Squash leaf curl virus (SLCV) incidence and severity on *Phaseolus Vulgars* in Egypt

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ABSTRACT

Squash leaf curl virus (SLCV) was isolated from naturally infected common bean plants grown in Egypt. Symptomatic leaf samples were collected from bean fields cultivated in different governorates and tested by PCR using Geminivirus degenerate primers and *Squash Leaf Curl Virus* (SLCV) specific primers. All bean varieties grown in surveyed governorates were found susceptible to Geminivirus infection and the dominant Geminivirus affecting bean fields was SLCV. Percentage of infection was higher at Nili season than that at the summer season. Six different commercial varieties of common bean were evaluated for SLCV infection at three different growth stages. The evaluation was performed using whitefly inoculation in insect proof green house. Two commercial varieties (Tema and Giza 6) were found to be tolerant to SLCV infection in all tested growth stages. The disease severity of the viral infection varied in the other four susceptible varieties at the different growth stages. It was observed that infection percentage and disease severity were decreased with increasing growth stage. A significant difference in the percentage of yield loss in inoculated plants at age 15d and 25d from planting compared with control was recorded. On the other hand percentage of yield loss in plants inoculated at 35d stage was non-significant. The coat protein gene of SLCV was PCR amplified from infected common bean plants. SLCV-CP was cloned in pJET cloning vector and directly sequenced. The sequence alignment and phylogenetic analysis showed a relatively high diversity among the three different isolates that the identity ranged from 89 to 94%.

Key words: Common bean, *Squash leaf curl virus*, virus incidence, PCR detection, cp sequence analysis, Egypt

INTRODUCTION

Squash leaf curl virus (SLCV) is a bipartite *Begomovirus* of family *Geminiviridae*. DNA A and DNA B of the bipartite genome are each approximately 2.6 kb in size. The virus is transmitted in a persistent circulative manner by the whitefly *Bemisia tabaci* (Fauquet *et al.,* 2008). Squash leaf curl disease (SLCD) was first observed in squash in California during 1977 and 1978 (Flock and Mayhew, 1981) and in cultivated buffalo gourd in Arizona at about the same time (Rosemeyer *et al.,* 1986) and recorded for the first time on *Phaseolus vulgars* plants in America and the Caribbean Basin by (Brown, 1990).Two isolates, termed *Squash leaf curl virus*-E (SLCV-E) and (SLCV-R) were previously identified as SLCD. These isolates differ in experimental host range. SLCV-E was reported to have a broader host range compared with that of SLCV-R (Lazarowitz, 1991; Lazarowitz and Lazdins, 1991). Until recently the

occurrence of SLCV was restricted to central and North America; however, in 2003, the first record on the occurrence of the SLCD in the western hemisphere was in Israel, where SLCV-E caused severe epidemics and disease incidence was almost 100% (Antignus *et al.,* 2003 and Idris *et al.,* 2006).).More recently, (Farrag *et al.,* 2005) reported that SLCV cause severe symptoms observed in squash fields in Egypt. In 2008 SLCV was recorded in the Jordanian valley(Al-Musa *et al.* 2008) on squash plants (*Cucurbitapepo*) and in 2010, it was detected on cucurbits plants cultivated at the coastal areas of Lebanon (Sobh *et al.,* 2012)..The virus cause leaf curling, yellow mottling, and reduced fruit set on squash plants (Idris *et al.,* 2006) and cause leaf curling, vein necrosis and stem necrosis on common beans plants (El-Dougdoug *et al.,* 2009).

Objective of this study was to detect *Geminivirus* associated to bean crops in Egypt, taking into account the persistent infections and the economic losses caused by SLCV on beans.

MATERIALS AND METHODS

1-Samples collection and Geminivirus incidence on Beans

A total of 513leaf samples were collected from bean fields cultivated in three different governorates in Egypt during 2009 to 2011. The suspected plants exhibited leaf malformation, mosaic, mottling, yellowing, leaf curling and stunting were collected. High population of whiteflies was also observed in the fields. Samples were tested by PCR using Geminivirus degenerate primers and Squash Leaf Curl Virus (SLCV) specific primers.

