COVERING LETTER

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I here with enclosed a research article,

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High impact of pagoda flower leaves to suppress zucchini yield loss due to zuchini yellow mosaic virus infection

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The high impact of pagoda leaf extract in suppressing Zucchini yellow mosaic virus (ZYMV) infection in zucchini plants was first reported in this study. The effect of application time and concentration of appropriate plant extracts in suppressing ZYMV infection is also a novelty in this study. Application of pagoda leaf extract one day before ZYMV inoculation with a concentration of 50% was able to reduce the incidence and severity of disease, accelerate flowering, and reduce yield loss of zucchini fruit. The results of this study enrich the information on the potential of pagoda flower leaves as agents for controlling plant viruses.

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Ni Putu Pandawani

High impact of pagoda flower leaves to suppress zucchini yield loss due to zucchini yellow mosaic virus infection

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Abstract. Zucchini yellow mosaic virus (ZYMV) is an important virus in zucchini plants. ZYMV infection is prevalent on zucchini plants in Bali and is notoriously tough to eradicate. This study aimed to determine the efficacy of pagoda flower leaf extract in reducing yield loss in zucchini plants. A ~~F~~factorial randomized block design with two variables was employed in this study. The time the extract was administered was the first variable, and the concentration was the second. Plant extracts were applied during seed immersion, one day before and after inoculation, and the same day as virus inoculation. The concentrations of plant extracts employed were 10%, 30%, and 50%. The crude extract was sprayed onto the zucchini plant's leaves. Plants treated with extracts one day after virus inoculation at 10%, 30%, and 50% concentrations exhibited yellowing, yellowing with cupping, mosaic, mosaic with cupping, and stunting. Meanwhile, extracts application one day before virus inoculation at 30% and 50% concentrations resulted in yellowing and yellowing with cupping signs. Plants infected with ZYMV flowered for the first time at 37 DAP. Meanwhile, plants treated with pagoda flower leaf extract one day before virus inoculation started flowering at 32 DAP. Application of pagoda flower leaf extract one day before ZYMV inoculation on zucchini plants effectively suppressed 40% yield loss. These findings enrich the information on the potential of pagoda flower leaves as a plant virus control agent through the mechanism of induction of systemic resistance of zucchini plants.

Key words: flowering, yield loss, potency of plant extracts, virus symptoms

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

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

INTRODUCTION

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 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 was 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 could result in symptoms consisting of mosaicism, yellowing, and eventually "shoeing" on the ~~leaf~~leaves. The fruit is stunted, twisted, and distorted, resulting in a loss in yield and the product's inability to be marketed, especially chayote zucchini (Massumi et al. 2011).

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 of viral infection depends on the time of infection and could cause yield losses of up to 100% (Coutts et al. 2011). Plants become very

susceptible to virus infection at a young plant age, affecting the high incidence of disease. When young plants are infected, the virus's incubation period is shorter, and the virus distribution and translocation process become faster (Coutts et al. 2013).

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 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 could also be transmitted mechanically easily through cutting tools that have been contaminated with the virus (Nasr-Eldin et al. 2016; Prendeville et al. 2012).

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 were *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).

Viral diseases can be controlled by eradicating insect vectors 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 to use resistant varieties, but there are not many commercial virus-resistant cultivars available.

Systemic resistance of a plant ~~will~~ 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 flower leaf (*Clerodendrum paniculatum*) were reported to induce systemic resistance to anthracnose and CMV pathogens in chili (Hersanti, 2003). Other plant extracts such as *Chenopodium amaranticolor* were 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 flower, thorn spinach, four o'clock flower, *C. amaranticolor*, and sambiloto plant extracts were able to suppress BCMV infection in yardlong bean plants (Kurnianingsih and Damayanti 2012). Therefore, it is necessary to explore potential plant species to control pathogens, including viruses. This study aimed to test the effectiveness of plant extracts to suppress ZYMV.

