High impact of pagoda leaf extract to suppress zucchini yellow mosaic virus infection in zucchini plants

Abstract. Zucchini yellow mosaic virus (ZYMV) is an important virus in zucchini plants. ZYMV infection is prevalent on zucchini plants in Bali and is extremely difficult to eradicate. This study aimed to determine the efficacy of pagoda leaf extract in reducing yield loss in zucchini plants. A factorial randomized block design with two variables was employed in this study. The application time of the pagoda extract was the first variable, and concentration was the second. Plant extract was applied during seed immersion, one day before and after inoculation, and on the same day of virus inoculation. The concentrations of pagoda extract employed were 10%, 30%, and 50%. The crude extract was sprayed onto the zucchini leaves. Plants treated with extract one day after virus inoculation at 10%, 30%, and 50% concentrations exhibited yellowing, yellowing with cupping, mosaic, mosaic with cupping, and stunting. While application of extract one day before virus inoculation at 30% and 50% concentrations resulted in yellowing and yellowing with cupping signs. Plants infected with ZYMV first showed flowering at 37 DAP, whereas plants treated with pagoda leaf extract one day before virus inoculation started flowering at 32 DAP. Application of pagoda leaf extract one day before ZYMV inoculation on zucchini plants effectively suppressed 40% yield loss. The findings of present investigation revealed information about the potential of pagoda leaf extract as a plant virus control agent through the mechanism of induction in zucchini plants.
Keywords: Flowering, pagoda leaf extract, yield loss, zucchini, ZYMV

Abbreviations (if any): Zucchini yellow mosaic virus (ZYMV)

RUNNING TITLE: High impact of pagoda flower leaves to suppress zucchini yellow mosaic virus infection

INTRODUCTION

ZYMV is one of the most critical viruses causing mosaic disease in Cucurbitaceae plants worldwide, which could cause yield reduction (Simmons et al. 2011; Simmons et al. 2013). The incidence of viral disease due to ZYMV infection in cucumber plants could reach 100%, with varying attack intensities (Tymchyshyn et al. 2017). The loss due to viral infection depends on the time of infection and can result in yield losses of up to 100% (Coutts et al. 2011). Plants become very susceptible to viral infection at young age, affecting the high incidence of disease. When young plants become infected, the incubation period of virus is shorter, and the virus distribution and translocation process is accelerated (Coutts et al. 2013). Host range is one way of determining the biological nature of a virus (Spadotti et al. 2015). The most frequently employed host range test plants for ZYMV are *Gomphrena globosa* L., *Chenopodium amaranticolor* Coste et Reyn, *C. quinoa* L., *Citrullus lanatus*, *Cucumis melo* L., *Cucumis sativus* L., *Luffa acutangula*, *Momordica charantia*, *Sechium edule*, *Phaseolus vulgaris* L., *Vigna sinensis*, *Capsicum annuum*, *Datura stramonium*, *Lycopersicon esculentum*,

Nicotiana tabacum L. cv. White barley, *N. benthamiana*, and *Physalis floridana* (Jaroszewska et al. 2013; Massumi et al. 2013; Spadotti et al. 2015).

Zucchini yellow mosaic virus (ZYMV) has a one-molecule positive senseRNA genome of approximately 10 kb and encodes a poly-protein that is proteolytically processed into mature protein; P1 (protease), HC (helper component/protease), P3, 6K1, CI (cylinder inclusion), 6K2, NIa (nuclear inclusion a), VPg (viral protein-associated genome), NIb (nuclear inclusion b) and CP (Gal-On 2007; Moradi et al. 2019). Until now, four out of ten potyviral proteins have been identified in ZYMV-infected plants, namely P1, HC-Pro, cylindrical inclusions, cylindrical inclusions (CI) pinwheel type 1, and CP (Maghamnia et al. 2018; Valli et al. 2017). Other proteins (Third protein (P3), 6K1, 6K2, viral genome-linked protein (VPg), Nuclear Inclusion A (NIa), and NIb replicas) have not been identified in ZYMV-infected plants and are based on potyvirus sequence homology.

