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Critical Phase of Zucchini Plant (*Cucurbitapepo L.*) to *Zucchini Yellow Mosaic Virus* (ZYMV) Infection

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Abstract: ***Zucchini Yellow Mosaic Virus (ZYMV) is one of the causes of mosaic disease in zucchini plants in Bali. Based on observations in four villages of zucchini cultivation center in BaturitiTabanan Bali area covering Pekarangan, ApitYeh, KembangMerta, and Angsri villages, the average incidence of mosaic disease was 89.74%. Serologic detection with ELISA and molecular identification with RT-PCR in zucchini plants showing mosaic symptoms, successfully detected that ZYMV is a symptom inducer of mosaic on zucchini plants. Determination of the critical phase of zucchini plants against ZYMV infection was done through experiments in greenhouses, by mechanical inoculation of zucchini at 2 weeks, 3 weeks, 4 weeks, 5 weeks and 6 weeks after planting with inoculums source from ZYMV sap. The variables observed were incubation period, disease incidence, disease severity, endurance level and yield.The results showed that ZYMV infection in plants of different ages had an effect on the incubation period, disease incidence, disease severity, endurance level and crop yield. If ZYMV infection occurs in older zucchini plants at 5 weeks after planting, the incubation period is slow, the incidence of the disease is lower and the severity of the disease is lower than that of ZYMV infection in younger zucchini plants. Zucchini infected plants ZYMV at the age of 4 weeks after planting the incidence of the disease reached 100% and can not produce fruit, zucchini infected ZYMV at age older than 4 weeks after planting, disease incidence reaches 50% and still can produce fruit, with yield decrease up to 41.216% compared with healthy zucchini plants. Zucchini plant age 4 weeks after planting, is a critical phase of ZYM infection.***

Keywords: critical phase, infection, mosaic, zucchini

1. Introduction

Zucchini Yellow Mosaic Virus (ZYMV) is one of the most important types of virus causing mosaic disease in cucurbitaceae plants worldwide, which can lead to decreased yield. (Lin *et al*. 2000; Simmons *et al*., 2011). The incidence of viral illness due to ZYMV infection in cucumber plants can reach 100%, with varying intensity of attack (Sumpena, 2012). Loss of infection depends on the time of infection, and can result in a yield loss, reaching 100%. The incidence of viral infections at different plant ages, indicating different responses to disease severity. The severity of the disease is strongly influenced by the growth phase and age of the plant. Plants are very susceptible to infection with viruses at young plant life, and have an effect on the high incidence of disease, because when young plants are infected with viruses, the virus incubation period is shorter, and the process of virus distribution and translocation will accelerate (Akhtar *et al.* 2004; Mandal *et al*., 2007).

Infection by ZYMV is one of the causes of mosaic disease in zucchini plants, in the zucchini planting center of BaturitiTabanan Bali, with the average incidence of mosaic disease reaching 89.74% and it is further found that 81.50% of all zucchini plants showing mosaic symptoms are Infected by Zucchini yellow mosaic virus (preliminary research result of the researcher).Symptoms appearing in ZYMV infected zucchini plants include, yellow mosaic symptoms, necrosis, malformations, blistering, shoes strings and vein bending. Other symptoms that occur in plants infected with ZYMV are yellow mottling on the leaves with stunting, distortion of leaves, fruits and flowers, and mottling of the fruit. The high incidence of mosaic disease due to ZYMV infection in zucchini plants has had an impact on yield reduction, it is suspected because the infection occurs in young plants. The incidence of such mosaic diseases is economically very detrimental, because it leads to a decrease in the quality and quantity of crops.

The incidence of mosaic disease resulting from ZYMV infection in zucchini cultivation in BaturitiTabanan Bali, has not been widely known and there has been no research oriented to disease incidence and disease severity. In this connection, it is necessary to do this research with the aim to know the critical phase of zucchini plant due to ZYMV infection, in order to know the vulnerable age of zucchini plants against ZYMV infection, so prevention and control of ZYMV infection can be done as early as possible and the greater yield loss can be avoided.

2. Material and Methods

Survey and Sample Collection

Zucchini plants were examined at each location. Individual representative leaf and plant specimens were collected from zucchini plants showing symptoms characterized by mosaic similar to those caused by virus. Samples, consisting of about 1 g fresh weight of young leaves or shoot tips showing disease symptoms were desiccated over anhydrous calcium chloride (about 7 g) in sealed, 25 ml plastic vials. Samples were stored at 4°C until fully desiccated. Laboratory examination included double antibody sandwich enzyme-linked immunosorbent assays (DAS-ELISA) and reverse transcription-polymerase chain reaction (RT-PCR).