MATERIALS AND METHODS

Zucchini Plant Preparation (*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. The grown seedlings were transplanted into plastic pots measuring 35 cm x 30 cm containing 5 kg of sterile planting medium, namely soil and manure (2:1 ratio). Each pot was planted with three seeds, and after one week of growth, one healthy plant was selected to be kept as the research object. Daily watering was performed with a volume suitable to the conditions.

ZYMV Inoculum Source Propagation

The ZYM inoculum was obtained from the Laboratory of the Faculty of Agriculture, Mahasaraswati University in Denpasar, which confirmed its nucleic acid sequence via DNA sequencing and designated it as the ZYMV-Zukini isolate Bali (Pandawani and Widnyana 2021) (Figure 1). The inoculum was propagated by mechanically inoculating ZYMV inoculum on zucchini plants. 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 that have been treated with 600 mesh carborundum. After inoculation, the 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 *C. paniculatum* Extract

The leaves of the pagoda flower (*C. paniculatum*) originated from the area around Baturiti Tabanan. *C. paniculatum* extract was prepared to obtain a simplicia form through a drying process. The simplicia was then macerated to produce a concentrated leaf extract with a concentration of 100%. Application of *C. paniculatum* extract to zucchini plants was carried out according to the treatment application time determined with a volume of 100 mL per plant. The application of *C. paniculatum* extract was carried out after the zucchini plants reached the age of three weeks after transplanting into research pots.

ZYMV Inoculation in Zukini Plants (*Cucurbita pepo* L.)

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Zucchini plants that were three weeks old after transplanting were started mechanically inoculated with ZYMV. Virus inoculum was prepared by grinding symptomatic young leaves of the zucchini plant (source of ZYMV inoculum) in 1:10 phosphate buffer. The virus was inoculated by applying the inoculum to the first leaves of the zucchini plant, which had been sprinkled with carborundum.

Experiment Design and Data Analysis

This study used a completely randomized design with a factorial pattern. The first factor was the time of application of the extract of the inducer, which consisted of five treatments, namely: 1) application of *C. paniculatum* extract during seed soaking (T1), 2) Application time of application of *C. paniculatum* extract one day before inoculation of ZYMV sap (T2), 3) Application of *C. paniculatum* extract on the same day as ZYMV application (T3), 4) Application of *C. paniculatum* extract one day after ZYMV (T4), and without application of *C. paniculatum* extract (T0) (control). The second factor was the concentration of the inducer extract, which consisted of three treatments, namely: 1) 10% *C. paniculatum* (C1), 2) 30% *C. paniculatum* (C2), and 3) 50% *C. Paniculatum* (C3). The treatments were combined to obtain (5 x 3) 15 combination treatments, and each combination treatment was repeated ten times so that 45 experimental units were obtained. Observation parameters included disease incidence, type of disease symptoms, disease severity, virus concentration, and loss of fruit yields. Data were analyzed using ANOVA according to a completely randomized design with a Factorial pattern (5 x 3 x 10) with Duncan's follow-up test (DMRT) at the 5% level.

Enzyme-Linked Immunosorbent Assays (ELISA)

The detection of the ZYMV virus was carried out on the leaves of the tested zucchini four weeks after inoculation. Serological virus detection was carried out by enzyme-linked immunosorbent assay (DAS-ELISA) using Potyvirus antiserum. Virus accumulation was quantitatively read using ELISA Reader. ELISA results are declared positive if the absorbance value of the sample is one and a half or two times greater than the absorbance value of the negative control.

RESULTS AND DISCUSSION

Incubation Period

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

Symptom Type

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

Disease Incidence

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

Disease Severity

Pagoda flower extract treatment significantly reduced the severity of the disease. Among the treatments for the time of application of plant extracts, the highest disease severity was indicated by the treatment of extract application one day after inoculation of the viral smear. In comparison, the lowest was indicated by applying the extract one day before the inoculation of the virus (Table 2). The best time for application of pagoda flower leaf extract is one day before ZYMV inoculation with a concentration of 50% because it could reduce disease severity up to 60.79% (Table 4). Treatment of plant extracts was able to suppress the incidence and severity of ZYMV infection. From the results, pagoda flower leaf extract affected ZYMV infection.

