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Squash Leaf Curl Virus: Species of Begomovirus as the Cause of Butternut Squash Yield Losses in Indonesia

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ABSTRACT

Curling symptom was found in pumpkin plants in Bali, and the PCR detection result using Begomovirus universal primers indicated Begomovirus infection. Further research was conducted to determine the distribution, molecular character of SLCV, and yield loss of the pumpkins. Sampling was carried out at pumpkin plantations in 9 districts in Bali. Detection and identification were carried out using PCR method, followed by cloning and DNA sequencing. DNA band with the measurement of 900 bp was successfully amplified from several pumpkin samples from Denpasar, Gianyar, and Buleleng. Homology analysis of nucleotide sequences using the database in GenBank of SLCV of Balinese isolates showed the highest homology and kinship of 97.3-98.4% and 98.4-99.3% respectively with East Timor isolates from pumpkin plants. The phylogeny analysis showed that SLCV Indonesian isolates were in the same group as Asian isolates. The result of this study is the first report on infection and molecular characterization of SLCV in pumpkin plants in Indonesia. The yield loss caused by curling disease on individual pumpkin plants was 56.3%, and the disease caused a decrease in the quality of harvest fruits. Yield loss estimation caused by the disease in fields with different levels of disease intensity ranged from 10.02 to 25.83%. It was equal to yield loss ranging from IDR 878,400.00 to IDR 10,826,400.00 depending on the disease intensity. It is found that the correlation between curling disease severity and yield loss was high.

1. Introduction

Begomovirus is one of the biggest plant virus group. It's groups have a circular, single stranded DNA (ssDNA) genome of roughly 2.7-5.2 kb typified in twinned icosahedral particles (Ali-Shtayeh *et al.* 2013). The International Committee on Taxonomy of Viruses (ICTV) classified Begomoviruses based on genome arrangement, host range, and insect vector into 193 species (Hanley-Bowdoin *et al.* 2013). The genome of bipartite Begomoviruses consists of two components, which are to as DNA A and DNA B. Insect vector of Begomovirus is whitefly *Bemisia tabaci* (Gennadius), and it was not transmitted by seed and mechanical inoculation. Begomovirus can be grouped based on their genomic organization and geographic location into 2 groups i.e. Old World (OW) and New World (NW). In the Old World, Begomoviruses can be monopartite or bipartite and associated with DNA-satellites, while in the New World, they are mostly bipartite (Malgarejo *et al.* 2013). For examples, the sweepoviruses (monopartite begomoviruses that affect sweet potato) in the OW groups associated with a specific host, while examples in the NW groups are legumoviruses (bipartite begomovirus that affects legumes) (Ilyas *et al.* 2010; Trenado *et al.* 2011). The species *Squash leaf curl virus* belongs to the New World group, bipartite begomovirus that infects squash (Lapidot *et al.* 2014).

Squash mottle and leaf curl diseases are prevalent in the world and they are caused by a *B. tabaci* transmitted Begomovirus, commonly known as *Squash leaf curl virus* (SLCV) (Navas-Castillo *et al.* 2011). SLCV was reported in *Phaseolus vulgaris, Chenopodium murale, Convolvulus* sp., *Prosporis farcta, Malva parviflora, Gossypium hirsutum*, and several members of the Cucurbitaceae, such as melon (*Cucumis melo*),

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watermelon (*Citrullus lanatus*), cucumber (*C. sativus*), squash and pumpkin (*Cucurbita* species) (Alisthayeh *et al.* 2013; Brown *et al.* 2005). SLCV causes symptom disease in the host, including mottled leaf, leaves having shortened petioles that cluster around the vines, and curled leaves with yellowed leaves (Antignus *et al.* 2003; Idris *et al.* 2006). During the last three decades, numerous SLCV incidents have caused huge economic losses and threatened crop production, especially in the tropics and subtropics.

