RT-PCR, a PCR product with size of about 892 bp was amplified.Lanes 1, 2, 3, 4: PAMV isolated from infected *S. tuberosum* cv. Cara leaves, Lane :*S tuberosum* cv. Cara healthy control leaves, Lane M: marker (50, 150, 300, 500, 750, 1000 bp).

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