Characterizations of the Egyptian isolate of *Onion yellow dwarf virus* Infecting onion and development of Virus-Free plantlets

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ABSTRACT

Onion yellow dwarf disease is an economically important disease of onion (*Allium cepa* L.) caused by *Onion yellow dwarf virus* (OYDV). In this research we aimed to identify and molecularly characterize the isolate of the OYDV infecting onion plants in Egypt and to obtain OYDV-free plants from infected onions through tissue culture techniques. To achieve our aim, the virus has been isolated from naturally infected onion plants grown in five Egyptian Governorates, Gharbia, Qalyobia, Giza, Fayoum and Beni-Suef then mechanically transmitted onto healthy onion plants in an insect proof greenhouse. The virus identification was done by indirect ELISA using specific antiserum and confirmed by Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) using virus-specific primers. The molecular characterization for the Egyptian isolate has been performed through cloning and sequencing for the OYDV CP gene which amplified by RT-PCR then cloned and sequenced in the TOPO cloning vector. The CP gene sequence has been submitted to the GenBank and compared to other available isolates of OYDV. Sequence alignment and phylogenetic analysis showed that the Egyptian isolate of the OYDV is related to other isolates from Japan, Netherlands, Israel, Brazil, China, India, and Poland with a similarity ranged from 70 to 93% among those isolates available on GenBank. The virus was eliminated from infected onion plants using cytokinin (kinetin) in a tissue culture line to obtain the OYDV-free plantlets. The effects of different concentration levels of kinetin as well as its efficacy on the OYDV elimination and regeneration of infected onion bulbs were determined.

Key words: Onion, *Onion yellow dwarf virus*, cloning, RT-PCR, ELISA, Tissue culture, Cytokinin.

INTRODUCTION

Onion (*Allium cepa* L.) is one of the most important field crops cultivated in many Governorates of Egypt primarily grown for both local consumption and export. Egypt is one of the biggest onion producers of the Mediterranean region with production of 1,903,000 tons in season 2013 (FAO, 2014). Onion was grown permanently in the open fields without protective shelter and exposed to injury with viral diseases, one of these is OYDV. *Onion yellow dwarf virus* is an aphid-borne *Potyvirus* and considered as a main viral pathogen, that causes substantial losses in *Allium* crops, in many countries including Egypt, Sudan, India and Brazil (Abd El Wahab *et al*., 2009, Fayad-André *et al*., 2011, Kumar *et al*., 2012 and Mohammed *et al*., 2013). OYDV is usually very serious and often reaches an epiphytotic level leads to great reduction of bulb yield and seed production (Conci *et al*., 2003). The natural infection in onion crop with OYDV results in a significant reduction of 27.42 % in pseudo-stem length, 29.14% in number of leaves, 31.9 % in plant weight and 41.8 % in weight of bulb (Elnagar *et al*., 2009). This virus was isolated for the first time in 1972 by

Allam and Ghalwash, the highest natural incidence (70.6%) occurred in onion fields was reported by Abdel Wahab *et al*. (2009), causes symptoms of mosaic, yellow streak, striping, dwarfing, curling and distortion of flower stems. The virus is transmitted by over 50 aphid species (Van Dijk, 1993), mechanically through sap inoculation and seeds (Hoa *et al*., 2003 and Abdel Wahab *et al*., 2009). Biological (mechanical inoculation, indexing), serological (enzyme-linked immunosorbent assay-ELISA), and molecular (polymerase chain reaction-PCR) methods are used for detection and identification of plant viruses (Taskin *et al*. 2013).

The genus *Potyvirus* is the largest genus of the family *Potyviridae*, with nearly 200 definite and tentative species (Berger *et al.,* 2005). Virions of potyviruses range in length from 700 to 900nm and encapsidate a monopartite, single-stranded RNA genome (10kb) characterized by 5` un-translated region (5` UTR), a single open reading frame (ORF) and a 3` UTR which has a polyadenylated (poly A) tail. The ORF encodes a single large polyprotein that is subsequently processed into ten functional proteins (Adams *et al.,* 2005).

Tissue culture techniques are the most viable methods for producing virus-free and uniform plants in a short period, regardless of season of the year. However, decreasing regeneration ability is the main factor limiting the efficiency of onion micropropagation (Kamstaityte and Stanys, 2004). Plant Growth Regulator (PGR) is a term which includes hormonal substances of natural occurrence (phytohormones) as well their synthetic analogues (Basra, 2000). PGRs can regulate a wide range of processes such as cell division, shoot initiation, growth, development, control of chloroplast division, modulation of metabolism and morphogenesis (Chernyad'ev, 2000; Kieber, 2002; Gray, 2004 and Hirose *et al*., 2007). It has been suggested that adding some PGRs to the medium may suppress viruses in infected cultures, and high concentrations stimulate host rather than viral protein synthesis (Walkey, 1991 and Clarke *et al*., 1998).

Therefore, this work aimed to (i) Identify and characterize OYDV in the onion plants in Egypt. (ii) Clone and sequence the coat protein gene of the Egyptian isolate of OYDV and comparing it with other OYDV isolates reported in the GenBank database. (iii) Study the influence of therapeutic doses of kinetin (6-Furfurylaminopurine) on production of virus-free onion plantlets and improve its regeneration ability through *in vitro* micropropagation.