The virus was detected for the primary time within the Mediterranean locale in 2003, followed by Central and North America. SLCV caused extreme epidemics and the disease frequencyin Israel was 100% (Antignus *et al.* 2003). In 2006, SLCV was reported to cause severe symptoms in pumpkins in Egypt (Idris *et al.* 2006). In 2008, the virus was reported in squash in Jordan and the disease incidence was up to 95% in the Dir Alla area (Al-Musa *et al.* 2008). Previously, SLCV was identified within the coastal regions in Lebanon and caused yield losses up to 80% (Sobh *et al.* 2012). The virus was detected in Palestina in 2013; samples collected from Nablus in the summer showed a disease incidence was 85%. It was 98% from Qilqilia in the autumn and 25% in the winter season (Ali-Shtayeh *et al.* 2013). SLCV was reported in the squash fields in Oman and the disease incidence was reported to reach the range between 35 and 55% (Shahid *et al.* 2020). Until now, SLCV has not been found in Indonesia.

In 2020, we detected SLCV in butternut squash in Denpasar, Gianyar, Tabanan, Karangasem, Jembrana, Klungkung, Buleleng, Badung, and Bangli regions in Bali Province, Indonesia. The disease symptoms including mottling, leaf curling, and yellowing were observed in Denpasar, Gianyar, and Buleleng regions. Accordingly, the research aim was to molecular characterize the causal agent of squash leaf curl disease and to estimate the yield loss. Furthermore, this research also focused on the distribution of the disease in butternut squash-growing regions in Bali, Indonesia and yield losses estimation caused by infection of SLCV.

2. Materials and Methods

2.1. Samples Collection

Some samples of leaves with virus like symptoms were collected from several butternut squash in Bali Province; Denpasar, Gianyar, Tabanan, Karangasem, Jembrana, Klungkung, Buleleng, Badung, and Bangli regions. Thirty symptomatic samples were collected by using purposive sampling method from all location. The complete sample obtained is 270 samples.

2.2. Determination of Disease Incidence and Virus Detection using Polymerase Chain Reaction (PCR)

The nucleic acid of the samples from each location was detected by using Polymerase Chain Reaction (PCR). The cetyltrimethylammonium bromide (CTAB) method was used for total DNA isolation (Doyle and Doyle 1987). After that, 50 µl of nuclease-free water was added to the total DNA.

Begomovirus primer pairs used for total DNA amplification are SPG1 (5'-CCCCKGTGCGWRAATCCAT-3') and SPG2 (5'-ATCCVAAYWTYCAGGGAGCTAA-3') with amplicon size ±900 bp (Li *et al.* 2004). The amplification reaction was 1 µl of virus DNA, 1 µl of 10 µM forward primer, 1 µl of 10 µM reverse primer, 12.5 µl GoTaq Green 2x (Thermo Scientific), and 9.5 µl nuclease free water. Nucleid acid staining dye FluoroVue™ (Smobio, Taiwan) added agarose for electrophoresis. Electrophoresis was performed at 100 volts for 30 minutes and DNA visualization was performed under a UV transilluminator. PCR results are used to determine the incidence of disease caused by Begomovirus (ID) with the following formula:

$$ID = \frac{\text{Number of infected samples}}{\text{Total detected samples}} \times 100\%$$

2.3. DNA Cloning

DNA cloning was performed using the pTZ57R/T vector plasmid (InsTAclone PCR Cloning Kit, Thermo Scientific). The stages of DNA cloning are ligation, transformation, and confirmation of plasmid DNA. The ligation steps were carried out according to the method provided by Thermo Scientific.

The transformation was carried out by chemical method (InsTAclone Kit, Thermo Scientific). The pellet of *E. coli* JM 107 bacteria was dissolved in 300 µl T-solution (a mixture of solutions A and B with a ratio of 1:1). After that, the bacterial cultures were incubated using ice for 5 minutes. Bacterial cultures were centrifuged at 3,000 rpm for 5 min and pellets were collected, further diluted in 120 µl T-solution. Subsequently, it was incubated on ice for 5 minutes.

