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IDENTIFICATION OF AN ISOLATE OF *ZUCCHINI YELLOW MOSAIC POTYVIRUS* INFECTING SQUASH IN EGYPT

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ABSTRACT

Squash is considered as one of the important vegetable crops worldwide including Egypt. *Zucchini yellow mosaic potyvirus* (ZYMV) is reported to be the most series viruses infecting cucurbits. In this study, an Egyptian isolate of ZYMV infecting squash plant (*Cucurbita pepo* cv. Eskandarani) was identified based on its biological, serological, and molecular properties. The isolate appeared severe mosaic, vein banding and deformation the infected squash plant under open field and greenhouse conditions. Positive reactions with polyclonal antibodies specific to ZYMV were obtained when samples were subjected to direct antigen coated (DAC)-enzyme-linked immunosorbent assay (ELISA). The electron microscopy of purified virus prepared from ZYMV-infected squash plants, showed the presence of filamentous virus-like particles measuring 750X13 nm. The viral isolate was confirmed to be belonging to Potyviruses group through producing cylindrical inclusions (pinwheels, scroll, and laminated aggregates) in the cytoplasm of cells infected with ZYMV. At the level of molecular characterization, the cylindrical inclusions (*CI*) protein and nuclear inclusions (*NIb*) genes of the ZYMV-EG isolate comprised 1902, and 1551 nucleotides, and encoding 634 and 517 amino acids protein, respectivelyand their similarities to some overseas isolates were addressed. The two genes appeared 100% homology compared to ZYMV TW-TN3 strain (AF127929).

Keywords: ZYMV, Squash, Identification, DAC-ELISA, cylindrical inclusions (*CI*) protein gene and nuclear inclusions (*NIb*) gene.

INTRODUCTION

Cucurbit species include a variety of high value crops as melons, watermelon, cucumber, summer squashes, and winter squashes that play important roles both in local diets and as export crops in many countries. Viral diseases are more serious for cucurbitaceous plants compared todiseases caused by other agents. Symptomsof viral infections on Cucurbitaceae are mosaic, yellowing, stunting, chlorosis, leaf and fruit de-formations (Lisa *et al*.,1981 and Lecoq *et al*., 1983). Presently, three Potyvirus species are most commonly reported in surveys of virus infecting cucurbits in different parts of the world like *Watermelon mosaic potyvirus* (WMV), *Papaya ring spot potyvirus* (PRSV) and *Zucchini yellow mosaic potyvirus* (ZYMV) (Ullman *et al*., 1991; Rivera *et al*., 1993; Luis-Arteaga *et al*., 1998; Kwon *et al*., 2005; Pereira *et al*., 2007; Coutts *et al*., 2011; Simmons *et al*., 2011 and Simmons *et al*., 2013). ZYMV is a member of genus Potyvirus in the family Potyviridae, was first reported in Italy in 1973 (Lisa *et al*., 1981) and at the same time it was also observed in France, where it was named as *Muskmelon yellow stunt potyvirus* (MYSV) (Lecoq *et al*., 1981 and 1983). It causes substantial economic losses in cucurbit cropsin Brazil (de Almeida Spadotti *et al*., 2015). It was firstly described in Egypt in 1983 (Provvidenti*et al*., 1984b). It has been observed in about 50 countries in both traditional and intensive growing conditions since its first report (Desbiez and Lecoq, 1997). *Zucchini yellow mosaic virus* (ZYMV; family *Potyviridae*) is a single-stranded positive-sense RNA virus that is an important pathogen of cucurbits (squash, melon and cucumbers). In addition to the distinctive yellow mottling of the leaves, the symptoms of ZYMV include stunting of the entire plant as well as fruit distortion and mottling (Desbiez and Lecoq, 1997). These symptoms can render the fruits unmarketable such that ZYMV can reduce agricultural yields by up to 94% (Blua and Perring, 1989). The Egyptian isolates of ZYMV (Provvidenti *et al*., 1984b and Abdel-Ghaffar *et al*., 1998) incited symptoms closely resembling those caused by European isolates of this virus (Lecoq *et al*., 1981 and Lisa *et al*., 1981) and the American strain ZYMV-Connecticut (ZYMV-CT) (Providenti *et al*., 1984a). ZYMV incertain circumstances may completely destroy cucumber (Sutarya and dan Sumpena, 1994), squash (Rakib *et al*., 2011 and Al-Saleh *et al*., 2014), and pumpkin and muskmelon (Al-Saleh

et al., 2014) plants.This study concerned to identify anEgyptian isolate of ZYMV using ELISA technique and electron microscopy. Alsoits morphological and cytopathological effects on the host plant was studied.