ZYMV was first reported in Italy in 1973 (Lisa et al. 1981) and then France (Lecoq et al. 1981). ZYMV distribution has been found in Africa, America, Asia, Europe, the Middle East, and Oceania (Spadotti et al. 2015; Bubici et al. 2020). In Egypt, ZYMV has been associated with significant yield losses in many cucurbit crops, including cucumbers, melons, pumpkins, marrow vegetables, watermelons, and especially zucchini (Aleem et al. 2021). ZYMV infection result in symptoms, such as mosaicism, yellowing, and eventually "shoeing" on the leaves. The fruit is stunted, twisted, and distorted, resulting in reduced yield and inability to market the product, especially chayote zucchini (Massumi et al. 2011). ZYMV is commonly transferred in two ways: horizontally via aphid vectors and vertically by transmission from the first generation of ZYMV-infected seeds to the following generation of ZYMV-infected seeds (Simmons et al. 2011; Wang and Li 2017). Several species of aphids are ZYMV vectors that transmit viruses non-persistently, such as *A. gossypii, Myzus persicae* (Maina et al. 2017; Romay et al. 2014), *Asyrthosiphon pisum, A. kondoi, Aphis craccivora, A. citricola, A. middletonii, A. spiraecola, Macrosiphum euphorbiae, Toxoptera aurantii*, and *Uroleucon ambrosiae* (Spadotti et al. 2015). ZYMV can also be transmitted mechanically easily through cutting tools that have been contaminated with the virus (Nasr-Eldin et al. 2016; Prendeville et al. 2012).

Viral diseases can be controlled by eradicating insect vectors, quarantining, and planting healthy and virus-free seeds. Additionally, the usage of antiviral compounds derived from plant extracts has been proven to be effective against a variety of viruses due to their ribosome-inactivating proteins (RIPs) (Gal-On 2007). One of the agents capable of inducing systemic resistance in plants is a plant extract (Elbeshehy 2017). The effective control of viral diseases is the use of resistant varieties, but there are not many commercial virus-resistant cultivars available.

Systemic resistance of a plant can be activated by inducing resistance genes present in plants by utilizing resistance-inducing agents (Elbeshehy 2017). One of the agents that induce systemic resistance of plants is plant extracts (Gal-On 2007). Spinach leaf extract (*Amaranthus spinosus*), four o'clock flower leaf (*Mirabilis jalapa*), and pagoda leaf (*Clerodendrum paniculatum*) were reported to induce systemic resistance to anthracnose and CMV pathogens in chili (Hersanti 2003). Other plant extract such as *Chenopodium amaranticolor* is reported to have antiviral activity for the tobacco mosaic virus (TMV) and antitumor for Ehrlich tumor (EA) (De Oliveira et al. 1993). Extracts of pagoda leaf, thorn spinach, four o'clock flower, *C. amaranticolor*, and sambiloto are able to suppress BCMV infection in yardlong bean plants (Kurnianingsih and Damayanti 2012).

The utilization of efficacious plant extracts have been used to benefit human health, but are still of little use in plant protection. Therefore, it is necessary to explore potential plant species to control pathogens, including viruses. The objective of this study was to test the effectiveness of plant extract to suppress ZYM

MATERIAL AND METHODS

Preparation of zucchini plant (*Cucurbita pepo* L.)

The seeds of the Carisa cultivar zucchini were sown on plastic composite trays containing sterile growth media of a mixture of soil and compost. After 15 days, the grown seedlings were transplanted into plastic pots measuring 35 cm × 30 cm containing 5 kg of sterile planting medium, i.e. soil and manure (2:1 ratio). Each pot was planted with three seeds, and after one week of growth, one healthy plant was selected for the research object. Plants were irrigated daily with appropriate volume.

Propagation of ZYMV inoculum

The ZYMV inoculum was obtained from the Laboratory of the Faculty of Agriculture, Mahasaraswati University in Denpasar, then confirmed its nucleic acid sequence via DNA sequencing and designated it as ZYMV-zucchini isolate Bali (Pandawani and Widnyana 2021) (Figure 1). The inoculum was propagated mechanically by inoculating ZYMV inoculum on zucchini plants two weeks after planting. Mechanical transmission of ZYMV was carried out by grinding sick leaves (source of inoculum) in phosphate buffer pH 7 with a ratio of 1:10 (w/v). The sap was then applied to the leaves of healthy plants which were treated with 600 mesh carborundum. After inoculation, leaves were rinsed with running water. The plants were then reared in a greenhouse, and symptomatic young leaves were used as a source of inoculum in this study.

Preparation and application of pagoda (*Clerodendrum Paniculatum*) extract

The leaves of pagoda plant (*C. paniculatum*) was obtained from the area around Baturiti Tabanan. Pagoda extract was prepared through a drying process to obtain a simplicia form. The simplicia was then macerated to produce a concentrated leaf extract with a concentration of 100% (Verma et al. 1998). Application of pagoda extract to zucchini plants was carried out according to the application time determined with a volume of 100 mL per plant. The pagoda extract was applied on zucchini leaves after three weeks of transplanting.