Virus Detection with ELISA

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ELISA absorbance values (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). The application of pagoda flower leaf extract during seed immersion, during virus sap inoculation, and one day after virus sap inoculation showed ELISA results containing the virus.

Flowering and Yield Loss

Infection with ZYMV inhibits flowering in zucchini plants. ZYMV-infected plants cause inhibits flowering and yield loss of 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 were at 32 DAP (Table 3). Treatment of pagoda flower leaf extract one day before virus inoculation on zucchini plants caused a yield loss of 19.99% - 23.50% compared with other treatments. It indicated that applying pagoda flower leaf extracts one day before ZYMV inoculation on zucchini plants effectively reduced yield loss by 40%.

Application of pagoda flower leaf extracts one day before virus inoculation increased the resistance of zucchini plants. Lower severity and accumulation of virus in this treatment indicated the potential of the pagoda flower inducer of systemic resistance of zucchini plants. 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. Ribosome-inactivating proteins 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).

When compared with its effectiveness against BCMV on long beans and CMV on chili plants, the pagoda flower leaf extract in this study showed 80% effectiveness in inhibiting ZYMV infection, while against BCMV, it reached 100%, and CMV reached 82.6% (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 the incidence and severity of disease, accelerate flowering, and reduce yield loss of zucchini fruit compared to the control treatment. The results of this study enrich the information on the potential of pagoda flower leaves as agents for controlling plant viruses.

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TABLES LIST

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

C.paniculatum flower leaf extract concentration (%)	The incubation period for ZYMV infection (DAP)					Average Effect of Extract Concentration
	No extract application (Positive control)	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 Effect of Extract Concentration	3.60 a	15.33 d	18.27 e	8.07 c	7.20 b	

Note: day after planting (DAP)

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

C.paniculatum flower leaf extract concentration (%)	Disease severity (%)					Average Effect of Extract Concentration
	No extract application (Positive control)	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 Effect of Extract Concentration	64.42 e	36.61 b	27.57 a	48.76 c	59.19 d	

Table 3. Flowers Begin to Appear (DAP)

C.paniculatum flower leaf extract concentration (%)	Flowers Begin to Appear (DAP)					Average Effect of Extract Concentration
	No extract application (Positive control)	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 Effect of Extract Concentration	37.00 c	33.20 a	32.47 a	34.53 b	36.40 c	

Note: day after planting (DAP)

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

Application time of *C.paniculatum* 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), 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

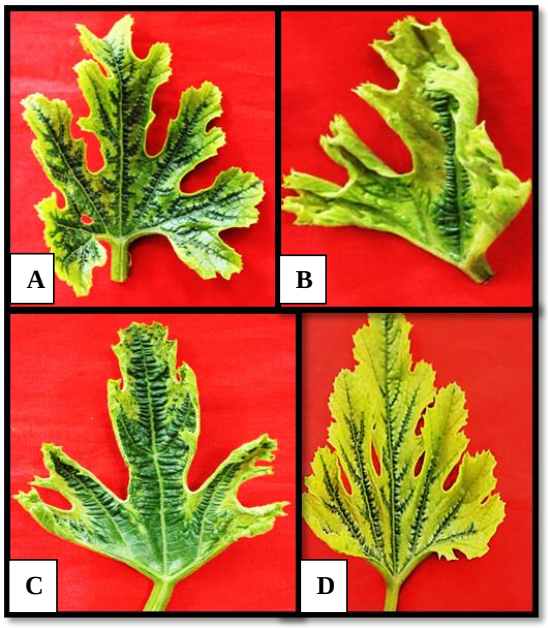

Figure 2. Variation in ZYMV isolates Bali symptoms on studied plants: A. mosaic, B. yellowing with cupping, C. mosaic with cupping, and D. yellowing

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• Full postal address (incl street name and number (location), city, postal code, state/province, country)	Perum Nuansa Anyelir II No. 4, Jl. Anyelir Denpasar 80235, Indonesia
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