2- Isolation of Squash Leaf Curl Virus (SLCV) and Whitefly maintenance

SLCV was isolated from natural infected bean plants grown in 3 different governorates (Qalubia, Giza & Fayoum).The identity of the virus was confirmed by PCR. The virus was inoculated into healthy bean plants *cv* Top Crop and *cv* Bronco using virus free whiteflies, *Bemisia tabaci*.

Adult whitefly (*Bemisia tabaci*) was collected from the open field and purified in glass cages held in an insect proof greenhouse by rearing on crops resistant to *SLCV* as (mulberry) for three generation, then the newly borne individuals were propagated on healthy tomato plants for one generation before acquisition feeding, as described by Ghanem *et al.,* (2001). Whiteflies were tested by PCR to confirm that it's a virus free insect. Non-viruliferous whiteflies were allowed an acquisition access period (AAP) of 48 h on SLCV infected beans, then were allowed a 48-h inoculation access period (IAP) on healthy bean plants using 20 individuals whiteflies per plant.

3- Effect of plant age and bean varieties on the percentage of infection

Six bean varieties were planted in 40 cm pots and inoculated using whitefly as a virus vector at three different growth stages i.e. 15, 25, 35 days from planting un-inoculated plants served as control. Each treatment contained three replicates (15 plants / replicate).Treated plants were sprayed by insectside (actelic) every three days and kept at insect proof greenhouse for three month. Percentage of infection and percentage of disease severity (Ds %) were recorded at flowering stage using disease severity index (DSI) (Fig.1). Percentage of disease severity was calculated using the following equation according to Yang *et al.,* (1997).

$$Ds\% = \sum \frac{Disease\,grade \times No\,of\,plant\,in\,Each\,grade}{total\,no\,of\,tested\,plants \times higest\,disease\,grade} \times 100$$

Total podweight/plant was recorded and percentage of yield loss were calculated using the following equation according to Fikre*et al., (*2011)

Yield loss% =

$$\frac{Yield\ of\ control - yield\ of\ treatment}{Yield\ of\ control} \times 100$$

4-Nucleic acid extraction:

The genomic DNA was extracted from infected common bean plants collected from three different governorates in Egypt at different seasons. The standard assay developed by Dellaporta was used to isolate the total DNA (Dellaporta*et.al.,* 1983).
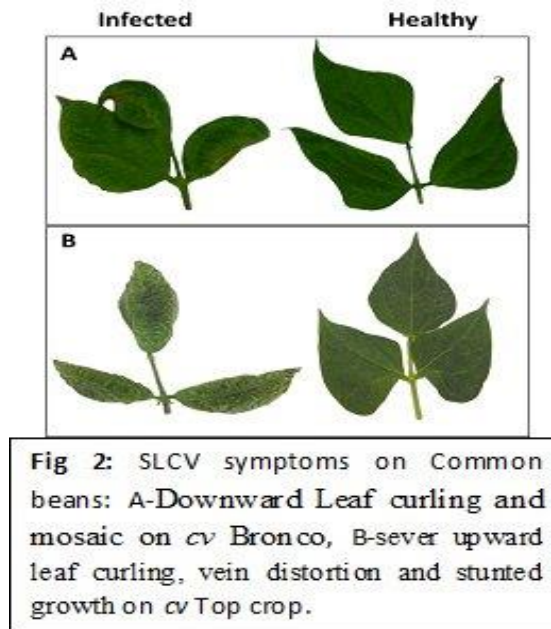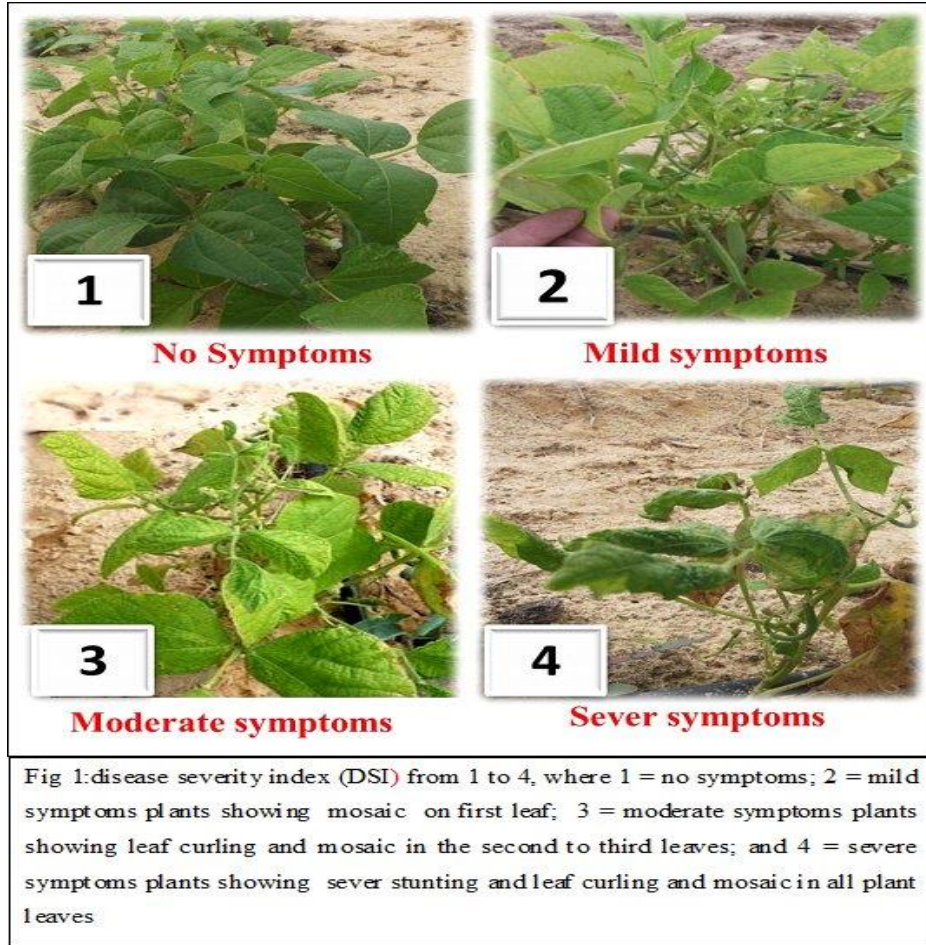
5-Detection of SLCV using Degenerate and Specific primers:

The viral DNA extracted from the infected common bean samples was used as a template for the PCR detection. Both degenerate and specific primers were used for the screening of SLCV infection. The degenerate primer pair AV-core: GCC HAT RTA YAG RAA GCC MAG RAT and AC-core: GGR TTD GAR GCA TGH GTA CAN GCC (Abdel-Salam *et al.* 2006) specific for Gemini viruses, were used for screening of viral infection in the collected samples. The DNA of the SLCV was detected in symptomatic samples through the specific amplification utilizing the primer pair; V268: CGG GGA CCA CAC ACA GCA C and C1166: ACA ATG GAT ACG CGC GCC. In both tests, the PCR reactions were performed in 25 μl volume containing 1 μl of DNA, 1.25 U Taq DNA Polymerase, 200 μM of each dNTP., 2.5 μl 10x Dream Taq Buffer, 25 pmol of each primer, 2.5 mM MgCl2 and 15.25 μl

of sterile water. The DNA extracted from healthy plant was used as negative control for all reactions.The amplification reaction was carried out in 0.2 ml micro Amp PCR tubes using T-Gradient thermal cycler (Biometra, Germany), and started with DNA denaturation at 94°C for 3min, followed by 35 cycles of denaturation at 94°C for 1 min, primer annealing at 60°C for 1min and extension at 72°C for 2min, followed by final extension step at 72°C for10 minutes. The PCR products were stained with gel star (Lonza, USA) and separated on 1% agarose gel with 1x TBE buffer then analyzed using (Gel Doc 2000 Bio.RAD).