MATERIALS AND METHODS

Virus isolation:

300 samples of naturally infected onion plants (*Allium cepa* L. cv. Giza 20) showing typical symptoms of OYDV were collected from five Governorates, Gharbia, Qalyobia, Giza, Fayoum and Beni-Suef in the winter vegetation periods during the growing season 2012/2013. The samples were labeled and brought to the laboratory in ice box. The identity of the causal agent was confirmed to be positive for OYDV by ELISA (Clark and Adams, 1977) then confirmed by RT-PCR. Positive samples, after biological purification through local lesion technique described by Kuhn, (1964), were used as a source plants for mechanical transmission and molecular characterization.

Plant materials and virus transmission:

Healthy onion seeds were obtained from Ministry of Agriculture

and land Reclamation, Central Administration for Seed Production, Giza, Egypt and cultivated in greenhouse under insect free conditions.

The seeds were disinfected before the start of the experiment. The disinfection was achieved with water and chlorine 1:1 for ten minutes, and then they were washed five times with distilled water. The seeds were planted in four seed trays (25 cells) containing sterile sandy clay soil with 2.5 cm deep, following with irrigation only three times in the first week, frequent irrigation every week and the irrigation is discontinued for a week before seedlings. Two-month old of the developed green leaves were inoculated with homogeneous mixture of virus infected onion leaves and 0.03M phosphate buffer (1:4w/v, pH8.0) dusted before with carborundum (400 mesh) then periodically observed for symptoms appearance. Half parts of onion plants (leaves and bulbs) were randomly selected and used for assessing the virus transmission status by indirect ELISA and the rest of the infected leaves and bulbs were used in molecular study and virus elimination, respectively.

Serological detection of OYDV

Indirect ELISA was applied According to method described by Clark and Adams 1977 to test leaf of onion plant samples using OYDV-specific antiserum which supplied by LOEWE Biochemica, GmbH, DSMZ, Germany.

Molecular characterization of the OYDV:

Total RNA extraction and primer design:

Total RNA was isolated from symptomatic onion plants using gene jet ™ RNA purification kit (fermentas, USA) according to manufacturer's manual.

To design PCR primers specific for the OYDV coat protein (CP) gene, the sequences of CP gene of different OYDV isolates were downloaded from GenBank and aligned together to get the conserved sequence. The forward primer was designed to begin with the start codon of the coat protein and its sequence was, OYDV-CPf: ATGATTGAAGCATGGGGTTA while the Reverse primer designed at the 3` end of the coat protein gene to be ended with the stop codon of the gene and its sequence was, OYDV-CPr: TTACATCTTAATGCCCAGCAG.

Reverse transcription- polymerase chain reaction (one step RT-PCR)

Total RNA extracted from the infected onion plants was used as a template for the one step RT-PCR amplification reactions using Verso TM one step RT- PCR kit (Thermo scientific). The RT-PCR was performed in 25 µl total volume containing 4.75 µl of nuclease - free water, 3ng/µl of total RNA, 12.5 µl of one step PCR master mix (2x), 3 µl of 10 µM of each primer,0.5 µl Verso enzyme mix and 1.25 µl RT-Enhancer. Reverse transcription reaction started with incubation at 50 ºC for 15 min, followed by denaturation at 95 ºC for 2min. The amplification reaction was performed by 35 cycles starting with denaturation at 95 ºC for 1min, primer annealing at 50 ºC for 1minute and extension at 72 ºC for 1 min. Additional extension step at 72 ºC for 7 minutes was added. The RT-PCR products were stained with gel star (Lonza, USA) and analyzed on 1% agarose gels in 0.5X TBE buffer then visualized by UV illumination using Gel Documentation System (Gel Doc 2000, Bio-Rad, USA).

Cloning and sequencing of OYDV-coat protein gene:

The RT-PCR fragment of the OYDV- CP gene was purified and ligated into pCR4-TOPO cloning vector (Invitrogen, Cat: K4530-40). The TOPO/OYDV-CP recombinant plasmid was transformed into DH5α- *E.Coli* competent cells using heat shock at 42 ºC for 45 sec (Ausubel *et al.,* 1999). The recombinant plasmid was amplified in the *E.Coli* liquid culture then isolated and purified using the QIAprep Spin Miniprep Kit (Qiagen, Germany). The obtained recombinant plasmid was analyzed with restriction digestions using *Eco*RI enzyme and verified by automated DNA sequencing**.** The forward and the reverse primers those previously used for RT-PCR were used for DNA sequencing. Blast search was performed at NCBI to search for sequence similarity. The nucleotide sequence was assembled using DNAMAN Sequence Analysis Software (Lynnon BioSoft. Quebec, Canada). To perform the phylogenetic tree; the nucleotide sequences of the OYDV-CP (1017 bp) were compared with sequences of the Coat protein gene from different OYDV isolates available in GenBank. The accession numbers of the isolates those compared to our isolate were: AB000472, AB000473, AB000474 and AB000837 from Japan, AF071226 from Israel, AF228414 from Brazil, AJ292224 from China, DQ519034 from India, HM473189 from Egypt (Garlic isolate), Y11826 from Netherlands, KF862685.1 (isolate 161), KF862683 (isolate 53), KF862684 (isolate 72), KF862686 (isolate OY206), KF862688 (isolate OY208), KF862689 (isolate OY209) and KF862691 (isolate 220).