Inoculation of ZYMV in zucchini plants (*Cucurbita pepo* L.)

Three-week-old zucchini plants were mechanically inoculated with ZYMV. Virus inoculum was prepared by grinding symptomatic young leaves of zucchini plant (source of ZYMV inoculum) in 1:10 (w/v) phosphate buffer. The virus was inoculated by applying the inoculum on the first leaf of zucchini plant, which was sprinkled with 600 mesh carborundum.

Experiment design and data analysis

This study was conducted in a completely randomized design with a factorial pattern. The first factor was the time of application of the inducer extract, which consisted of five treatments, namely: 1) application of pagoda extract during seed soaking (T1), 2) application time of pagoda extract one day before inoculation of ZYMV sap (T2), 3) application of pagoda extract on the same day as ZYMV applied (T3), 4) application of pagoda extract one day after ZYMV (T4), and no application of pagoda extract (T0) (control). The second factor was the concentration of inducer extract, which consisted three treatments, namely: 1) 10% of pagoda extract (C1), 2) 30% of pagoda extract (C2), and 3) 50% of pagoda extract (C3). The treatments were combined to obtain (5 × 3) 15 combination treatments, and each combination treatment was repeated ten times to obtain 45 experimental units. Observation parameters included disease incidence, type of disease symptoms, disease severity, virus concentration, and loss of fruit yield. Data were analyzed using ANOVA according to a completely randomized design with a factorial pattern (5 × 3 × 10) with Duncan's follow-up test (DMRT) at 5% level.

Enzyme-linked immunosorbent assays (ELISA)

The detection of ZYMV virus was performed on the leaves of zucchini plant four weeks after inoculation. Serological virus detection was carried out by enzyme-linked immunosorbent assay (DAS-ELISA) using Potyvirus antiserum (DSMZ). Virus accumulation was quantitatively read using ELISA Reader model 550 (Bio-Rad, USA) at a wavelength of 405 nm.

RESULTS AND DISCUSSION

Incubation period

The incubation period of ZYMV in plants treated with pagoda leaf extract was the most prolonged average incubation period (18.27 DAP) compared to the treatment without pagoda leaf extract (C+) (3.60 DAP). Both factors (inoculation time and concentration of pagoda leaf extract) exhibited a significantly longer incubation period when compared with the control treatment. The application of 50% pagoda leaf extract one day before virus inoculation significantly slowed the incubation period of virus (21.00 DAP) compared with other treatments (Table 1). Kurnianingsih and Damayanti (2012) found that pagoda leaf extract could slow the incubation period of BCMV in yardlong beans.

Symptoms of infection

ZYMV inoculation produced various symptoms, such as yellowing, yellowing with cupping, mosaic, mosaic with cupping, and stunting (Figure 2 and Table 1). Plants treated with leaf extract one day after virus inoculation at concentrations of 10%, 30%, and 50% showed symptoms of yellowing, yellowing with cupping, mosaic, mosaic with cupping, and stunting. The application of extract one day before inoculation of viral sap at concentrations of 30% and 50% only produced yellowing and yellowing with cupping symptoms.

Disease incidence

Plants treated with pagoda leaf extract one day before sap virus inoculation showed the lowest (20%) disease incidence until the end of observation. The other treatments resulted in 40% - 60% disease prevalence. In control plants where no pagoda leaf extract was applied, disease incidence reached 100% (Table 4).

Disease severity

Pagoda extract treatment significantly reduced the severity of disease. Among the treatments, the highest disease severity was recorded in extract application treatment one day after inoculation of viral smear. In comparison, the lowest was observed in the application of extract one day before the inoculation of virus (Table 2). The best time for application of pagoda leaf extract was one day before ZYMV inoculation with a concentration of 50% because it could reduce disease severity up to 60.79% (Table 4). It was observed that pagoda leaf extract suppressed the disease incidence and severity of

ZYMV infection. This is in accordance with Damayanti and Pebriyeni (2015), Kurnianingsih and Damayanti (2012), who reported that pagoda leaf extract can suppress BCMV infection reaching 100% in yardlong beans.

Virus detection with ELISA

ELISA absorbance value (NAE) showed that no virus was detected in the samples given pagoda flower leaf extract at a concentration of 30% and 50% one day before virus inoculation. NAE was not significantly different from the negative control (Table 4). Based on ELISA test, zucchini plants showed presence (positive) of ZYMV in all treatments, except one day before virus sap inoculation with a concentration of 30 and 50%, zucchini plants showed absence (negative) of ZYMV.