6-Cloning and sequencing:

The partial coat protein gene of the SLCV that amplified from three different SLCV infected samples was cloned in XL1Blue *E.Cloi* into the pJET 1.2/plunt cloning vector (Fermentas). The DNA Blunting Enzyme was used for blunting the 3'dA overhangs generated by Taq DNA polymerase. The blunting reaction was performed at18 μl total volume and contained 0.2 pmols of the PCR product (1 μl), 10 μl of the 2X Reaction Buffer, 6 μl nuclease free Water and 1 μl of blunting enzyme then incubated at 70°C for 5 min. The ligation reaction was set on ice by adding 1 μl pJET1.2/blunt Cloning Vector (50ng/μl) and 1 μl T4 DNA Ligase into the blunting reaction mixture then incubated at room temperature (22±2°C) for 10 min.3 μl of the ligation reaction were used to transform the XL1 Blue competent cells using heat chock at 42°C for 45 seconds. The pJET recombinant plasmid carrying the partial coat protein fragment of the SLCV was purified from selected single colonies using standard Mini-Prep protocol (Maniaties *et al.,* 1982).

Fig 1:disease severity index (DSI) from 1 to 4, where 1 = no symptoms; 2 = mild symptoms plants showing mosaic on first leaf; 3 = moderate symptoms plants showing leaf curling and mosaic in the second to third leaves; and 4 = severe symptoms plants showing sever stunting and leaf curling and mosaic in all plant leaves

Fig 2: SLCV symptoms on Common beans: A-Downward Leaf curling and mosaic on *cv* Bronco, B-sever upward leaf curling, vein distortion and stunted growth on *cv* Top crop.

The coat protein gene fragment was liberated from 1µg recombinant plasmid using BgIII restriction endonuclease. The digestion reaction was performed in 10 µl total volume at 37 C°. The digested plasmid was stained with Gel Star and electrophoresed on 0.7 % (wt/v) agarose mini gel to verify the size of insert.

The selected clones those liberated fragments at the correct insert size after the digestion analysis were directly sequenced using automated DNA sequencing at Macrogen Inc.; South Korea. Three different clones of the SLCV-CP gene fragment those representing the three different governorates were sequenced individually. The universal primers pJET1.2F: CGA CTC ACT ATA GGG AGA GCG GC and pJET1.2R: AAG AAC ATC GAT TTT CCA TGG CAG specific for the pJET cloning vector were used for the DNA sequencing. The nucleotide sequence of the three clones was analyzed using DNAMAN Sequence Analysis Software (LynnonBioSoft. Quebec, Canada) and compared with each other and with the sequence of the coat protein gene of different isolates of the SLCV available in GenBank.

RESULTS

1-Geminivirus incidence on Bean fields

513 symptomatic bean samples were collected during 2009 to 2011 to determine the incidence of geminivirus and SLCV in different beans fields at different governorates. Results in Table 1 reveal that 157of 513 samples were infected with SLCV and only 10 samples were infected with other giminvirus. The highest percentage of infection with SLCV was at Qaliubia governorate .The highest percentage of infection was recorded in September and October.

2- Isolation of *Squash Leaf Curl Virus* (SLCV):

Whitefly (*Bemisia tabaci*) was able to transmit SLCV into two common beans cultivars (Top crop and Bronco) after 2-3 weeks post inoculation. Downward Leaf curling and mosaic symptoms were developed on *cv* bronco while *cv* Top crop developed sever upward leaf curling, vein distortion and stunted growth (Fig. 2).Results were confirmed by PCR using SLCV specific primer.

3-Effect of bean plant growth stage and varieties on Squash Leaf Curl virus infection

An experiment was conducted to study the reaction of six bean varieties (Neprasca, Bronco, Tema, Paulista, Smenta and Giza 6) to SLCV infection at three different growth stages 15, 25 and 35 days from planting. Results in table 2 revealed that a highest percentage of infection were observed in cultivars Paulista, Smenta, Neprasca, Bronco and the lowest percentage of infection was observed in cultivars Giza 6 and Tema in all growth stages.disease severity percentage was higher in cultivar Smentaand was moderate in cultivar Bronco.The lowest disease severity percentage was observed in cultivars Giza 6 and Tema in all growth stages.