Virus elimination in a tissue culture line:

Infected onion bulbs collected from greenhouse were washed several times with tap water. The protective skin and outer two fleshy sheaths were removed from each bulb, then disinfected by immersion in 20% commercial sodium hypochlorite solution with one drop of tween 20 for twenty minutes and sequentially rinsed with sterilized distilled water.

Sterilized bulbs were further pared down by 3 sheaths in addition; 1mm from the stem base was excised and discarded. The resulting basal disc explants were re-sterilized by 70% (v/v) ethanol solution for ten second and then vertically divided into two equidistant sections along the stem bases then plated on regeneration MS medium (Murashige and Skoog, 1962) supplemented with $30gL^{-1}$ of sucrose and $8gL^{-1}$ agar, pH 5.7, without kinetin (as a control). Other group was supplemented with different concentrations $10mg/L^{-1}$, $20mg/L^{-1}$ and $30mg/L^{-1}$ of kinetin to study the antiviral activity on virus elimination. Thirty sections of basal disc were used in each treatment. After regeneration, the virus-free plantlets from the rapid growth treatment were divided as mentioned above and multiplied on control MS media. In all *in vitro* stages, the cultures were incubated at 28°C under 18h photoperiod with 3000 Lux light intensity for four weeks (Mohamed-Yasseen *et al*., 1993).

Therapy Efficiency (TE):

At the end of each *in vitro* treatment, therapy efficiency (TE %) was determined according to the rate of success in removing virus and percentage of surviving plantlets as follows:

$$TE = \frac{\text{Number of survival virus-free plants}}{\text{Total number of plants in each treatment}} \times 100$$

RESULTS:

Virus isolation and symptomatology:

Onion yellow dwarf virus was successfully isolated from onion plants (Giza 20) in five Governorates, *i.e.* Gharbia, Qalyobia, Giza, Fayoum and Beni-Suef. Naturally infected onion plants showed symptoms of OYDV such as leaf curling, stunting, malformation and yellow striping (Fig. 1).

After some difficulties, the OYDV was successfully transmitted using mechanical inoculation from the infected source plants to the healthy ones. The high percentage of OYDV transmission occurred when infected green leaves were used as a source of inoculum, diluted with phosphate buffer (pH8.0) and using heavily rubbing during the inoculation process without leaf injured. The successful transmission was confirmed utilizing back inoculation and/or ELISA. All healthy plants inoculated with OYDV showed symptoms of dwarfing and yellowing on leaves after 17 days from inoculation.

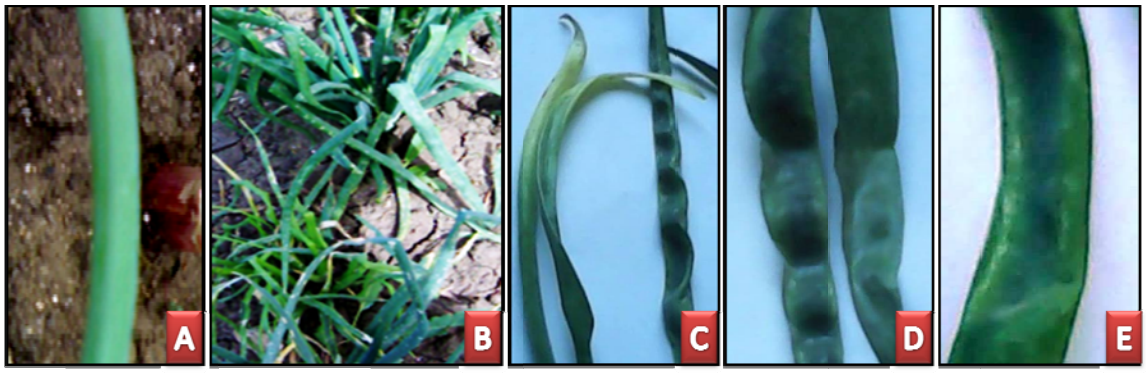

Fig. 1: Natural infection as well as mechanical transmission of OYDV on onion leafs. A: Healthy onion leaf (*Allium cepa* L.). B, C and D: Naturally infected onion plants from several locations showing stunting symptoms (B); chlorotic, white necrotic and yellowing (C); leaf curling, yellow striping and malformation (D). E: Mechanically inoculated onion plant showing yellow striping and leaf curling as OYDV symptoms.

Indirect ELISA:

Both naturally infected and mechanically inoculated samples of leaves and bulbs were indexed for virus detection by indirect ELISA using specific antiserum. Results showed that, all samples were positive for *Onion yellow dwarf virus* after 30 min of adding substrate. The absorbance value greater than three times fold of a negative control value (> 0.30) and with a visually detectable yellow color was rated as a positive reaction as indicated in table 1.

Table 1: Detection of OYDV by indirect enzyme-linked immune-sorbent assay

Sample	ELISA value (*A*405)					
	Healthy	1	2	3	4	5
Leave	0.11	0.35	0.33	0.41	0.34	0.37
Bulb		0.34	0.40	0.35	0.38	0.43

Molecular characterization of the OYDV-CP:

Amplification of the full coat protein gene:

The RNA extracted from both naturally infected and mechanically inoculated onion plants were used as template for the RT-PCR amplification of the OYDV-CP gene. To molecularly characterize the OYDV-CP, specific PCR primers were designed to amplify the full sequence of the coat protein gene. Electrophoresis analysis of RT-PCR product showed a single band at ~1017 bp representing the expected size for the amplified fragment of the OYDV-CP for either the naturally infected or the mechanically transmitted onion samples (Fig.2). No fragments were amplified from the RNA extracted from healthy plant controls.