MATERIALS AND METHODS

Virus isolation, confirmation and maintenance: Leaves exhibited ZYMV-like symptoms suspect- ted to be virus naturally infected were collected in January 2006 from *Cucurbita pepo* (Zucchini squash) cv. Eskandarani from commercial fields of Kafr Saad City, Damietta Governorate, Egypt. These samples were found by direct antigen coating (DAC)-ELISA (Converse and Martin, 1990) to be devoid of other viruses infecting cucurbits except ZYMV. The ZYMV isolate was maintained and propagated under greenhouse conditions in Zucchini squash (*C. pepo* cv. Eskandarani) by mechanical inoculation.

Virus purification: ZYMV was purified from *C. pepo* cv. Escandarani using polyethylene glycol (PEG) as described by Abdel-Halim *et al*., (2000). The virus pellets were then immediately resuspended in 2 ml of 0.02M sodium phosphate buffer (SPB), pH 7.2 and kept overnight at 4˚C. Density-gradient centrifugation was carried out as described by Delgado and Grogan (1966) and Abdel-Halim *et al.*, (2000). The final pellet was then resuspended in 500µl of 2mM SPB, pH 7.4.

Negative staining: The method Milne and Lesemann (1984) was applied for negatively staining of the purified virus preparation.

Inclusion bodies: The ultrathin sections of ZYMV-infected leaves were prepared four weeks post mechanically inoculated *C. pepo*cv. Escandarani with ZYMV infectious sap as described by Abdel-Ghaffar *et al*., (1998).

Electron microscopy: Both of gold carbon coated grids carries each of purified virus or ultrathin sections, stained with a mixture of 2% uranyl acetate were examined by electron microscope, JEOLJEM-100S in the Electron Microscope Unit, Egyptian Organization for Biological Products and Vaccines (VACSERA), Agouza, Giza, Egypt.

Molecular identification: The nucleotides sequencing of PCR products of ZYMV-EG *CI* and *NIb* genes were determined using Genetic Analyzer (ABI Prism 310, version 3.4, Semi Adaptive, version 3.2). DNA sequences were translated to protein using EditSeq of DNA star software program. DNA and protein sequences of the ZYMV-Eg *CI* and *NIb* genes were aligned and compared using Mega Align of DNA star program with 14 isolates or strains of ZYMV and a strain of PVY^Nrecorded in GenBank. Multiple comparison was based on Jotun Hein algorithmus (Hein, 1990) and the method given by Higgins and Sharp (1989). Substitutions of both of *CI* and *NIb* genes of ZYMV-EG compared to 14 ZYMV isolates and a strain of PVY^N available in the GenBank were also determined in phylogenetic trees.

RESULTS AND DISCUSSIONS

ZYMV disease is a major constraintin the production of cucurbits world-wide.The virus can cause massive damage (total loss) to cucurbit crops, and preventsthe growth of some cucurbit crops incertain areas (Francki *et al*., 1985; Zechmann *et al*., 2003 and Gal-On, 2007). In this study, the presence of ZYMV in sampled leaves of zucchini squash was detected *via* DAC-ELISA with positive absorbance reading at 405 nm in the range of 0.181 to 0.319 while healthy leaf extract had reading of 0.090. The naturally infected leaves as well as the mechanically inoculated leaves developed severe mosaic, vein banding, chlorosis, yellows and crinkling (Fig.1). Similarresults were obtained by Abdel-Ghaffar *et al*., (1998) and Mahmoud *et al*., (2004), who reported that the characteristic observed symptoms were severe mosaicand malformation. Prieto *et al*., (2001) also reported that infected zucchini samples showed yellow mosaic and severe leaf blistering. Also, the experimental results are in harmony with that found by Lisa *et al*., (1981); Siaw *et al*., (1985); Riechmann *et al*.,(1989); Murphy *et al*., (1990); Desbiez and Lecoq (1997); Svoboda and Polák (2002) and Verma *et al*., (2006). Recently, Al-Saleh *et al*. (2014) diagnosed the presence of ZYMV among 11 out of 33 samples collected from squash, pumpkin and muskmelon plants showing virus-like symptoms in Riyadh and Al-Madena regions of Saudi Arabia *via* double antibody sandwich ELISA (DAS-ELISA).