Flowering and yield loss

Infection with ZYMV inhibits flowering in zucchini plants. ZYMV-infected plants showed reduced flowering and yield loss in zucchini (Kheder et al. 2017; Kone et al. 2017; Maghamnia et al. 2018; Maina et al. 2017). Plants infected with ZYMV first flowered at 37 DAP, while plants treated with pagoda leaf extract one day before virus inoculation was flowered at 32 DAP (Table 3). Treatment of pagoda leaf extract one day before virus inoculation on zucchini plants resulted in a yield loss of 19.99% - 23.50% compared with other treatments. This indicated that applying pagoda leaf extracts one day before ZYMV inoculation on zucchini plants effectively reduced yield loss by 40%. Application of pagoda leaf extract one day before virus inoculation increased the resistance of zucchini plants. The low severity and virus accumulation in this treatment indicated the potential of the pagoda leaf as an inducer of systemic resistance of zucchini plants. The mechanism of induction of systemic resistance of zucchini by pagoda leaf extract has been reported by Verma et al. (1998). The active compound in the form of 34 kDa protein in pagoda flower leaf extract causes tobacco leaves to become immune to viruses. In addition, pagoda flower leaf extract can suppress BCMV because it contains virus inhibitors and has antiviral activity (De Oliveira et al. 1993). The active compounds of the pagoda flower are known as antiviral proteins and are known as ribosome-inactivating proteins (RIPs). RIPs are also present in the root and leaf extract of *M. jalapa* and are referred to as Mirabilis antiviral protein (MAP) (Verma et al. 1998). MAP can reach the ribosome's active site earlier than the virus to prevent viral infection at an early stage before the virus undergoes encapsidation (Vivanco et al. 1999).

In the present study pagoda leaf extract showed 80% effectiveness in inhibiting ZYMV infection, whereas against BCMV and CMV, inhibition was observed to be 100% and 82.6%, respectively (Hersanti 2003; Kurnianingsih and Damayanti 2012). It indicates that the same plant extract showed different effectiveness against different viruses.

Application of pagoda leaf extract one day before ZYMV inoculation with a concentration of 50% was able to reduce disease incidence and severity of, accelerate flowering, and reduce yield loss of zucchini fruit compared to the control treatment. The results of this study enrich the information about the potential of pagoda leaves as agent to control plant viruses.

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Table 1. Incubation period in ZYMV-infected zucchini plants in response to differences in application time and concentration of pagoda leaf extract (*C. paniculatum*)

Pagoda leaf extract concentration (%)	Incubation period of ZYMV infection (DAP)					Average effect of extract concentration
	No extract application (Positive control, C+)	Extract application during seed soaking	Extract application one day before virus sap inoculation	Extract application concurrently with viral sap inoculation	Extract application one day after viral sap inoculation	
10	3.40 a	14.0 e	16.00 fg	6.20 b	6.00 b	9.12 a
30	3.60 a	15.2 f	17.80 h	8.60 d	7.20 c	10.48 b
50	3.80 a	16.8 g	21.00 i	9.40 d	8.40 d	11.88 c
Average of extract concentration	3.60 a	15.33 d	18.27 e	8.07 c	7.20 b	

Note: day after planting (DAP); Different letters in the same row show a significant effect compared to the positive control (C+)

Table 2. Disease severity in ZYMV-infected zucchini plants in response to differences in application time and concentration of pagoda leaf extract (*C. paniculatum*)

Pagoda leaf extract concentration (%)	Disease severity (%)					Average effect of extract concentration
	No extract application (Positive control, C+)	Extract application during seed soaking	Extract application one day before virus sap inoculation	Extract application concurrently with viral sap inoculation	Extract application one day after viral sap inoculation	
10	66.37 j	39.96 e	31.80 bc	51.42 g	61.93 hij	50.30 c
30	64.30 ij	37.01 cd	27.33 ab	49.71 fg	58.78 hi	47.43 b
50	62.59 j	32.87 bc	23.59 a	45.14 ef	56.88 h	44.21 a
Average of extract concentration	64.42 e	36.61 b	27.57 a	48.76 c	59.19 d	

Note: Different letters in the same row show a significant effect compared to the positive control (C+)

Table 3. Flowering in zucchini plants in response to differences in application time and concentration of pagoda leaf extract

Pagoda leaf extract concentration (%)	Appearance of flower (DAP)					Average effect of extract concentration
	No extract application (Positive control, C+)	Extract application during seed soaking	Extract application one day before virus sap inoculation	Extract application concurrently with viral sap inoculation	Extract application one day after viral sap inoculation	
10	37.00 f	33.60 bcd	32.80 b	35.80 e	36.80 f	35.20 b
30	37.20 f	32.60 b	33.80 bcd	33.20 bc	37.40 f	34.84 b
50	36.80 f	33.40 bc	30.80 a	34.60 cd	35.00 de	34.12 a
Average of extract concentration	37.00 c	33.20 a	32.47 a	34.53 b	36.40 c	