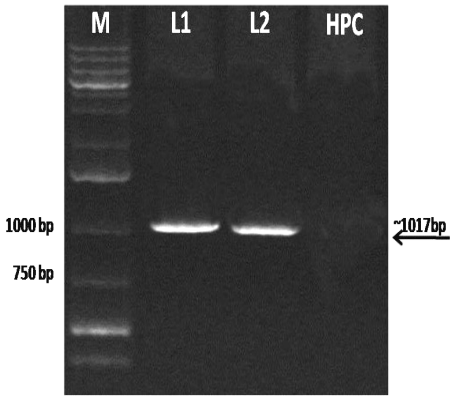

Fig. 2: Electrophoresis analysis for the RT-PCR product of the full coat protein gene of OYDV. M: 1kb DNA ladder. L1: Naturally infected onion sample. L2: Mechanically inoculated sample. HPC: Healthy plant control.

Restriction analysis:

The selected clones containing the recombinant OYDV-CP plasmid were digested using the *EcoRI* restriction enzyme. The electrophoresis analysis for the digested clones showed a clear band at the expected size of the coat protein gene of OYDV (1017bp) as well as a characteristic band at the size of the digested TOPO vector (4000 bp) that showed in figure 3 .

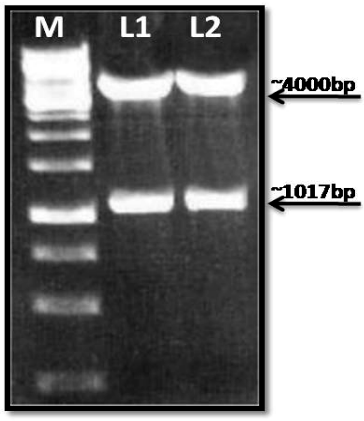

Fig. 3: Restriction analysis for TOPO/OYDV-CP positive clone. L1 and L2: Digestion of the plasmid using *EcoRI* showing the release of the OYDV-CP gene at 1017 bp the digested TOPO vector at ~4000 bp. M: 1 kb molecular weight DNA ladder.

Sequence analysis:

The purified plasmids those showed positive results for the digestion analysis were automatically sequenced. The complete nucleotide sequence for the coat protein gene was submitted to the GenBank as the Egyptian isolate of OYDV infecting onion. The nucleotide sequence of the Egyptian isolate was aligned with the corresponding sequences of other isolates from Japan, Netherlad, Chaina, India and Brazil (Fig. 4). The tree was generated using the DNAMAN software. Table 2 shows the percent of identity between Egyptian OYDV (onion isolate) and the other isolates. The comparison of the coat protein gene sequences showed a relatively high degree of diversity among the available sequences on the GenBank. The percent of similarity was ranged from ~ 70% as lowest identity

with Japanese isolate and ~93 % as a biggest one with Poland isolate. The highest degree of identity in OYDV-CP gene was ranged from 89.9 % and 93.02 % those recorded when the OYDV-CP sequence was compared with the OYDV-CP (Garlic isolate) from Egypt and the Isolate 72 from Poland.