Enzyme-Linked Immunosorbent Assays (ELISA)

About 5% Zucchini showing mosaic symptom samples from all surveys were tested for virus infection using double antibody sandwich (DAS)-ELISA . DAS-ELISA was performed for the detection of *Zucchini yellow mosaic virus*

(ZYMV) in the collected plant samples. ZYMV -specific antibodies along with alkaline phosphatase-linked antibodies procured from DSMZ (Germany) were subjected for ELISA as manufacturer's instruction and as method where ELISA tests were performed using ZYMV-specific polyclonal antibodies to detect ZYMV, CMV-specific polyclonal antibodies to detect CMV and dan PRSV-specific polyclonal antibodies to detect PRSV. All ELISA test samples were considered positive when absorbance values exceeded three times the mean of appropriate healthy controls that were included on each microtiter test plate. Zucchini sample samples showing positive reactions to ZYMV antiserum were further identified molecularly by reverse transcription-polymerase chain reaction (RT-PCR).

Total RNAs Extraction

Total RNAs was isolated from mosaic symptom shown Zucchini leaf samples following manufacturer protocol (Thermo Scientific, Lithuania). Fresh tissue (0.1 g) was ground in liquid Nitrogen to powder, 500 µl of Plant RNA lysis Solution was added and the sap was transferred to 1.5 ml clean tube, and then was vortexed 10 – 20 s thoroughly. The sap was incubated in water bath at 56° C for 3 min, then centrifuged at 14 000 rpm for 5 min. The supernatant was pipetted to 1.5 ml clean tube, 96% ethanol was added and mixed by pipetting. The liquid was transferred to a purification column inserted in a collection tube and was centrifuged at 12 000 rpm for 1 min and then discarded flow-through. The purification column was added 700 µl of Wash Buffer WB 1 then was centrifuged at 12 000 rpm for 1 min and then discarded flow-through. The purification column was placed into a clean 2 ml collection tube and was added 500 µl Buffer 2 then was centrifuged at 12 000 rpm for 1 min. The solution was discarded flow-through and repeat the additional of 500 µl Buffer 2 using maximum speed of centrifugation. The collection tube was discarded flow-through and the purification column was transferred to aRNase-free 1.5 ml collection tube. The purification column was added 50 µl of nuclease free water to elute the RNA. The column was centrifuged at 12 000 rpm for 1 min and then discarded the purification column. The RNA which was kept on a collection tube was used as template in RT-PCR.

Reverse Transcriptase- Polymerase Chain Reaction (RT-PCR)

RT-PCR was performed using the primary oligo d (T) and the resulting cDNA was used for the amplification reaction. Amplification was performed using a *Potyvirus* universal primer, *Poty* MJ-1 (5'-ATGGTHTGGTGTGYATHGARAAYGG-3 ') and *Poty* MJ-2 (5'-TGCTGCKGCYT TCATYTG-3'), which will amplify the genes of coat protein members of the genus *Potyvirus* by ~ 320 bp (Marie-Jeanne *et al*., 2000).Amplification was conducted using one-step RT-PCR method. RT-PCR reaction contains 12.5 µl Go Taq Green PCR master mix (Fermentas, US), 10 µM each of primer, 2.0 µl DTT 50 mM, 0.1 µlRNAse Inhibitor, 0.1 µlMMuLV, 0.5 µlMgCl, 2.0 µl RNA total, and the reaction was adjusted to 25 µl with nuclease free water. Amplifications was performed in GeneAmp PCR System 9700 machine with 60 min at 42.0°C and 2 min at 94.0°C for RT, 5 min at 94.0°C for pre-heating, followed by 30 cycles of denaturation (3 min at 94.0°C), annealing (1 min at 61.0°C), and extension (2 min at 72.0°C). The last cycle was ended at 72.0°C for 3 min and cooled down to 4.0°C. Electrophoresis was done using 1% Agarose gel in 0.5 x TBE (Tris-Boric acid-EDTA) buffer, run at 50 V for 50 min. Following electrophoresis, agarose gel then was soaked on to 0.1% EtBr for 5 min, washed with H_2O, and visualized under UV transilluminator.