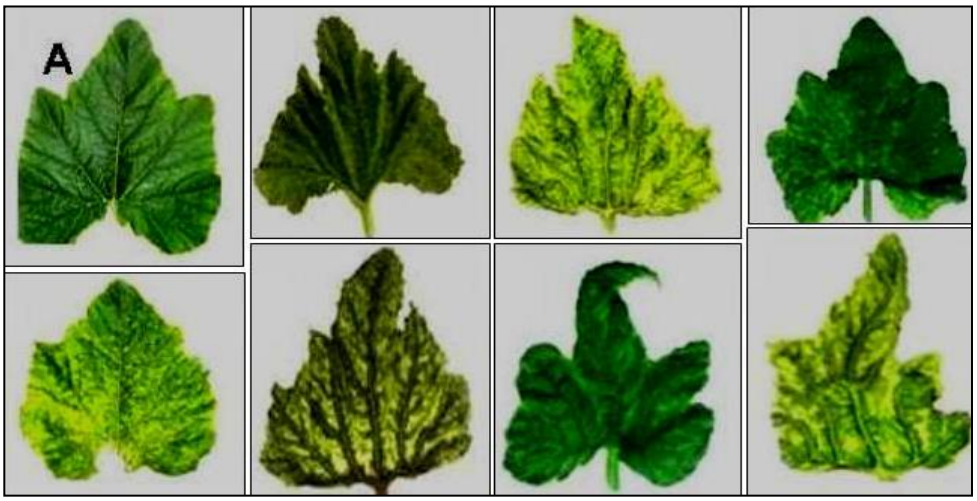

Figure-1: External symptoms caused by ZYMV-EG in *C. pepo* cv. Escandarani. A) control.

The electron micrograph of negatively stained partially purified virus preparations shows the presence of flexuous filamentous virus particles with dimensions 750 X 13 nm (Fig. 2). Similar results wereobtained by Abdel-Ghaffar *et al*., (1998) and Mahmoud *et al*. (2004). Also, theseresults are similar to that reported by Gal-On (2007) who reported that ZYMV virions are flexuous filaments of 11-13 nm in diameter. Members of *Potyvirus* group are characterized by inducing CCI in the cytoplasm of virus-infected cells duringthe infection cycle (Lesemann, 1988; Edwardson and Christie, 1996). The induction of such CCI formation by virus encoded protein is the most important phenotypic criterion for assigning virusesto the potyviruses (Edwardson *et al*., 1984; Milne, 1988). In this work, the electron micrographs of ultrathin sections prepared from control sampled leaves zucchini squash appeared the absence of cytoplasmic inclusions in the cytoplasm of tested cells (Data not shown). On the other hand, cylindrical inclusions in the form of pin wheel, scrolls, and laminated aggregates (Fig. 3) were observed in the positive-ELISA leaf samples (Virus infected leaves). This result is in agreement with that found by Abdel-Ghaffar *et al*., (1998). Kitajima and Lovisolo (1972) found that aggregated mitochondria have been observed in *Datura* cells infected with a *Potyvirus*. Data here in indicate that the infection of zucchini leaf cells with ZYMV induced several ultrastructure changes. It was obvious that chloroplast, mitochondria and nucleus were severely affected by ZYMV-infection (Un-shown data). The development of abnormal membrane system within mitochondria has been described for several virus infections (Francki, 1987).

