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COVERING LETTER

Dear Editor-in-Chief,

I here with enclosed a research article,

12 Title:

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

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19 For possibility publication on the journal:

20 (fill in Biodiversitas or Nusantara Bioscience or mention the others)

Biodiversitas

Novelty: 22 23

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(state your claimed novelty of the findings versus current knowledge)

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.

24 25 Statements:

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

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59 Abstract. Zucchini yellow mosaic virus (ZYMV) is an important virus in zucchini plants. ZYMV infection is prevalent on 60 zucchini plants in Bali and is notoriously tough to eradicate. This study aimed to determine the efficacy of pagoda flower 61 leaf extract in reducing yield loss in zucchini plants. A factorial randomized block design with two variables was 62 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 63 64 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% 65 concentrations exhibited yellowing, yellowing with cupping, mosaic, mosaic with cupping, and stunting. Meanwhile, 66 extracts application one day before virus inoculation at 30% and 50% concentrations resulted in yellowing and yellowing 67 with cupping signs. Plants infected with ZYMV flowered for the first time at 37 DAP. Meanwhile, plants treated with 68 pagoda flower leaf extract one day before virus inoculation started flowering at 32 DAP. Application of pagoda flower leaf 69 70 extract one day before ZYMV inoculation on zucchini plants effectively suppressed 40% yield loss. These findings enrich 71 the information on the potential of pagoda flower leaves as a plant virus control agent through the mechanism of induction 72 of systemic resistance of zucchini plants.

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

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

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

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INTRODUCTION

78 Zucchini yellow mosaic virus (ZYMV) has a one-molecule positive senseRNA genome of approximately 10 kb 79 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 80 81 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 82 83 (CI) pinwheel type 1, and CP (Maghamnia et al. 2018; Valli et al. 2017). Other proteins (Third protein (P3), 6K1, 6K2, 84 viral genome-linked protein (VPg), Nuclear Inclusion A (NIa), and NIb replicas) have not been identified in ZYMV-85 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 leaves. The fruit is stunted, twisted, and

Comment [A1]: arrange based on alphab

Comment [A2]: It is better if author describe the important of the zucchini and biotic constraint of its production in Indone before explanation about the virus.

Comment [A3]: Rewrite this section about the basic information of ZYMV; taxonomy, genome, particles, transmission, host range one paragraph

The symptom on te next paragraph

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distorted, resulting in a loss in yield and the product's inability to be marketed, especially chayote zucchini (Massumi et al.
 2011).

25 27MV 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 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 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, 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 to use 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 118 119 resistance-inducing agents (Elbeshehy 2017). One of the agents that induce systemic resistance of plants is plant extracts 120 (Gal-On 2007). Spinach leaf extract (Amaranthus spinosus), four o'clock flower leaf (Mirabilis jalapa), and pagoda flower 121 leaf (Clerodendrum paniculatum) were reported to induce systemic resistance to anthracnose and CMV pathogens in chili 122 (Hersanti, 2003). Other plant extracts such as Chenopodium amaranticolor were reported to have antiviral activity for the 123 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 124 125 in yardlong bean plants (Kurnianingsih and Damayanti 2012).

The utilization of efficacious plant extracts has been carried out to benefit human health, but still little is used in plant protection. 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.

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MATERIALS AND METHODS

130 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. After 15 days, 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.

137 ZYMV Inoculum Source Propagation

The ZYMV inoculum was obtained from the Laboratory of the Faculty of Agriculture, Mahasaraswati University 138 139 in Denpasar, which confirmed its nucleic acid sequence via DNA sequencing and designated it as the ZYMV-Zukini 140 isolate Bali (Pandawani and Widnyana 2021) (Figure 1). The inoculum was propagated by mechanically inoculating 141 ZYMV inoculum on zucchini plants two weeks after planting. Mechanical transmission of ZYMV was carried out by 142 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 143 144 with running water. The plants were then reared in a greenhouse, and symptomatic young leaves were used as a source of 145 inoculum in this study.