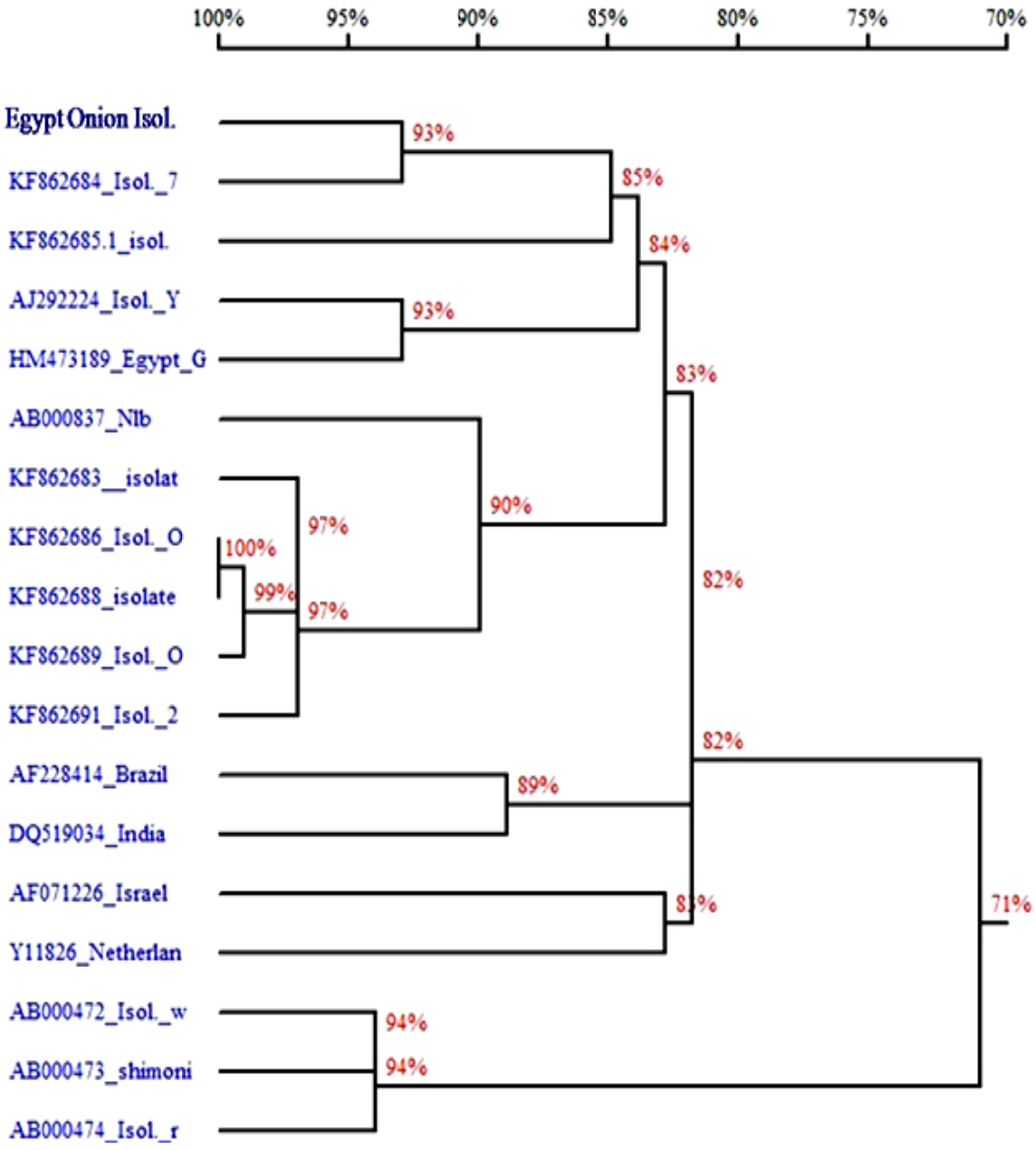

Fig. 4: Phylogenetic trees based on a multiple alignment of the complete sequence for the coat protein gene of the OYDV-Egyptian isolate and other isolates available in the GenBank. Horizontal distances are proportional to sequence distances.

Table 2. Percentage of nucleotide sequence identities (%) between OYDV-Egyptian isolate and other OYDV-CP gene isolates available in the GenBank.

	OYDV isolate	Country	Accession number	Identity (%)
1		Netherlands	Y11826	**80.95**
2	OYDV- Garlic isolate	Egypt	HM473189	**89.90**
3	OYDV	India	DQ519034	**81.62**
4	Isolate YH3	China	AJ292224	**85.45**
5	OYDV	Brazil	AF228414	**83.20**
6	OYDV	Israel	AF071226	**82.23**
7	Wakenegi	Japan	AB000472	**71.39**
8	Shimonitanegi	Japan	AB000473	**70.39**
9	Isolate Rakkyo	Japan	AB000474	**71.98**
10	OYDV	Japan	AB000837	**82.40**
11	Isolate 161	Poland	KF862685	**83.97**
12	Isolate OY206	Poland	KF862686	**84.96**
13	Isolate OY208	Poland	KF862688	**84.96**
14	Isolate OY209	Poland	KF862689	**84.96**
15	Isolate 53	Poland	KF862683	**84.17**
16	Isolate 72	Poland	KF862684	**93.02**
17	Isolate 220	Poland	KF862691	**83.78**

Elimination of *Onion yellow dwarf virus:*

The *in vitro* data was tabulated in table (3) showed the effect of different concentrations of kinetin on percentage of surviving and production of virus-free onion plantlets (Table 3). All explants propagated as control on a medium without kinetin were positive for *OYDV*, and resulted in 100% of regenerated explants with symptoms of dwarfing (Fig.5A). Incorporation of $10mgL^{-1}$, $20mgL^{-1}$ and $30mgL^{-1}$ of kinetin into MS medium resulted in (96.6, 100 and 96.6%) of survived explants as well as (65.5, 76.6 and 86.3%) of virus-free plantlets respectively.

On the other hand, observation on the behavior of propagated explants on different concentrations of kinetin showed that $10mgL^{-1}$ enhanced the regeneration of explants, and leading to maximum shoots within 30 days of culture (Fig.5B) while, increasing the concentration to 20 and 30 reduced the shoot proliferation, and some of them elongated as single shoot (Fig.5C,D). Roots and complete *in vitro* onion plantlets were formed within 4 weeks (Fig.5E) after multiplication of *OYDV*-free explants resulting from kinetin ($10mgL^{-1}$) as indicator to rapid growth treatment.

Table 3: Effect of kinetin on the production of *OYDV*-free onion plantlets.