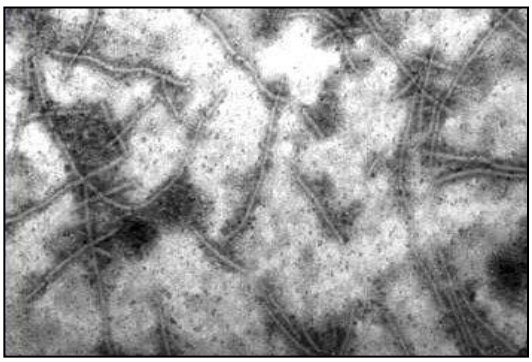

Figure-2: Electron micrograph of partially purified ZYMV-EG negatively stained with 2% uranyl acetate.

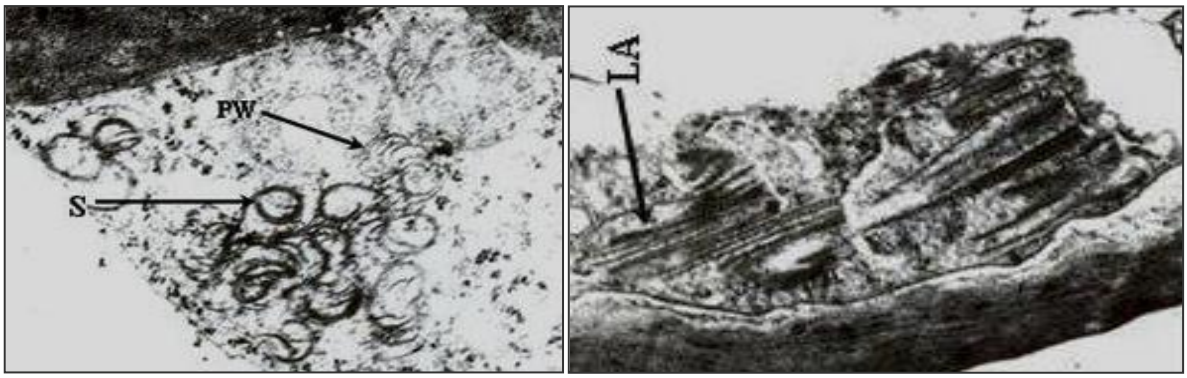

Figure-3: Electron micrographs of ultrathin sections from ZYMV-EG infected leaves of *C. pepo* cv. Eskandarani showing the presence of cylindrical inclusions as pinwheel (PW), scroll (S) and laminated aggregates (LA) in the cytoplasm.

The nucleotide sequences of the major ORFs of *CI* (Fig. 4) and *NIb* (Fig. 5) genes of ZYMV-EG under investigation comprised 1902 and 1551 nucleotides, encoding 634 and 517 amino acids proteins were determined. Comparative analysis of both of nucleotide sequences and amino acids of ZYMV-EG showed 100.0% homology between ZYMV-EG and strain ZYMV TW-TN3 (AF127929). These results were confirmed by the divergence percentages of both of nucleotide and amino acid sequences of ZYMV-EG*CI* gene compared to 14 ZYMV isolates or strains available in the GenBank (Table 1) and phylogenetic trees (Figs. 6 and 7). Al-Saleh *et al*. (2014) showed that comparative analysis of the nucleotide sequences of *cp* gene from the Saudi isolates and other ZYMV isolates obtained from NCBI, showed a relatively high nucleotide sequence similarity that ranged between 92.0 -98.8%. de Almeida Spadotti *et al*. (2015) reported that the nucleotide sequence variability of the coat protein gene ranged from 82-99 % compared to the corresponding sequences of ZYMV isolates from different geographical locations.