The ligated plasmid DNA was taken 2.5 µl and added 50 µl of competent cells. After that, the mixture was homogenized and incubated using ice for 5 minutes. The DNA transformants were isolated in luria broth agar (LB) containing 50 µl X-gal 2%, 10 µl IPTG 100 mM, and ampicillin (100 mg/l). Afterward, it was incubated at 37°C for 16 hours.

Universal primer M13F (-20) and M13R (-26) pUC was used to confirm DNA plasmid carrying recombinant. The positive DNA plasmid containing DNA target was then cultured in LB containing ampicillin and incubated at 37°C at 150 rpm on shaker for overnight. Isolation of recombinant plasmid DNA was carried out following the protocol by Crosa *et al.* (1994).

2.4. Sequences Analysis

DNA sequencing on recombinant plasmid DNA was carried out at First Base Malaysia. The nucleotide sequences of the gene were aligned with those of corresponding virus sequences deposited in GenBank database by using Clustal-W software. Sequences homology analysis of the gene was performed using Bio Edit version 7.05 software, and phylogenetic tree was constructed using MEGA 6.0 software with the neighbor-joining algorithm and 1.000 bootstrap replications (Tamura *et al.* 2013).

2.5. Yield Losses Estimation

Yield losses were calculated based on individual observation data plants and the observation of different levels of attack. Crop yields symptomatic and asymptomatic plants observed and measured include: the number of tubers produced, the weight of each tuber per plant, the total weight of the tubers cropping, tuber quality, and percentage of yield losses at the end observation. The percentage of yield losses can be obtained by using the following formula:

$$\text{Yield losses} = \frac{\text{Optimum results-actual results}}{\text{Optimum results}} \times 100\%$$

The optimum result is the result obtained in the absence of an attack pathogen, whereas actual yield is the result obtained in the event of a pathogen attack and control measures have been taken. After obtaining the percentage of disease severity, the area under disease curve (AUDPC) was calculated, and the observations were conducted every 2 weeks for 5 observations. At the end of the observations, the yield of each was calculated.

2.6. Data Analysis

The data obtained from the observations of plants in the field were tested with the chi-square method to compare between the number of tubers, weights per tubers and total tuber weight in the observation area per individual plant. Analysis correlation regression was used to evaluate the relationship between yield losses at various disease severities.

3. Results

3.1. Symptoms of Disease in the Field

Symptoms of the disease in pumpkin plants found in the fields included mottling, leaf curling, yellowing, and chlorosis (Table 1, Figure 1). These symptoms are very common in viral infections; therefore, they are only examined for an early diagnosis of a viral infection. The symptoms positive for Begomovirus included mottling, leaf curling, and yellowing (Figures 1B, C, D).

3.2. Identification and Molecular Characterization of SLCV

A phylogenetic tree based on Nei's coefficient groThe Begomovirus specific DNA band with the measurement of ±900 bp was successfully amplified using Begomovirus primers from several field samples (Figure 2). On the other hand, DNA band with amplification using Begomovirus primers was not obtained in samples from Badung, Tabanan, Karangasem, Klungkung, Bali, and Jembrana. The results of detection by the PCR method using Begomovirus universal primers showed that 53.33%, 36.66%, and 26.66% of Begomovirus infected pumpkin plants were found in leaf samples from Denpasar, Gianyar, and Buleleng. The detection results indicated a very high frequency of Begomovirus in pumpkin plants in Denpasar.

The amplified Begomovirus DNA fragments of samples from Denpasar, Gianyar, and Buleleng were successfully cloned into the TA vector pTZ57R/T and transformed into competent bacterial cells of *E. coli* JM 107. The recombinant DNA plasmid with the measurement of ±3,800 bp was successfully amplified using M13F (-20) / M13R (-26) pUC primers and obtained a DNA band with the measurement of ±1,060 bp (G). The PCR results proved that the recombinant DNA plasmid contained the insertion of the target DNA.