Kientin (Conc. mgL^{-1})	No. of survival	Virus free	Infected	Survival (%)	Virus elimination (%)	Therapy Efficiency (TE%)
10	29	19	10	96.6	65.5	63.3
20	30	23	7	100	76.6	76.6
30	29	25	4	96.6	86.2	83.3
Control	30	0	30	100	0	

30 infected explants/treatment - Data are based on indirect-ELISA detection

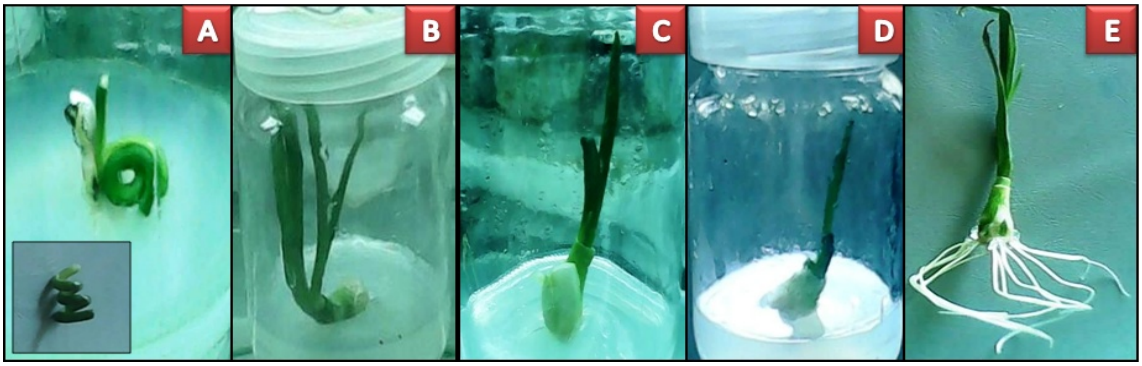

Fig.5: Dwarfing caused by *onion yellow dwarf virus* on explant leaves (A). Onion shoots development after using 10, 20 and 30 mgL^{-1} of kinetin respectively (B,C and D). Complete *in vitro* un-infected plantlets development (E).

DISCUSSION

The virus isolated from naturally infected onion plants grown in different Governorates, showing symptoms seems to be identical to OYDV. Its identity was confirmed by symptomatology, serology and molecular biology. Results showed that the virus isolate was successfully transmitted mechanically to the healthy onion plants.

All samples those collected from the five different Governorates showed positive ELISA and RT-PCR results confirming the high incidence of OYDV in Egypt. The infected leaves and harvested bulbs after mechanical inoculation with OYDV were 100% as estimated by ELISA using *OYDV* specific antiserum and RT-PCR using specific primers for the coat protein gene of the virus. In addition, symptoms under greenhouse conditions were similar to *OYDV* symptoms in the open fields. Symptomatology was reported as a successful approach for *potyviruses* infection (Shukla *et al*., 1989) and the application of enzyme-linked immunosorbent assay (ELISA) is one of the most popular techniques used to confirm virus infection to avoid misidentification of virus diseases (Trigiano *et al*., 2003, Mavric *et al*., 1999).

In our study, we found that all tested onion plants those previously showed symptoms characteristic for OYDV were positive for the presence of viral RNA using RT-PCR in either naturally infected or mechanically inoculated plant samples; indicating that

the primers designed, in this study, for OYDV detection were effective for the amplification of the full coat protein gene in onion. This proved that RT-PCR-based detection method using specific primers from conserved region of OYDV is a very sensitive and specific technique for the detection of the virus which was confirmed by the clear band obtained at the expected size (1017 bp) of the coat protein gene.

The Egyptian isolate of OYDV infecting onion was molecularly characterized through the amplification and phylogenetic analysis of the full nucleotide sequence of the coat protein gene. The coat protein gene was isolated using RT-PCR and successfully cloned into the TOPO cloning vector. The recombinant plasmids carrying the full coat protein gene was sequenced. The complete nucleotide sequence of the coat protein gene was compared with corresponding sequences for 17 different OYDV isolates those recorded in different countries such as Netherlands, Japan, China, India, Brazil, Poland and Egypt (garlic isolate). The maximum sequence identity was shared with an isolate of OYDV from Poland.

The phylogenetic analysis reflected a high degree of diversity (from 70 to 93 % of identity) between the different isolates of the OYDV-CP that due to the variability in the N-terminal region of coat protein (CP) of different isolates as previously proved by Arya *et al*. (2006). The viral genotypic polymorphism found between countries, and between the two isolates of OYDV within Egypt itself, may have implications both on diagnosis, where RT-PCR primers and monoclonal antibodies for one genotype may not necessarily be suitable for detecting another, and on the world-wide trade of new cultivars. To the best of our knowledge this is the first report of the molecular characterization for the OYDV infecting onion in Egypt.

Tissue culture technique combined with cytokinin (kinetin) was evaluated for the ability to eliminate *OYDV* from infected onion bulbs. The effectiveness of this technique was influenced by the concentration that was used. Antiviral activity and weak growth was more pronounced in the concentration $30mgL^{-1}$ of kinetin as evidenced by the reduction of shoot proliferation. Increasing the concentrations of kinetin typically increases the effectiveness of virus elimination. This is in agreement with Mokbel *et al*. (1999) who succeeded in elimination *potato virus Y* from 63%, 71% and 88% of infected potato plantlets using 10 mgL^{-1}, 20 mgL^{-1} and 30 mgL^{-1} of kinetin respectively.

Taking in account that, *OYDV* is a member of family Potyviridae, genus Potyvirus as the type species with potato virus Y (*PVY*) that characterized by a monopartite, positive-sense, single stranded RNA genome encapsidated in flexuous filaments particles (Brunt *et al*. 1996) and there are no previous reports on kinetin therapy for onion viral diseases. In contrast, Simpkins *et al*. (1981) showed that kinetin treatments enhanced the concentration of virus. However, Plavsic *et al*. (1988) in their studies on infected tomato with mycoplasma like organisms by using electron microscopy showed that kinetin treatment caused destructive changes in the prokaryotic microbe and reversion of other pathogenic effects.