Determination of Critical Zucchini Phase Against ZYMV Infection

The critical phase test of zucchini plants against ZYMV infection is done by mechanically sap inoculation from infected plants ZYMV, in healthy zucchini plants as test plants. The test plants were classified into five levels of age at ZYMV inoculation: ZYMV inoculation at 2 weeks, 3 weeks, 4 weeks, 5 weeks and 6 weeks after planting and control (zucchini plants without ZYMV inoculation). Observations were made on variables: incubation period, disease incidence, disease severity and crop yield.

The severity of the disease was calculated on each test crop with the score category. Scores used are (score 0): plants do not show mosaic symptoms; (Score 1): plants show very mild mosaic symptoms; (Score 2): the plants show moderate mosaic symptoms but the leaves do not show any change in shape; (Score 3): the plant exhibits moderate mosaic symptoms and on its leaves indicates a change in shape; (Score 4): the plant exhibits severe mosaic symptoms and leaves many changes in leaf shape. The measured value of the score is converted to disease severity by Townsend and Heüberger (Agrios 2005):

$$DS = \frac{\sum_{i=1}^{k}(n_i \times v_i)}{N \times V} \times 100,$$

Description:
DS = Disease Severity
n_i= number of plants with an i-score
v_i = value of disease with an i-score
N = number of plants observed
V= highest score

Table 1: Resistance Rate of Cucumber to infection ZYMV

Rate of resistance	Intensity of virus symptom
Immun	0%
Very resistant	0 - 10%
Hold	10 - 25%
Medium Hold	25 - 40%
Medium Sensitive	40 - 50%
Sensitive	50 - 70%
Very Sensitive	> 70%

3. Result and Discussion

The Incidence of Mosaic Disease and Mosaic Symptoms on Zucchini Plants in Bali

The incidence of mosaic disease encountered during the study at the zucchini planting center in Bali ranged from 87.58% to 92.72%, as shown in Table 2. Variation of symptoms of mosaic disease found during observation in

zucchini plants is, leaf malformation in zucchini plants which is still young, vein banding on the leaves of mature plants and distortions and fruit malformations occur in infected plants during production, as shown in Figure 1.

Table 2: The incidence of mosaic disease in zucchini cultivation centers in Bail

Location zucchini cultivation centers	Population Plants	Plants symptomatic mosaic	Percentage of disease incidence (%)
Pekarangan village	3.140	2.750	87,58
ApitYeh village	3.090	2.865	92,72
KembangMerta village	4.395	3.865	87,94
Angsri village	3.740	3.392	90,70

Description: Survey results from July to August 2016

Figure 1: Variation of mosaic symptoms in zucchini plants infected with ZYMV Malformation; (B) Vein banding; (C) distortions and fruit malformations

ZYMV Induced Mosaic Disease in Zucchini Plant

Results of DAS-ELISA test with ZYMV, CMV, and PRSV specific antiserum was found that all samples for ZYMV antiserum showed positive reaction, except sample from ApitYeh village (2) and Angsri (2), while for CMV antiserum only sample from Pekarangan Village (2) , ApitYeh Village (2) and Angsri Village (2) which showed a positive reaction. For PRSV antiserum all samples showed a negative reaction, as shown in Table 3.

Zucchini plants infected by single ZYMV virus, serologically detected on sempel from five sample source sites are from Pekarangan Village (1), ApitYeh Village (1), KembangMerta Village (1), KembangMerta Village (2) and Angsri Village (1). Zucchini plants are infected with ZYMV singly, then molecularly identified with RT-PCR technique, to obtain more precise identification results.

Table 3: DAS-ELISA results from zucchini plants with mosaic symptoms, using a specific antiserum ZYMV, CMV, and PRSV

Sample source (Village)	The result of detection using Specific antiserum		
	ZYMV	CMV	PRSV
Pekarangan (1)	+	-	-
Pekarangan (2)	+	+	-
ApitYeh (1)	+	-	-
ApitYeh (2)	-	+	-
KembangMerta (1)	+	-	-
KembangMerta (2)	+	-	-
Angsri (1)	+	-	-
Angsri (2)	-	+	-

Description: The DAS-ELISA reaction is positive if the sample absorbance value is equal twice or greater than the negative control or buffer absorbance value.