GGACTTGAAG ATATTGAGAG CTTGGAGGAC GATAAGAGAC TCACAATTGA CTTTGATATT AACACGAATG AGGCTCAGTC
GTCGACAACG TTTGATGTTC ATTTTGATGA TTGGTGGAAT CGGCAGCTGC AGCAAAATCG CACAGTTCCA CATTACAGGA
CCACAGGTAA ATTCCTCGAA TTTACCAGAA ACACTGCAGC TTTTGTGGCC AATGAAATAG CATCATCAAG TGAAGGAGAG
TTTTTAGTCA GAGGAGCAGT GGGTTCTGGA AAATCAACGA GCTTGCCCGC ACATCTTGCC AAGAAGGGTA AGGTACTGTT
ACTTGAACCT ACACGCCCCT TGGCGGAGAA TGTCAGTAGA CAGTTGGCAG GCGATCCTTT TTTCCAAAAC GTCACACTCA
GAATGAGAGG GCTAAATTGC TTTGGTTCAA GTAACATTAC AGTGATGACG AGTGGATTTG CTTTTCACTA TTATGTTAAC
AATCCACATC AATTAATGGA ATTTGACTTT GTTATAATAG ACGAGTGCCA TGTCACGGAC AGTGCGACTA TAGCTTTCAA
TTGTGCGCTT AAGGAGTATA ACTTTGCTGG CAAATTGATT AAAGTGTCTG CAACGCCGCC AGGGAGAGAG TGTGATTTCG
ATACGCAATT CGCGGTTAAA GTCAAAACGG AGGACCACCT TTCATTCCAT GCATTCGTTG GCGCACAGAA GACCGGTTCA
AATGCTGACA TGGTTCAGCA TGGCAATAAC ATACTTGTGT ATGTTGCAAG TTACAACGAA GTGGACATGC TTTCCAAGTT
ACTCACTGAG CGACAATTTT CAGTGACGAA GGTAGATGGG CGAACAATGC AACTTGGGAA AACTACCATT GAAACGCATG
GAACTAGCCA AAAGCCTCAT TTCATAGTAG CTAGAAACAT CATCGAAAAT GGAGTGACGT TGGATGTTGA GTGTGTTGTT
GATTTTGGAC TGAAAGTGGT CGCAGAATTA GACAGCGAAA ATCGGTGTGT GCGCTACAAC AAGAAATCAG TTAGTTATGG
GGAAAGGATT CAGCGGCTAG GGAGAGTGGG GAGATCTAAG CCTGGAACGG CATTGCGTAT AGGGCACACA GAAAAAGGCA
TCGAGAGCAT TCCTGAATTC ATTGCCACAG AAGCAGCAGC CCTATCGTTT GCCTATGGGC TTCCAGTCAC TACGCATGGG
GTTTCCACAA ATATACTCGG AAAGTGCACA GTCAAGCAGA TGAGATGTGC TTTGAATTTC GAGCTAACTC CTTTCTTCAC
CACTCATCTA ATCCGTCATG ATGGCAGTAT GCACCCATTG ATACACGAAG AATTAAAACA ATTCAAACTC AGGGATTCAG
AAATGGTGCT CAACAAGGTT GCATTACCTC ACCAATTTGT GAGTCAATGG ATGGATCAAA GTGAGTATGA ACGCATTGGA
GTGCACGTTC AATGTCATGA GAGCACACGC ATACCTTTCT ACACAAATGG AGTGCCTGAC AAGGTCTATG AGAAAATTTG
GAAGTGCATA CAAGAAAACA AGAATGATGC GGTTTTTGGT AAGCTCTCAA GCGCTTGTTC GACTAAGGTC AGTTATACAC
TCAGCACTGA CCCAGCAGCA TTACCCAGAA CCATTGCAAT CATCGACCAC CTGCTTGCCG AGGAAATGAT GAAGCGGAAT
CACTTCGACA CGATTAGCTC AGCTGTGACG GGTTATTCAT TTTCCCTCGC TGGAATTGCT GATTCTTTTA GGAAGAGATA
CATGCGTGAT TACACAGCGC ACAACATTGC AATTCTTCAA CAAGCACGTG CCCAGCTGCT CGAGTTCAAT AGCAAAAATG
TGAACATCAA CAACCTGTCC GATCTGGAAG GAATTGGAGT TATTAAGTCG GTGGTGTTGC AA