Virus inhibition during the present study may due to that kinetin

caused breaking of virus RNA and inhibits protein synthesis this agreed with Schuster and Huber (1990) and/or speed up the division and differentiation of cells to such an extent that virus was unable to reach newly developed shoot at time of their exciting as suggested by Svobodova (1964) and/or regulate metabolism with enhancing the defensive capacity of tissues because of plant disease resulting from virus infection is thought to be caused by metabolic changes in particular sets of plant cells during the establishment and replication of the virus (Goodman *et al*., 1986). On the other hand, the successful shoot proliferation may be evident at low concentration of kinetin compared to other concentrations. Our results are in agreement with Tanikawa, *et al*. (1996) who mentioned that the onion plantlets regeneration increases by increasing the concentration of kinetin from 1-10 mg/L^{-1}. Similarly, Jules *et al*. (1981) reported that PGRs influence plant growth and development at low concentration but inhibit the plant development at high concentration, this can explains why kinetin at $10mgL^{-1}$ effective in *OYDV* elimination with higher shoot proliferation than other concentrations which can be lethal to the plant under virus elimination.

Finally, we agree with Shiboleth *et al. (*2001) in that; virus free plants can now be used to conduct epidemiological and pathological experiments to determine symptoms and yield effects of the different viruses causing disease in onion. Research may be conducted to elucidate virus/virus and virus/vector interactions such as transencapsidation, synergism or antagonism with the different virus combinations available. The information gained from this research paves the way for the production of virus-free onion in Egypt, and offers new insights into the biology of OYDV.

REFERENCES

Abdel Wahab, A.S., Elnagar, S., El-Sheikh, M.A.K. (2009). Incidence of aphid-borne *Onion yellow dwarf virus* (OYDV) in *Alliacea* crops and associated weeds in Egypt. In: 4th conference on recent technologies in agriculture. P.21-33.

Adams M.J., Antoniw, J.F. and Fauquet, C.M. (2005). Molecular criteria for genus and species discrimination within the family Potyviridae. Archives of Virology 150:459-479.

Allam, E.K. and Ghalwash, F. (1972). A study of onion yellow dwarf virus disease and its transmission in Egypt. U.A.R. J. Microbial. 5(1): 29-41.

Arya, M., Baranwal, V. K., Ahlawat, Y.S. and Singh, L. (2006). RT-PCR detection and molecular characterization of *Onion yellow dwarf virus* associated with garlic and onion. CURRENT SCIENCE 91 (9): 1230-1234. http://www.currentscience.ac.in/php/toc.php?vol=091&issue=09.

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D. (1999). Short protocols in molecular biology. John Wiley & Sons 4th edition; NY.

Basra, A.S. (2000). Plant growth regulators in agriculture and horticulture: their role and

commercial uses. Harworth Press, Philadelphia. 264pp.

Berger, P.H., Adams, M.J., Barnett, O.W., Brunt, A.A., Hammond, J., Hill, J.H., Jordan, R.L., Kashiwazaki, S., Rybicki, E., Spence, N., (2005). *Potyviridae* In: Fauquet, C.M., Mayo, M.A., Maniloff, J., Desselberger, U., Ball, L.A., editors. Virus taxonomy. VIIIth Report of the International Committee on Taxonomy of Viruses. London: Elsevier/Academic Press; p. 819-841.

Brunt, A.A., Crabtree, K., Dallwitz, M.J., Gibbs, A.J., and Watson, L. (1996). Viruses of Plants. Descriptions and Lists from the VIDE Database. 2nd Edn., CAB International, Wallingford, UK. 1484pp.

Chernyad'Ev, I.I. (2000). Ontogenetic changes in the photosynthetic apparatus and effects of cytokinins - Review. Appl. Biochem. Micro 36 (6): 527-539.

Clark, M.F. and Adams, A.N. (1977). Characteristics of the microplate method of the enzyme linked immunosorbent assay for the detection of plant viruses. J. Gen. Virol. 34:475-483.

Clarke, S.F., Burritt, D.J. and Guy, P.L. (1998). Influence of plant hormones on virus replication and pathogenesis-related proteins in *Phaseolus vulgaris* L. infected with *white clover mosaic potexvirus*. Physiological and Molecular Plant Pathology 53(4):195-207.

Conci, V.C., Canavelli, A. and Lunello, P. (2003). Yield losses associated with virus-infected garlic plants during five successive years. Plant Dis. 87:1411-1415.

Elnagar, S., El-Sheikh, M.A.K. and Abd El-Wahab, A.S. (2011). Effect of Natural infection with Onion yellow dwarf virus (OYDV) on yield of onion and garlic crops in Egypt. Journal of Life Science 5:634-638.

FAO, (2014) Food and Agricultural Organization of the United Nations. Statistics Division, http://faostat3.fao.org/faostat-gateway/go/to/browse/Q/QC/E.

Fayad-André, M., Dusi, A.N. and Resende, R.O. (2011). Spread of viruses in garlic fields cultivated under different agricultural production systems in Brazil. Tropical Plant Pathology 36:341-349.

Goodman, R.N., Kiraly, Z. and Wood, K.R. (1986). The Biochemistry and Physiology of plant Disease. University of Missouri Press, Columbia.