Analysis of nucleotide and amino acid sequences showed high homology of 92.0-98.4% and 93.2-99.3%

respectively with SLCV sequences in the database in GenBank (Table 2). SLCV among Bali isolates had nucleotide and amino acid homology ranged from 98.5-99.6% and 99.4-100%, respectively. SLCV sequences from Denpasar, Gianyar, and Buleleng showed the highest homology with East Timor isolates (KY652743) of 97.3-98.4% and 98.4-99.3% respectively (Table 2).

Phylogeny analysis showed that SLCV was divided into 2 groups: Group I and Group II and was separated from the TYLCV group as an outgroup. Group I consisted of isolates from Southeast Asia and

Table 1. The frequency of Begomovirus on butternut squash in Bali Province based on PCR

Location	Variety	Type of symptoms disease	Begomovirus frequency (%)
Denpasar	Labu Madu F1	Mottling, leaf curling, and yellowing	26/30 (86.66)
Badung	Labu Madu F1, Jacqueline F1 Enza Zaden	Mosaic and yellowing	0/30 (0)
Gianyar	Labu Madu F1, Hawk F1	Mottling, leaf curling, and yellowing	21/30 (70)
Tabanan	Labu Madu F1, Jacqueline F1 Enza Zaden	Mosaic, chlorosis, and yellowing	0/30 (0)
Buleleng	Hawk F1, Jacqueline F1 Enza Zaden	Mottling, leaf curling, and yellowing	19/30 (63.33)
Karangasem	Hawk F1, Labu Madu F1	Mosaic, chlorosis, and yellowing	0/30 (0)
Klungkung	Jacqueline F1 Enza Zaden	Mosaic and yellowing	0/30 (0)
Bangli	Hawk F1, Labu Madu F1	Chlorosis, and yellowing	0/30 (0)
Jembrana	Labu Madu F1	Mosaic, chlorosis, and yellowing	0/30 (0)