Molecular identification by RT-PCR technique, using a universal primer of *Potyvirus*, successfully amplifies DNA bands of ~ 320 bp. DNA fragments measuring ~ 320 bp (Figure 2) were successfully amplified in zucchiniplant samples from KembangMerta Village and Angsri Village . These results indicate that the virus is a *potyvirus*group, and there is no indication of other viruses. This result occurs because the primary pair used can amplify DNA from RNA all viruses belonging to the *Potyvirus* group, including ZYMV which is a virus from the *Potyvirus*group.

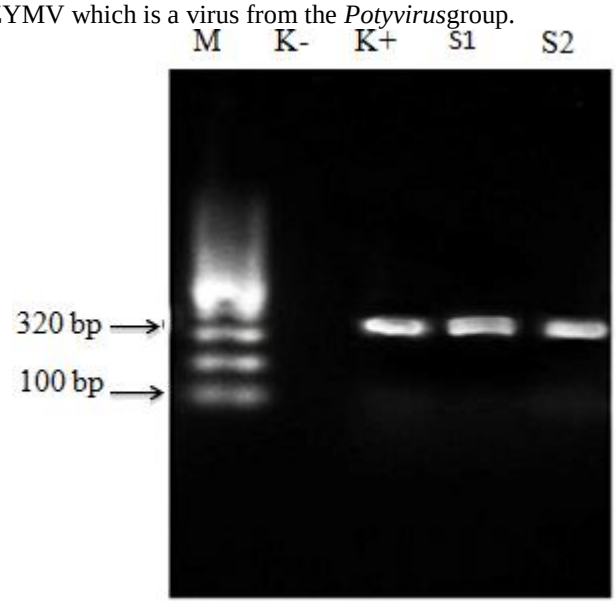

Figure 2: Visualization of results from DNA amplification using a *Potyvirus* universal primer. M = DNA marker (100

bp) (Biorad, USA); K- = negative control ; K + = positive control (DNA *Potyvirus*); S1= sample of KembangMerta village; S2 = sample Pekarangan village.

The Incubation Period and The Incidence of Mosaic Disease in Zucchini Plants areInfected with ZYMV

ZYMV infection at the age of different zucchini plants, it affects the incubation period, the incidence and severity of the disease. If infection occurs at the age of older plants at 5 weeks of age and 6 weeks after planting, longer incubation periods, low incidence of disease and disease severity are also low, when compared with infections occurring at younger ages (Fig. 3). Infection occurring at plant age 2 to 4 weeks after planting resulted in an incubation period ranging from 9.30 days to 10.30 days and the incidence of the disease reached 100%. Infections that occur in older plants when the age of 5 weeks after planting showed a slower incubation period of the virus that is 14.50 days, and resulted in 50% disease incidence. The incubation period is closely related to the ability of the virus to spread from local infection to other parts of the plant and then show symptoms. The virus is able to spread to the young plants quickly because young plants do not yet have a strong defense system against viral infections (Agrios, 2005).

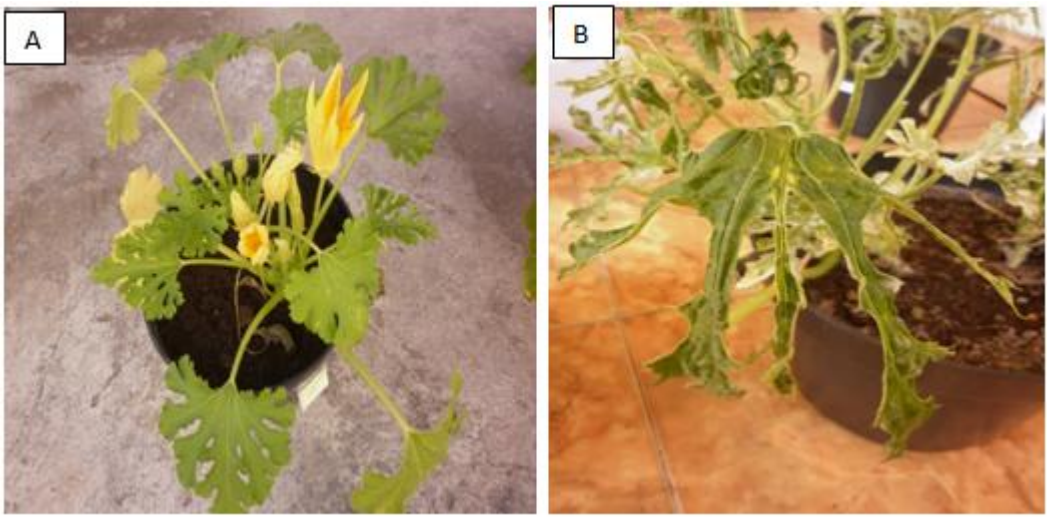

Figure 3: (A). Zucchiniplants infected with ZYMV at 6 weeks after planting (symptomatic light) (B). Zucchiniplants infected with ZYMV at 4 weeks after planting (symptomatic weight)