Gray, W.M. (2004). Hormonal regulation of plant growth and development. PLoS Biol 2(9): 1270-1273.

Hirose, N., Takei, K., Kuroha, T., Kamada-Nobusada, T., Hayashi, H. and Sakakibara, H. (2007). Regulation of cytokinin biosynthesis, compartmentalization, and translocation. J. Exp. Bot. 14:1-9.

Hoa, N.V., Ahlawat, Y.S., and Pant, R.P. (2003). Partial characterization of Onion yellow dwarf virus from onion in India. Indian Phytopathol. 56:276-282.

Jules, J., Rober, W.S., Frank, W.N. and Varnon, W.R. (1981). Plant Science. An Introduction to World Crops. W.H. freeman and Co. New York, P.55-64, 162-192.

Kamstaityte, D. and Stanys, V. (2004). Micropropagation of onion (*Allium cepa* L.), Acta Universitatis Latviensis Biology, 676:173-176.

Kieber, J.J. (2002). Cytokinin. In: The Arabidopsis Book. Somerville, C. Meyerowitz, E. (eds): American Society of Plant Biologists. Doi/10.1199/tab. 0063, http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3243406/

Kuhn, C. W. (1964). Separation of Cowpea virus mixture. Phytopathology 54: 739-740.

Kumar, P., Dhawan, P. and Mehra, R. (2012). Symptoms and losses caused by Onion yellow dwarf virus and Iris yellow spot virus diseases of onion crop in Northen India. Journal of Mycology and Plant Pathology, 42 (1):153-160.

Mavric, I., Mirkovic, V. and Ravnikar, M. (1999). Virus infections of *Allium*. Zbornik predavanj in referatov 4. Slovenskega Posvetovanja o Varstvu Rastlin v Portorozu od3.do4. Marca. P.45-50.

Mohamed-Yasseen, Y., Walter, E.S. and Richard, E.L. (1993). *In Vitro* Bulb Formation and Plant Recovery from Onion Inflorescences. Institute of Food and Agricultural Sciences, University of Florida, Agr. Exp. Sta. j. 28(10):1052.

Mohammed, H.S., Zicca, S., Manglli, A., Mohamed, M.E., El Siddig, M.A., El Hussein, A.A. and Tomassoli, L. (2013). Occurrence and phylogenetic analysis of potyviruses, carlaviruses and allexiviruses in garlic in Sudan. Journal of Phytopathology, 161(9): 642-650.

Mokbel, A.S. (1999). The use of certain treatment for induction of virus-free potato plants through tissue culture. M.S. Thesis. Plant Pathology Department, Fac. Agric., Cairo University. 89pp.

Murashige, T. and Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. Plant Physiol. 15:473-497.

Plavsic, B., Krivokapic, K., Eric, Z., Maramorosch, K. and Raychaudhuri, S.P. (1988). Kinetin treatment of stolbur diseased plants and possibility of its application in chemotherapy. Mycoplasma disease of Crop Basic and Applied Aspects. P.417-430.

Schuster, G. and Huber, S. (1990). Kinetin inhibits the replication cycle of potato virus-X at a distinct event. Biochemie and Physiologie der Pflanzen. 186(5-6):403-407.

Shiboleth, Y.M., GAL-ON, A., Koch, M., Rabinowitch, H.D. and Salomon, R. (2001). Molecular characterisation of *Onion yellow dwarf virus* (OYDV) infecting garlic (*Allium sativum* L.) in Israel. *Ann. appl. Biol.* 138: 1-9.

Shukla, D.D., Tosic, M., Jilka, J., Ford, R.E., Toler, R.W. and Langham, M.A.C. (1989). Taxonomy of potyviruses infecting maize, sorghum and sugarcane in Australia and the United States as determined by reactivity of polyclonal antibodies directed towards virus-specific N-termini of coat proteins, Phytopathology. 79:223-229.

Simpkins, I., walkey, D.G.A. and Neely, H.A. (1981). Chemical suppression of virus in cultured plant tissue. Ann. Appl. Biol. 99:161-169.

Svobodova, J. (1964). Spread of virus-Y through tobacco callus tissue into

shoot developed from those tissues, "In, Host-parasite Relations. In Plant Pathology, pp.65-76. Ministry of Agric. The Hungarian Academy of Sciences, Hunary".

Tanikawa, T., Takagi, M. and Ichii, M. (1996). "Plant regeneration from suspension cultures of onion (*Allium cepa* L.)". Plant Tissue Culture Letters. 13:259-264.

Taskin, H., Baktemur, G., Kurul, M. and Büyükalaca, S. (2013). Use of Tissue Culture Techniques for Producing Virus-Free Plant in Garlic and Their Identification through Real-Time PCR. The Scientific World Journal. http://dx.doi.org/10.1155/2013/781282

Trigiano, T.N., Windham, M.T. and Windham, A.S. (2003). Plant Pathology: Concepts and Laboratory Exercises. CRC Press, Florida.

Van Dijk, P. (1993). Survey and characterization of potyviruses and their strains of *Allium* species. Neth. J. Plant Pathol. 99:1-48.

Walkey, D.G.A. (1991). Production of virus-free plants. In: Applied Plant Virology (Chapman and Hall, eds), St Edmundsbury Press, Great Britain. P. 270-292.