GLEDIESLEDDKRLTIDFDINTNEAQSSTTFDVHFDDWWNRQLQQNRTVPHYRTTGKFLEFTRNTAAFVANEIASSSEGEFLVRGAVGSGKST
SLPAHLAKKGKVLLLEPTRPLAENVSRQLAGDPFFQNVTLRMRGLNCFGSSNITVMTSGFAFHYYVNNPHQLMEFDFVIIDECHVTDSATIAFN
CALKEYNFAGKLIKVSATPPGRECDFDTQFAVKVKTEDHLSFHAFVGAQKTGSNADMVQHGNNILVYVASYNEVDMLSKLLTERQFSVTKVDGR
TMQLGKTTIETHGTSQKPHFIVARNIIENGVTLDVECVVDFGLKVVAELDSENRCVRYNKKSVSYGERIQRLGRVGRSKPGTALRIGHTEKGIE
SIPEFIATEAAALSFAYGLPVTTHGVSTNILGKCTVKQMRCALNFELTPFFTTHLIRHDGSMHPLIHEELKQFKLRDSEMVLNKVALPHQFVSQ
WMDQSEYERIGVHVQCHESTRIPFYTNGVPDKVYEKIWKCIQENKNDAVFGKLSSACSTKVSYTLSTDPAALPRTIAIIDHLLAEEMMKRNHFD
TISSAVTGYSFSLAGIADSFRKRYMRDYTAHNIAILQQARAQLLEFNSKNVNINNLSDLEGIGVIKSVVLQ

Figure -4: Nucleotide sequences of *CI* gene of ZYMV-EG (1902 nts) and its ORF number 1 in reading frame 1 on the direct strand extends from base 1 to base 1902 and encoding 634 amino acids.

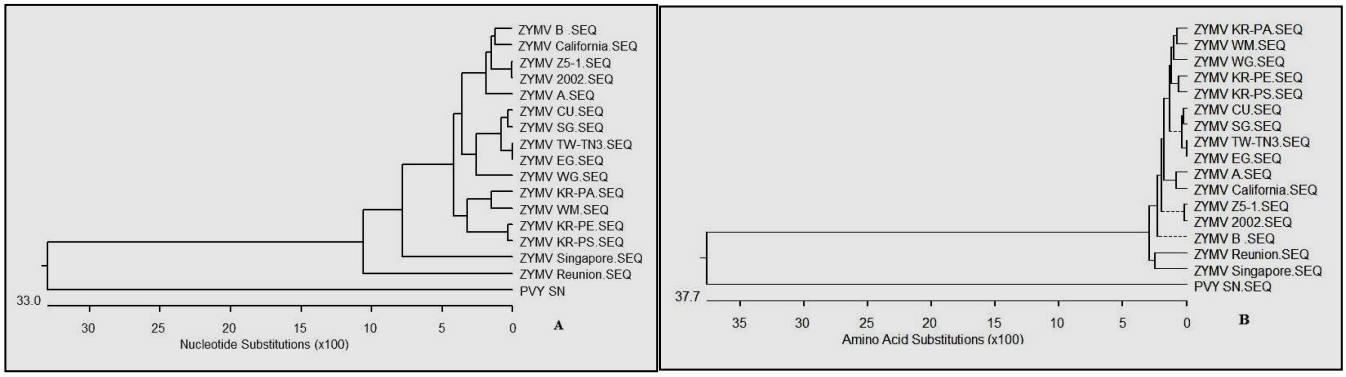

Figure -5: Phylogenetic trees of the nucleotide (A) and amino acid (B) show substitutions of ZYMV-EG *CI* gene compared to 14 ZYMV isolates and a strain of PVY^{N} available in the GenBank. The isolate (ZYMV-EG) was fell in one cluster with ZYMV TW-TN3 strain (AF127929).