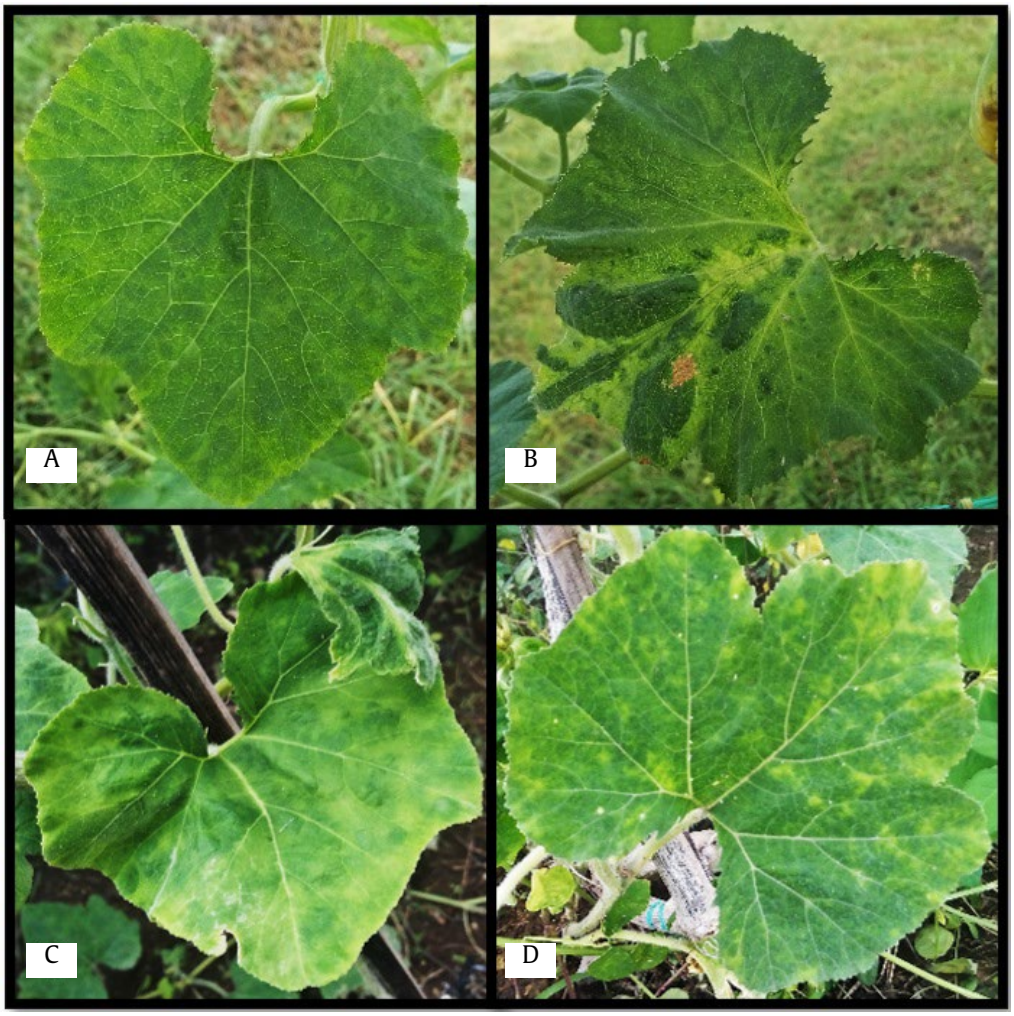

Figure 1. The symptoms disease on butternut squash in Bali: mosaic (A), mottle with curling (B), yellowing (C), and chlorosis (D)