4-Effect of Squash Leaf Curl virus infection on pod yield

Plant age at time of inoculation had significant effect on bean yield. Results in (table 3) revealed that when 15-days old plants were inoculated, the percent of yield loss was higher in varieties Paulista (23%), Bronco (19%), Neprasca (17.7%) and

Smenta (13.9%) compared with control plants, while the lowest percentage of yield loss were observed in cultivars Tema (6.5%) and Giza 6 (7.5%) respectively. When 30 days old plant were inoculated the percentage of yield loss was higher in varieties Bronco(7%), Smenta, (6.3%), Neprasca (5.4%), Paulista (4.9%) compared with control plants, while the lowest percentage of yield loss were observed in cultivars Tema (1.2%) and Giza 6 (0.6%) respectively. There weren't significant difference between the yield losses of the six varieties compared with control when plants were inoculated at 35 days from planting.

5-Detection of SLCV using Degenerate and Specific primers

The naturally infected common bean plants those collected from three different governorates, as well as the artificially inoculated plants were tested for SLCV infection. The total DNA isolated from each sample was used as a template for the PCR amplification. Electrophoresis analysis of PCR product showed a single amplified fragment of 550 bp (Fig.3A) when the AV/AC degenerate primers were used, while it showed 900 bp fragment (Fig.3B) when the V268/C1166 specific primers were used. No fragments were amplified from the DNA extracted from healthy plants.

6-sequance analysis

The partial coat protein gene fragment (550 bp) was PCR amplified using AC/AV primers and the PCR product was blunted using blunting enzyme to generate blunt overhangs compatible with the pJET 1.2 blunt cloning vector. The blunt end PCR product was cloned into the pJET cloning vector. The digestion of the recombinant plasmid carrying the SLCV-CP partial sequence using BgIII restriction enzyme showed a single band at the expected size of the insert fragment (550 bp) as well as another band at the expected size of the digested vector (~ 3000 bp) as shown in (Fig.4). The SLCV-CP gene fragment was sequenced in the pJET recombinant plasmid using the universal sequencing primers pJET1.2F/R. Three different clones were sequenced representing the three samples collected from three different governorates. The resulted nucleotide sequences obtained from the three clones were analyzed individually and aligned to each other and to 11 different isolates of the SLCV available on the GenBank. The Phylogenetic tree was constructed with each other as well as for the alignment of the three clones with the corresponding SLCV sequences available in GenBank (Fig.5). The accession numbers for the SLCV isolates those used for the phylogenetic analysis are: AY206998, GQ273922, HM368373, HQ184436, FJ455514, FJ030877, EF532620, GQ273921, AF081531, DQ273921, AF081531, DQ285016 and M38183. The alignment of the three Egyptian SLCV sequences showed that, two of them(SLCV-2 and SLCV-3) are closed to each other with 95% identity while the third (SLCV-1)one is relatively different with 89% identity. The alignments of the three sequences with the 11 different SLCV isolates available in the GenBank showed relatively high degree of diversity ranged from 93% to 89%.
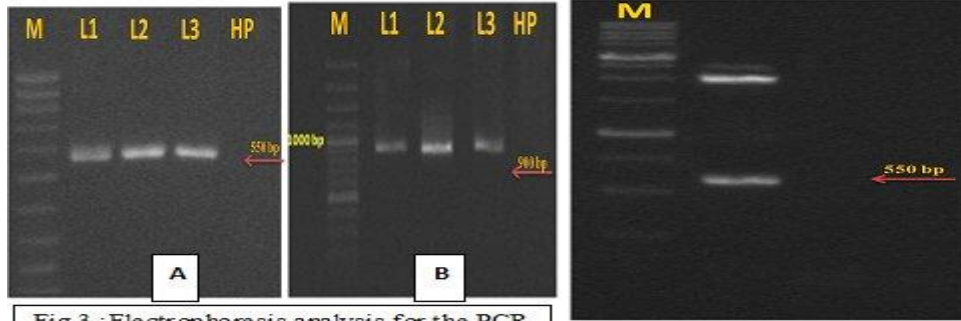
Fig 3 : Electrophoresis analysis for the PCR amplification of the SLCV partial coat protein gene using AV/AC primers [A] and V268/C1166 [B]. M: 100 bp DNA ladder. L1, L2 and L3 are three different bean samples infected with the SLCV. HP: Healthy Plant.