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147 Preparation and Application of C. paniculatum Extract

148 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.

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

157 Zucchini plants that were three weeks old after transplanting were started mechanically inoculated with ZYMV.
 158 Virus inoculum was prepared by grinding symptomatic young leaves of the zucchini plant (source of ZYMV inoculum) in
 159 1:10 w/v phosphate buffer. The virus was inoculated by applying the inoculum to the first leaves of the zucchini plant,
 160 which had been sprinkled with 600 mesh carborundum.

162 Experiment Design and Data Analysis

163 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 164 165 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 166 of C. paniculatum extract one day after ZYMV (T4), and without application of C. paniculatum extract (T0) (control). The 167 168 second factor was the concentration of the inducer extract, which consisted of three treatments, namely: 1) 10% C. 169 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 170 171 units were obtained. Observation parameters included disease incidence, type of disease symptoms, disease severity, virus 172 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. 173

175 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 (DSMZ). Virus accumulation was quantitatively read using ELISA Reader model 550 (Bio-Rad, USA) at a wavelength of 405 nm. 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.

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RESULTS AND DISCUSSION

183 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 (inculation 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.

192 Symptom Type

193 ZYMV inoculation produced various symptoms, such as yellowing, yellowing with cupping, mosaic, mosaic with 194 cupping, and stunting (Figure 2; Table 1). Plants treated with plant extracts one day after virus inoculation at 195 concentrations of 10%, 30%, and 50% showed symptoms of yellowing, yellowing with cupping, mosaic, mosaic with 196 cupping, and stunting. The extract application treatment one day before inoculation of viral sap at concentrations of 30% 197 and 50% only caused yellowing and yellowing with cupping symptoms.

199 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).

204 Disease Severity

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205 Pagoda flower extract treatment significantly reduced the severity of the disease. Among the treatments for the 206 time of application of plant extracts, the highest disease severity was indicated by the treatment of extract application one 207 day after inoculation of the viral smear. In comparison, the lowest was indicated by applying the extract one day before the 208 inoculation of the virus (Table 2). The best time for application of pagoda flower leaf extract is one day before ZYMV 209 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 of this study, pagoda flower leaf extract affected ZYMV infection. It is in accordance with Damayanti and Pebriyeni (2015), Kurnianingsih and Damayanti (2012) who stated that pagoda flower leaf extract could suppress BCMV infection in yardlong beans with inhibition reaching 100%.

Virus Detection with ELISA

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

222 Infection with ZYMV inhibits flowering in zucchini plants. ZYMV-infected plants cause inhibits flowering and 223 yield loss of zucchini (Kheder et al. 2017; Kone et al. 2017; Maghamnia et al. 2018; Maina et al. 2017). Plants infected 224 with ZYMV first flowered at 37 DAP, while plants treated with pagoda leaf extract one day before virus inoculation were 225 at 32 DAP (Table 3). Treatment of pagoda flower leaf extract one day before virus inoculation on zucchini plants caused a 226 yield loss of 19.99% - 23.50% compared with other treatments. It indicated that applying pagoda flower leaf extracts one 227 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 228 229 plants. Lower severity and accumulation of virus in this treatment indicated the potential of the pagoda flower as an 230 inducer of systemic resistance of zucchini plants. The mechanism of induction of systemic resistance of zucchini by 231 pagoda flower leaf extract has been reported by Verma et al. (1998). The active compound in the form of 34 kDa protein in 232 pagoda flower leaf extract causes tobacco leaves to become immune to viruses. In addition, pagoda flower leaf extract can 233 suppress BCMV because it contains virus inhibitors and has antiviral activity (De Oliveira et al. 1993). The active 234 compounds of the pagoda flower are known as antiviral proteins and are known as ribosome-inactivating proteins (RIPs). 235 RIPs are also present in the root and leaf extract of M. jalapa and are referred to as Mirabilis antiviral protein (MAP) 236 (Verma et al. 1998). MAP can reach the ribosome's active site earlier than the virus to prevent viral infection at an early 237 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 238 239 leaf extract in this study showed 80% effectiveness in inhibiting ZYMV infection, while against BCMV, it reached 100%, 240 and CMV reached 82.6% (Hersanti 2003; Kurnianingsih and Damayanti 2012). It indicates that the same plant extract 241 showed different effectiveness against different viruses.