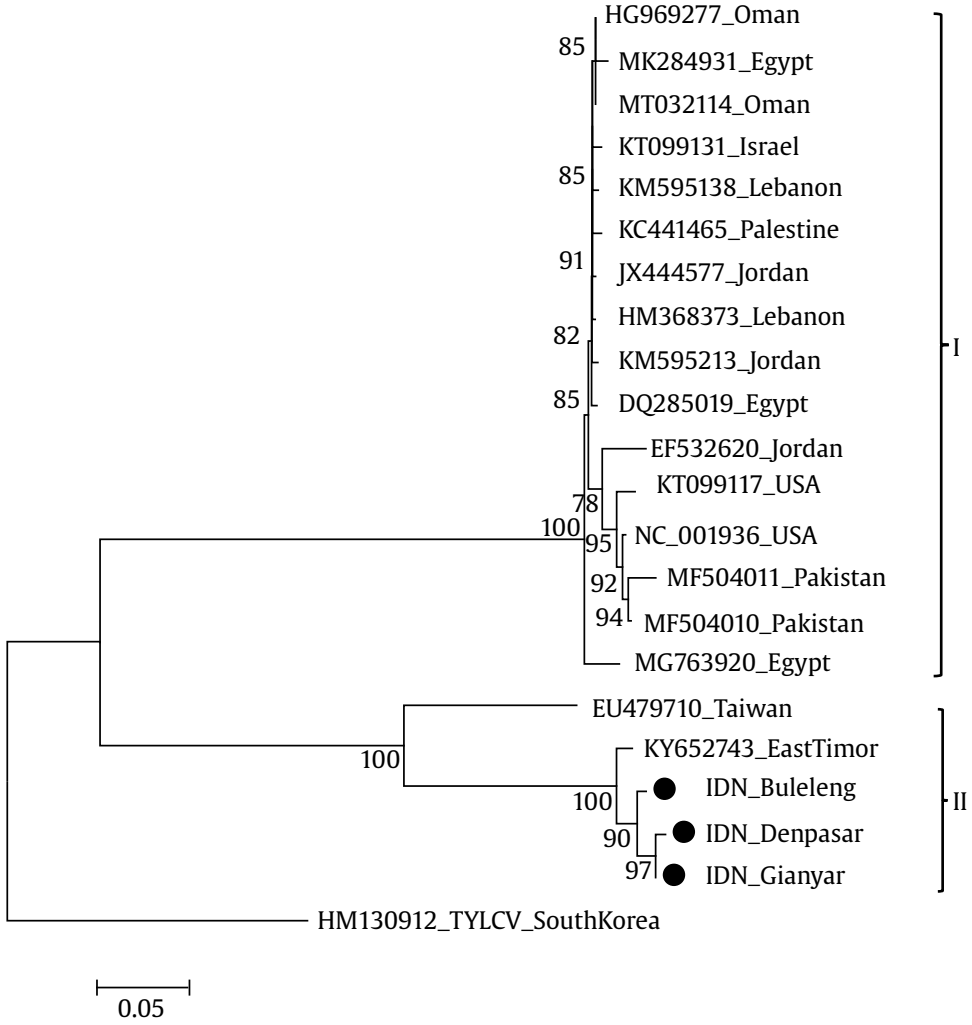

Figure 2. Phylogenetic tree based on the AC1 and AC2 gene from SLCV Bali isolates. Tomato yellow leaf curl virus (TYLCV) was used as outgroups. IDN (Indonesia), isolates marked with black dots are Bali isolates. Bootstraps values greater than 70% based on 1000 replicates are shown on tree branches. Scale bar below tree indicates 0.05 nucleotide substitutions per site

Table 2. Nucleotide (nt) and amino acid (aa) AC1 and AC2 sequence identity between SLCV Bali isolates and isolates from other countries with sequences deposited in GenBank

Isolates	Homology nt (aa) (%) SLCV_IDN_			Accession number	Host
	Denpasar	Gianyar	Buleleng		
East Timor	97.5 (98.6)	97.3 (98.4)	98.4 (99.3)	KY652743	Pumpkin
Palestine	93.2 (94.8)	93.4 (94.8)	93.5 (94.8)	KC441465	Squash
Taiwan	96.6 (97.2)	96.8 (97.3)	97.2 (98.4)	EU479710	Squash
Oman	93.2 (94.8)	93.4 (94.8)	93.7 (95.0)	MT032114	Squash
Egypt	92.9 (93.5)	93.2 (94.8)	93.4 (94.8)	MK284931	Squash
Pakistan	92.2 (93.5)	92.5 (93.5)	92.6 (93.5)	MF504011	Upland cotton
Egypt	92.8 (93.5)	93.1 (94.8)	93.2 (94.8)	MG763920	Tomato
Pakistan	92.9 (93.5)	93.2 (94.8)	93.3 (94.8)	MF504010	Upland cotton
Jordan	93.4 (94.8)	93.7 (95.0)	93.8 (95.1)	JX444577	Tomato
Jordan	92.0 (93.2)	92.2 (93.5)	92.4 (93.5)	EF532620	Cheeseweed mallow
Egypt	93.2 (94.8)	93.4 (94.8)	93.5 (94.8)	DQ285019	Squash
Lebanon	93.4 (94.8)	93.7 (95.0)	93.8 (95.1)	HM368373	Zucchini
USA	92.8 (93.5)	93.1 (94.8)	93.2 (94.8)	NC_001936	Squash
Oman	93.2 (94.8)	93.4 (94.8)	93.7 (95.0)	HG969277	Squash
USA	92.9 (93.5)	93.2 (94.8)	93.3 (94.8)	KT099117	Squash
Israel	93.2 (94.8)	93.4 (94.8)	93.5 (94.8)	KT099131	Squash
Lebanon	93.3 (94.8)	93.5 (94.8)	93.7 (95.0)	KM595138	Squash
Jordan	93.3 (94.8)	93.5 (94.8)	93.7 (95.0)	KM595213	Squash
TYLCV_South Korea	75.6 (76.1)	75.5 (76.0)	75.9 (76.3)	HM130912	Tomato

nt (nucleotide), aa (amino acid), IDN (Indonesia), TYLCV (Tomato yellow leaf curl virus) was used as outgroups

East Asia, while group II consisted of isolates from West Asia, Africa, South Asia, and America. The SLCV isolates studied coming from Denpasar, Gianyar, and Buleleng were in the same group with isolates from East Timor and Taiwan (Figure 2).