Fig 4 : Digestion analysis for thepJET-SLCV-CP recombinant plasmid. M: 1Kb DNA ladder. L1 is the plasmid digested using BgIII restriction enzyme.
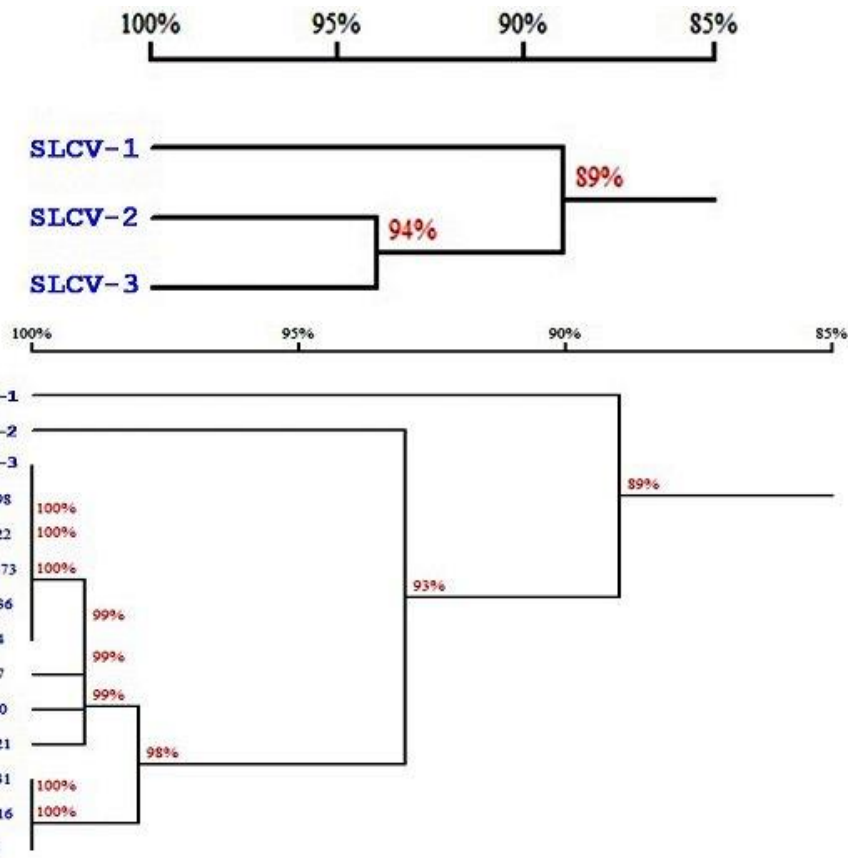

Fig5: Phylogenetic analysis for the three different clones of the SLCV when aligned with each other [A] and when aligned with 11 different isolates of SLCV available in GenBank[B].

Table 1: incidence of*Squash Leaf Curl Virus* (SLCV) in different bean fields at different governorates checked by PCR.

Location	Date of Planting	Year														
		2009					2010					2011				
		No. Of tested plants	infection				No. Of tested plants	infection				No. Of tested plants	infection			
			SLCV	% of SLCV	Other geminivirus	%		SLCV	% of SLCV	Other geminivirus	%		SLCV	% of SLCV	Other geminivirus	%
Monofia (KafrAshma)	March	40	4	10.0	1	2.5	20	1	5	2	10	30	4	13.3	0	0
Qaliobia (Kaha)	October	50	26	52.0	0	0	33	14	42.4	0	0	40	10	25	1	2.5
Fayoum(basion village)	September	40	17	42.5	2	5.0	25	7	28	1	4	30	4	13.3	2	6.7
Giza (Elsaf)	August	30	6	20.0	0	0	20	3	15	0	0	25	9	36	0	0
Giza (El Ayat)	September	70	30	42.9	0	0	30	12	40	1	3.3	30	10	33.3	0	0
Total		230	83	36.1	3	1.3	128	37	28.9	4	3.1	155	37	23.9	3	1.9

Table 2: Effect of SLCV infection on bean plants at three different growth stages from sowing expressed as % of infection and disease severity.