AGCAAGCGAG AAAGATGGGT CTACGAAAGC TGTGAAGGGA ACCTTCGAGC TGTTGGAACT GCACAATCAG CGCTAGTCAC CAAACATGTT
GTAAAAGGCA AGTGTCCTTT CTTCGAAGAA TACTTGCAAA CTCACGCAGA AGCGAGCGCT TACTTCAGAC CCTTGATGGG AGAATACCAG
CCTAGCAAGT TGAACAAAGA GGCCTTTAAA AAGGATTTCT TCAAATACAA TAAACCCGTC ACTGTTAATC AATTGGATCA CGATAAATTC
TTGGAAGCAG TTGATGGGGT TATACGTATG ATGTGCGACT TTGAATTCAA TGAGTGCCGA TTCATTACAG ACCCCGAGGA AATTTATAAC
TCTTTGAACA TGAAAGCAGC AATTGGAGCC CAGTATAGAG GAAAGAAGAA AGAGTATTTT GAAGGGCTAG ATGATTTTGA TCGAGAGCGA
CTTTTATTTC AGAGTTGTGA AAGGTTGTTT AATGGCTACA AAGGTCTGTG GAATGGATCT TTAAAGGCCG AGCTCAGGCC GCTTGAGAAA
GTCAGGGCCA ACAAAACACG AACTTTTACA GCAGCGCCCA TTGATACATT GCTCGGAGCT AAAGTTTGCG TGGATGATTT CAATAATGAA
TTTTATAGCA AAAACCTCAA GTGTCCATGG ACGGTTGGCA TGACGAAATT TTATGGTGGT TGGGATAGAT TGATGAGATC ATTACCTGAT
GGTTGGTTAT ATTGTCATGC TGATGGATCA CAGTTTGACA GTTCATTGAC CCCAGCCTTA CTGAATGCAG TGCTTATAAT CCGATCATTT
TATATGGAGG ATTGGTGGGT CGGTCAAGAG ATGCTTGAAA ATCTTTATGC TGAGATTGTG TACACTCCAA TTCTTGCTCC GGATGGAACA
ATTTTCAAGA AATTTAGAGG TAACAACAGT GGGCAACCCT CAACAGTGGT GGATAACACA CTAATGGTTG TGATCTCTAT TTACTATGCG
TGCATGAAAT TTGGTTGGAA TTGCGAGGAA ATTGAGAATA GACTTATCTT CTTTGCAAAT GGAGATGATC TGATACTTGC AGTCAAAGAT
GAGGATAGCG GCTTACTTGA TAACATGTCA GCTTCTTTTT CCGAACTCGG ACTGAATTAT GATTTTTCAG AACGCACGCA CAAAAGAGAA
GATCTTTGGT TCATGTCCCA CCAAGCAATG TTAGTTGATG GAATGTACAT TCCAAAACTC GAGAAAGAAA GAATTGTTTC AATTCTAGAG
TGGGATAGAA GCAAAGAAAT CATGCACCGA ACAGAGGCTA TTTGCGCTGC GATGATTGAG GCATGGGGGC ACACCGAGCT TTTGCAAGAA
ATCAGAAAGT TTTACCTATG GTTCGTTGAG AAAGAGGAAG TGCGAGAATT AGCTGCCCTC GGAAAAGCTC CATACATAGC TGAGACAGCA
CTTCGCAAGT TATATACTGA CAAAGGAGCG GAAACAAGTG AATTGGCACG CTACCTACAA GCCCTCCATC AAGATATCTT CTTTGAACAA
GGAGACACCG TAATGCTCCA A

SKRERWVYESCEGNLRAVGTAQSALVTKHVVKGKCPFFEEYLQTHAEASAYFRPLMGEYQPSKLNKEAFKKDFFKYNKPVTVNQLDHDKFLEAV
DGVIRMMCDFEFNECRFITDPEEIYNSLNMKAAIGAQYRGKKKEYFEGLDDFDRERLLFQSCERLFNGYKGLWNGSLKAELRPLEKVRANKTRT
FTAAPIDTLLGAKVCVDDFNNEFYSKNLKCPWTVGMTKFYGGWDRLMRSLPDGWLYCHADGSQFDSSLTPALLNAVLIIRSFYMEDWWVGQEML
ENLYAEIVYTPILAPDGTIFKKFRGNNSGQPSTVVDNTLMVVISIYYACMKFGWNCEEIENRLIFFANGDDLILAVKDEDSGLLDNMSASFSEL
GLNYDFSERTHKREDLWFMSHQAMLVDGMYIPKLEKERIVSILEWDRSKEIMHRTEAICAAMIEAWGHTELLQEIRKFYLWFVEKEEVRELAAL
GKAPYIAETALRKLYTDKGAETSELARYLQALHQDIFFEQGDTVMLQ

Figure-6: Nucleotide sequences of *NIb* gene of ZYMV-EG (1551 nts) and its of ORF number 1 in reading frame 1 on the direct strandand encoding 517 amino acids.