3.3. Yield Losses Estimation

3.3.1. Yield and Estimated Loss of Individual Crop Yields

The yields of healthy pumpkins and those with curling symptom showed that there were significant differences in the weight per fruit and fruit weight per plant (Table 3); however, the number of fruits per plant was not significantly different. This means that the curling disease in the pumpkin reduces the weight per fruit and fruit weight per plant. Pumpkin crop yields are influenced by the quality and quantity of fruits produced; therefore, if more fruits are produced, the yields will become higher. The difference in fruit weight produced between pumpkin plants with no symptoms and with yellow symptoms can significantly reduce yields. The actual loss of yield based on the comparison between the pumpkin plants with curling system and the asymptomatic pumpkin plants was 56.3%. SLCV was identified in coastal areas of Lebanon in 2012, and yield losses were reported to range between 70% and 80%.

The highest number of fruit per plant in plants without curling symptoms was 3 to 5, while in plants with curling symptoms, the highest number of fruit was 1 to 4. The highest weight per fruit in asymptomatic plants was more than 2.5 kg, while the weight per fruit in curling-symptomatic plants was between 0.5 to 2.0 kg. These results indicate that curling disease can reduce fruit production per plant.

Loss of crop yields can have direct or indirect impacts. The direct impact can be in the form of an economic loss of yield that is disadvantageous to farmers and buyers, while the indirect loss of yield is influenced by a decrease in the quality of the harvest. A decrease in the quality of the harvest will have an impact on the decreasing selling price. Pumpkin yields on plants with curling symptoms showed a decrease in fruit quality in the form of shape malformations and color changes, and color changes are accompanied by shape malformations (Table 4). The highest number occurred in a decrease in color changes accompanied by shape malformations with a percentage of 15.5%.

3.3.2. Relaxation of Yield Losses in Various Disease Intensities

Pumpkin productivity was significantly influenced by the incidence by the incidence of curling disease. The higher the disease incidence rate, the lower the pumpkin fruit production. Yield reduction was up to 25.83% in fields with a disease severity of 32% (Table 5). The yield with the highest disease severity was found on land with AUDPC 742 producing a yield of 2,397.8 kg/1,000 m^2. This correlates with the symptoms produced by symptomatic fruits i.e. the size of the fruit was smaller than that of the fruit from a healthy plant. The size and quality of the harvests affect the selling price of pumpkins in the market. If the harvested fruits are large and have good quality but no physical defects, the selling prices will be high. The selling price of pumpkins in the market is approximately IDR 15,000.00 per kg, while the average selling price from farmers is IDR 7,000.00 per kg. Curling disease in pumpkins affects the selling price of pumpkins in the market, and the pumpkin yields produced from symptomatic plants will cause a decrease in yields which will affect the income generated by farmers. The biggest loss occurred on land with AUDPC 742 causing a yield loss of IDR 10,826,400.00 (Table 5).

Table 3. The yields of butternut squash harvest on healthy and curly symptom plants

Plants	Agronomic variables			Harvest (g)
	Number of fruits per plant (fruits)	Weight per fruits (g)	Fruits weight per plant (g)	
Symptomless	4.4 a	1,703 a	7,524 a	3,978,045 a
Curling symptoms	3.7 a	858 b	3,356 b	1,739,420 b
Yield losses (%)	15.9	49.6	55.4	56.3

Numbers in one column followed by different letters indicate the difference with the chi-square test

Table 4. The quality of pumpkin yields on yellow symptomatic plants and healthy plants

Fruit quality	Symptomless		Curling	
	Number (fruits)	Percentage (%)	Number (fruits)	Percentage (%)
Healthy	328	91.8	235	74.1
Malformation	14	3.9	17	5.4
Discoloration	12	3.4	16	5.1
Discoloration and malformation	3	0.8	49	15.5