Days from sawing	15 Days		25 Days		35 Days	
Cultivar	DS %	% of infection	DS %	% of infection	DS %	% of infection
Neprasca	45.8 a	68.9 b	29.9 c	57.8 b	16.7 ab	37.8 a
Bronco	25.0 b	48.9 c	18.8 d	42.2 c	11.2 c	24.4 b
Tema	11.0 c	24.4 d	9.6 e	17.8 d	8.4 d	11.1 c
Paulista	45.4 a	86.7 a	42.2 a	71.1 a	17.3 a	37.8 a
Smenta	47.6 a	68.9 b	36.3 b	75.6 a	14.7 b	33.3 a
Giza 6	10.1 c	20.0 d	9.6 e	17.8 d	8.0 d	8.9 c

Table 3: Effect of SLCV infection at three different growth stages from sowingon pod yield (g. /plant)

Days from sawing	15 Day		25 Day		35 Day		Control
Cultivar	Average Yield(g)/Plant	% yield loss	Average Yield(g)/Plant	% yield loss	Average Yield(g)/Plant	% yield loss	Average Yield(g)/Plant
Neprasca	28.28 b	17.7 b	32.50 b	5.4 a	34.21 b	0.5 ab	34.37
Bronco	23.94 d	19.0 b	27.50 c	7.0 a	29.39 c	0.6 ab	29.56
Tema	32.24 a	6.2 d	33.60 ab	2.2 bc	34.16 ab	0.6 ab	34.37
Paulista	28.17 b	23.0 a	34.78 a	4.9 ab	36.49 a	0.2 b	36.57
Smenta	29.90 b	13.9 c	32.54 b	6.3 a	34.48 b	0.7 a	34.72
Giza 6	26.23 c	7.5 d	28.18 c	0.6 c	28.23c	0.5 ab	28.36

DISCUSSION

All bean cultivars inspected were found susceptible to Geminivirus infection.The major Geminivirus affecting beans fields was SLCV.Infection was higher at Nili season than summer season due to the high population of *Bemisia tabaci*which considered the main vector of the disease. Significant effect of plant growth stage during infection was recorded. It was observed that incidence and severity of the disease decreased with the Increasing plant age at time of inoculation. These results agreed with (Lapidot, 2002) who recorded the success rate of TYLCV infection to common beans was highly dependent on bean plant age, the high rate of infection with SLCV was occurred when the plants were 15 days old at inoculation. Also there was a significant different yield loss in inoculated plants at age 15d and 25d compared with control .However there wasn't a significant difference between the control plants and infected plants at 35d from planting. These results were agreed with (Levy and Lapidot 2008) they recorded that plant age had a significant effect on the yield reduction due to inoculation tomato plants with TYLCV. these results revealed that plants are susceptible to virus infection when they are at seedling stage. When studying the effect of SLCV on bean yield was studied, it was clearly appeared that The lowest yield was recorded on plants inoculated at 14 days after sowing. Results showed that cultivars Giza 6 and Tema were tolerant in all ages compared with other varieties and control. We recommend spraying bean fields with insecticides at the first month from planting to reduce the population of the insect vector *Bemisiatabaci* and thus reducing spread of SLCV infection. All common bean samples which showed SLCV symptoms and collected from different Governorates showed positive PCR results when tested with either AV/AC degenerate primers or V268/C1166 specific ones that confirmed the high incidence of SLCV infecting common bean in Egypt. Specific bands at 550 bp, when using degenerate primers, and 900 bp when using specific primers these results agreed with Sobh *et al.* (2012).

The presented results were related to the occurrence of high population of whiteflies that efficiently transmitted SLCV onto the common bean. The sequence analysis and phylogenetic trees of the partial nucleotide sequences of the SLCV-CP gene showed a relatively high diversity in the coat protein gene among three different samples representing different governorates. These results are very interesting and confirm the presence of at least two different isolates of the SLCV infecting the common bean and unlike the SLCV infecting squash in Egypt (Varsani *et al.,* 2014).

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