Note: day after planting (DAP); Different letters in the same row show a significant effect compared to the positive control (C+)

Table 4. Effect of application time and concentration of pagoda leaf extract (*C. paniculatum*) on disease incidence, type of symptoms, suppression of ZYMV disease severity, and yield loss

Application time of pagoda leaf extract	Extract concentration (%)	Disease incidence (%)	Type of disease symptom*	Disease severity suppression (%)	ELISA results		Yield loss (%)
					Average of NAE**	Reaction	
C+	10	100 (10/10)	M, Mc, St	-	0.418	+	58.42
	30	100 (10/10)	M, Mc, St	-	0.43	+	55.90
	50	60 (6/10)	Y, Yc, M	-	0.395	+	55.29
Extract application during seed soaking	10	60 (6/10)	M, Mc, St	39.77	0.557	+	35.56
	30	40 (4/10)	Y, Yc, Mc, St	42.58	0.486	+	33.69
	50	40 (4/10)	Y, Yc Mc, St	47.70	0.458	+	37.41
Extract application one day before virus sap inoculation	10	40 (4/10)	Y, Yc	52.04	0.426	+	23.50
	30	20 (2/10)	Y, Yc	58.99	0.184	-	22.18
	50	20 (2/10)	Y, Yc	60.79	0.112	-	19.99
Extract application concurrently with viral sap inoculation	10	60 (6/10)	M, Mc, St	22.57	0.608	+	55.31
	30	40 (4/10)	M	23.25	0.515	+	40.74
	50	40 (4/10)	Y, Yc	26.96	0.489	+	43.93
Extract application one day after viral sap inoculation	10	60 (6/10)	Y, Yc, M, Mc, St	6.68	0.638	+	55.44
	30	60 (6/10)	M	8.96	0.588	+	55.19
	50	40 (4/10)	M	8.90	0.550	+	54.63

Note: C+ (without extract application)
Type of disease symptom*: Y = yellowing, Yc = yellowing with cupping, M = mosaic, Mc = mosaic with cupping, St = stunting
Negative control: healthy plants (without virus inoculation and extract application)
Positive control: sick plants (virus inoculation and without extract application)
**NAE: Elisa Absorbance value

FIGURES LIST

Figure 1. Inoculum source of ZYMV isolates Bali on zucchini plants

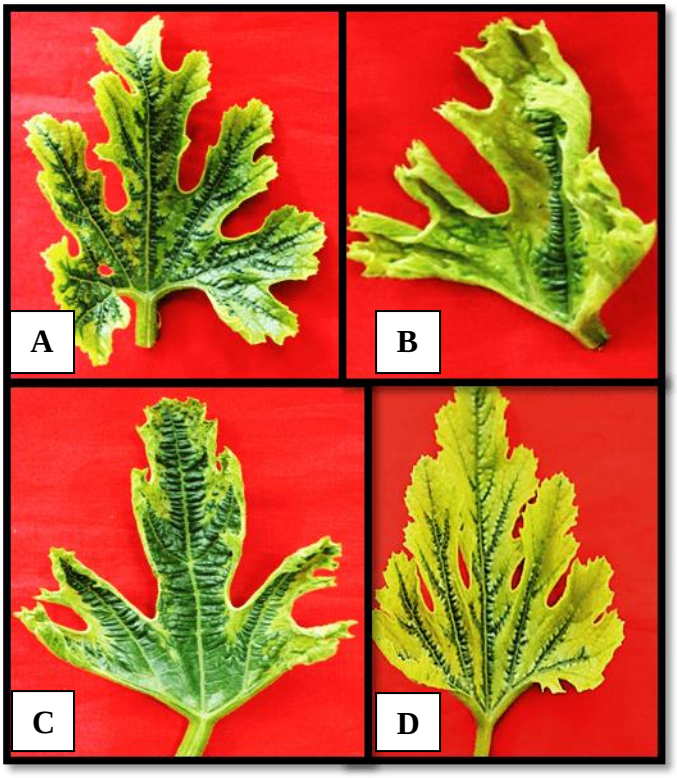

Figure 2. Disease symptoms of ZYMV isolates Bali on zucchini plants: A. mosaic, B. yellowing with cupping, C. mosaic with cupping, and D. yellowing