Table 5. Yield loss on some fields

AUDPC DS*	Yields (kg/1,000 m²)	Yield loss (%)	Yield loss (IDR)
357	3,226.8	10.02	878,400.00
581	2,967.9	15.23	3,985,200.00
624	2,683.5	19.61	7,398,000.00
679	2,572.6	22.72	8,728,800.00
742	2,397.8	25.83	10,826,400.00

4. Discussion

Symptoms found in pumpkin plants in the fields vary widely in which mottle and curling are the most common symptoms. Variations in disease symptoms can be caused by differences in cultivars, plant age, and environmental conditions (Ali-Shtayeh *et al.* 2013). The variation in symptoms due to SLCV infection was also reported by Diaz-Nazera *et al.* 2020 on cucurbit plants. The results of molecular detection indicate that SLCV infection in pumpkin plants has not widely spread in Bali. The detection of SLCV in Bali is the first report of SLCV infection in Indonesia.

Based on the data on the distribution of SLCV on the pumpkin fields in Bali, there were three regions invaded by the virus, and this is the first report made on this virus. Transplant transfer activities between Indonesia and its neighboring countries including East Timor and Taiwan are assumed to cause the development of virus in Indonesia, or it probably because viruliferous *B. tabaci* moved from infected cucurbit plantations in Timor Leste and Taiwan to cucurbit plantations in Indonesia. Maina *et al.* (2017) stated that SLCV invaded the regions of East Timor and Taiwan; however, it was not found in all samples even though the majority of the tested cucurbit sample in this research were from the symptomatic plants. Apparently, other viruses infected the cucurbits with SLCV negative symptom.

Based on the results of the survey utilizing the PCR, high incidence of SLVC occurred in the three-surveyed regions during the dry seasons in particular where a great number of whiteflies were monitored during the study. There might be a number of reasons why this high incidence of SLVC occurred in these regions i.e. low knowledge of the farmers regarding the etiology of the disease, ineffective and inefficient control measures against the whitefly vector and improper cultural practices including crop overlapping causing continuous sources of SLCV.

Since a high incidence of SLVC occurred in these three surveyed regions, Ali-Shtayeh *et al.* (2013) examined the sources of this infection i.e. a number of weed species existing in the cucurbit fields. Moreover, SLCV could be detected in *Convolvulus* sp., *Chenopodium murale, Prosopis farcta*. It was also reported by Al-Musa *et al.* (2008) and Antignus *et al.* (2003) that respectively SLCV could infect *M. parviflora* in Jordan and *M. nicaeensis* and *Ecballium elaterium* (Cucurbitaceae) in Israel.

From the epidemiological perspective, these findings are crucial since *Convolvulus* sp., and *C. murale* are one of the most common weed species found in cucurbits in Indonesia. It can be predicted that these weed species function as reservoir for SLCV the whole year; therefore, it is expected that high incidence of SLCV in Indonesia occurs throughout the year where high population of whitefly exists.

The DNA-A sequences obtained showed that these sequences had a high identity and the same cluster as the Timor Leste isolates, which indicated that they were isolates of the same species based on the species demarcation criteria. (Fauquet *et al.* 2008). Previous studies indicated that high homology of Palestine isolates with isolates from Jordan and Israel was caused by the transfer of SLCV from its neighboring countries of Israel and Jordan to Palestine either by *B. tabaci* or by the switch of the seeds where a number of viroids and viruses have been definitely dispersed all over the world by the exchanges of undetected infected seeds (Ali-Shtayeh *et al.* 2013; Abudy *et al.* 2010; Al-Musa *et al.* 2008).

Special attention should be given to this virus since there have been high yield losses due to SLCV and the presence of the endemic population of vector *B. tabaci* in in Indonesia; otherwise, this virus can invade fields in this country and cause incredible yield losses to other crops. Despite the low incidence of SLCV in some surveyed regions, intensive efforts are required to be made to evade further spread of the disease to other cucurbit-growing areas particularly during the dry season where the population of the insect vector is typically high.

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