Application of pagoda leaf extract one day before ZYMV inoculation with a concentration of 50% was able to 242 243 reduce the incidence and severity of disease, accelerate flowering, and reduce yield loss of zucchini fruit compared to the 244 control treatment. The results of this study enrich the information on the potential of pagoda flower leaves as agents for 245 controlling plant viruses.

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TABLES LIST

325 326 327 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)

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The incubation period for ZYMV infection (DAP)						
<i>C.paniculatum</i> flower leaf	No extract application	Extract application	Extract application	Extract application	Extract application	Average Effect of
extract	(Positive	during	one day	concurrently	one day	Extract
concentration	control)	seed	before	with viral	after viral	Concentration
(%)		soaking	inoculation	sap inoculation	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	3.60 a	15.33 d	18.27 e	8.07 c	7.20 b	
Extract						
Concentration						

Note: day after planting (DAP)

334

 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)

Disease severity (%)						
<i>C.paniculatum</i> flower leaf extract concentration	No extract application (Positive control)	Extract application during seed soaking	Extract application one day before virus	Extract application concurrently with viral	Extract application one day after viral	Average Effect of Extract Concentration
(%)			sap	sap	sap	
			inoculation	inoculation	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	64.42 e	36.61 b	27.57 a	48.76 c	59.19 d	

Table 3. Flowers Begin to Appear (DAP)

		Flowers	Begin to App	ear (DAP)		
C.paniculatum	No extract	Extract	Extract	Extract	Extract	Average
flower leaf	application	application	application	application	application	Effect of
extract	(Positive	during	one day	concurrently	one day	Extract
concentration	control)	seed	before	with viral	after viral	Concentration
(%)		soaking	virus sap	sap	sap	
			inoculation	inoculation	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	37.00 c	33.20 a	32.47 a	34.53 b	36.40 c	
Concentration						

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	Extract	Disease	Type of	Disease	ELISA	Results	Yield loss
<i>C.paniculatum</i> leaf extract	concentration (%)	incidence (%)	disease symptom*	severity suppression (%)	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	10	60 (6/10)	M, Mc, St	39.77	0.557	+	35.56
during seed soaking	30	40 (4/10)	Y, Yc, Mc, St	42.58	0.486	+	33.69
0	50	40 (4/10)	Y, Yc Mc, St	47.70	0.458	+	37.41
Extract application	10	40 (4/10)	Y, Yc	52.04	0.426	+	23.50
one day before	30	20 (2/10)	Y, Yc	58.99	0.184	-	22.18
virus sap inoculation	50	20 (2/10)	Y, Yc	60.79	0.112	-	19.99
Extract application	10	60 (6/10)	M, Mc, St	22.57	0.608	+	55.31
concurrently with	30	40 (4/10)	Μ	23.25	0.515	+	40.74
viral sap inoculation	50	40 (4/10)	Y, Yc	26.96	0.489	+	43.93
Extract application one day after viral	10	60 (6/10)	Y, Yc, M, Mc, St	6.68	0.638	+	55.44
sap inoculation	30	60 (6/10)	Μ	8.96	0.588	+	55.19
	50	40 (4/10)	Μ	8.90	0.550	+	54.63

Note: C+ (without extract application), Y = yellowing, Yc = yellowing with cupping, M = mosaic, Mc = mosaic with

346

cupping, St = stunting Negative control: healthy plants (without virus inoculation and extract application) 347 348

Positive control: sick plants (virus inoculation and without extract application)

349 350 351 352 353 *NAE: Elisa Absorbance value

FIGURES LIST



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

342 343 344



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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