ZYMV infection at different plant ages, affecting the severity of the disease, there are the younger plants infected ZYMV the severity of disease is higher. The results of this study indicate that the severity of the disease at age plant of 2 weeks is 72.50% and decreased to 35% at plant age 6 weeks after planting (Table 4). These results indicate that if infection occurs earlier then the severity of the disease becomes higher. Viral infections with mosaic symptoms in plants can lead to a decrease in the amount of chlorophyll, carotenoids, carbohydrates, proteins, and amino acids. As a result of further growth disorders will increase, along with the increasing age of plants that have been infected with the virus (Hemida, 2005). ZYMV infection at younger plant age leads to a decrease in chlorophyll earlier than infected plants at older ages, resulting in infected young plants impacting growth and plant resistance systems against viral infections. As a result of this incident will cause the severity of the disease is higher because young plants do not yet have a strong resistance system, when the infection occurs from the virus (Hull, 2002).

Table 3: Incubation period, incidence and severity of disease in zucchini plants infected with ZYMV

Plant age Inoculation(*)	Incubation period (**)	Incidence of disease (%)	Disease severity (%)
2	9,3	100	72,50
3	9,8	100	67,50
4	10,3	100	65,00
5	14,5	60	47,50
6	15,6	50	35,00

Description: (*) weeks after planting, (**) days after inoculation.

The Degree of Sensitivity of Plants and Zucchini Crops is Infected with ZYMV

The younger of zucchini plants are infected with ZYMV, the more plants are unable to produce the fruit, compared to plants infected with the virus at an older age. ZYMV infection in plants aged 2 weeks to 4 weeks after planting causes plants can not produce fruit. These results occur because, ZYMV infection in young zucchini plants, there will be severe mosaic symptoms that affect the decrease in the amount of plant chlorophyll that plants occur earlier, so that in young plants infected ZYMV fruit formation process will be disrupted and even no process occurs Fruit formation. In Zucchini plants infected with ZYMV at 5 weeks to 6 weeks after planting, can still produce fruit, although a decrease of 41.26% compared with healthy plants as shown in Table 5.

Table 4: Results and extent of zucchiniplant resistance to ZYMV infection

Age at ZYMV Inoculation (*)	Sensitivity	Loss Crop yield (**) (%)
2	Very sensitive	100
3	sensitive	100
4	sensitive	100
5	Moderately sensitive	41,26
6	Resistant	13,33

Description: (*) weeks after planting, (**) of healthy plants

ZYMV infections occurring at different plant ages affect the level of plant resistance and crop yield. Viral infections of zucchini plants occurring at the age of 2 weeks to 4 weeks after transplanting, show a susceptibility response at a sensitive to extremely sensitive level. The severity of the disease is strongly influenced by the growth phase and age of the plant when it is infected with the virus. Plants are highly susceptible to viral infections at a young age and have an effect on the high incidence of disease. Young plants infected with the virus, the incubation period of the virus becomes shorter and the process of virus distribution and translocation in plants will be faster (Akhtar *et al*. 2004; Mandal *et al*. 2007).

4. Conclusion

ZYMV infections at different ages of zucchini plants turned out to be the incubation period, disease incidence, disease severity and crop yield. If ZYMV is infected at older plant age at 5 weeks after planting, virus incubation period is slow, disease incidence is lower and disease severity is lower when compared with ZYMV infection in younger zucchini plants. Zucchini plant starts at planting until age 4 weeks after planting shows 100% disease incidence and plants can not produce fruit. Zucchini damaged ZYMV at age older than 4 weeks after planting shows 50% disease incidence and the plant can still Results of fruit, can occur up to 41.216% results when compared with healthy zucchini plants. From the time of planting until the age of 4 weeks after planting, zucchini plants are sensitive to ZYMV infection, so it can be concluded, the critical phase of zucchini plant against ZYMV disease, occurred from the time of planting until the age of 4 weeks after planting.

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