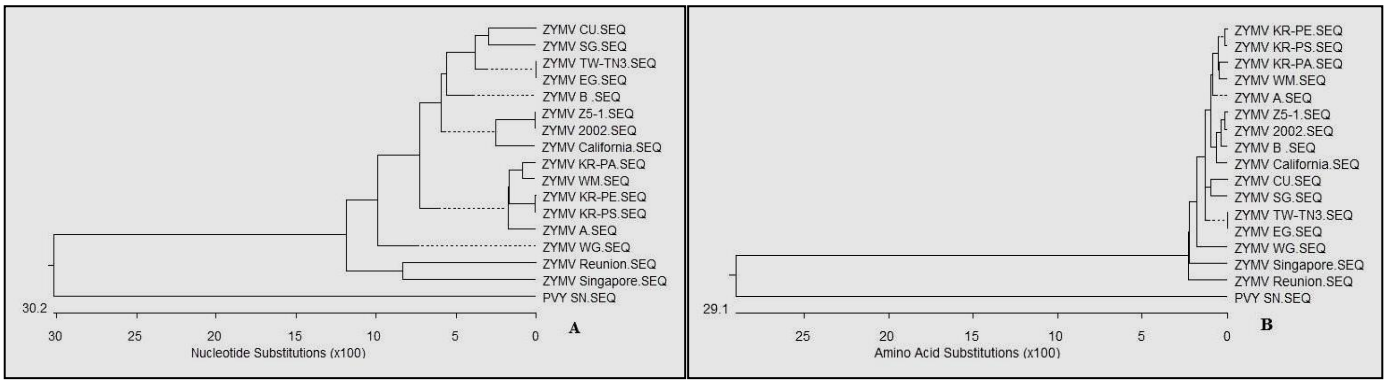

Figure-7: Phylogenetic tree of the nucleotide (A) and amino acid (B) substitutions of ZYMV-EG *NIb* gene compared to 14 ZYMV isolates and PVY^N strain available in the GenBank. The isolate (ZYMV-EG) was fell in one cluster with ZYMV TW-TN3 strain (AF127929).

Table-1: Homology percentages of both of nucleotides (nts) and amino acids (aa) sequences of ZYMV-EG *CI* and *NIb* genes compared to 14 ZYMV isolates and PVY^N strain available in the GenBank.

ZYMV isolates or strains	Accession numbers	*CI* gene		*NIb* gene	
		Homology (%)		Homology (%)	
		nts	aa	nts	aa
ZYMV A	AJ429071	97.30	93.10	94.30	98.50
ZYMV B*	AY188994	98.70	92.70	94.60	98.60
ZYMV California	L31350	97.30	92.80	94.50	98.30
ZYMV CU	AJ307036	99.40	98.60	94.40	97.90
ZYMV KR-PA	AY278998	98.30	91.70	92.50	97.90
ZYMV KR-PE	AY278999	98.10	92.00	92.30	98.60
ZYMV KR-PS	AY279000	98.70	92.30	92.10	98.50
ZYMV SG	AJ316228	99.20	98.40	83.80	96.30
ZYMV Singapore	AF014811	96.10	86.40	96.60	99.00
YMV TW-TN3	AF127929	100.0	100.0	83.90	96.10
ZYMV WG	AJ316229	98.40	95.00	100.0	100.0
ZYMV WM	AJ515911	98.60	92.40	91.60	96.90
ZYMV Z5-1	AB188115	98.90	93.60	92.40	98.10
ZYMV 2002	AB188116	98.90	93.60	94.50	98.10
PVY^N	X97895	56.60	52.10	52.10	59.20

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