Inheritance of Resistance Gene to Cucumber Mosaic Virus in Maize

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ABSTRACT. Maize (*Zea mays* L.) is one of important crop in the world. In Indonesian, maize production has been increasing, due to high consumption. Madura island is the largest areal for maize production in East Java and Indonesia. The island has also some local maize. Virus infection, including *Cucumber Mosaic virus* (CMV) could reduced maize production until 62.2%. Hence, it is important to improve the quality of Madura's local maize by resistant breeding. The aim of this research was to study on the inheritance of resistance gene to CMV in crosses between Guluk-guluk, a Madura's local cultivar, and Srikandi Kuning 1. Guluk-guluk was provided by Assessment Institute for Agricultural Technology Malang, East Java Indonesia, while maize Srikandi Kuning 1 was provided by Indonesian Cereals Research Institute Maros, South Sulawesi. Resiprocal breeding experiments were conducted between Guluk-guluk and Srikandi to obtain F_1 population. To produce F_2 population, F_1 individuals were selfed. On the other hand, other F_1 individuals were crossed to Srikandi Kuning 1 to produce testcross population. The resistance to CMV in maize population was evaluated using symptoms observation and Enzyme-linked Immunosorbent Assay (ELISA) analysis. Furthermore, the data of resistance to CMV in maize was analyzed using chi-square. Results of this research showed that Guluk-guluk exhibits resistance to CMV, while Srikandi Kuning 1 was susceptible. All F_1 plants showed resistance to CMV, while F_2 and testcross populations showed segregation with ratio 3 : 1 (F_2) and 1 : 1 (testcross). Seventy two individuals of progeny (F_2) showed resistances and 27 were susceptible. Ten individuals of testcross are resistance, while 9 plants are susceptible. Based on chi-square analysis, it indicates that F_2 (72:27) and testcross (10:9) demonstrated Mendel's law. The results of the study revealed that the inheritance of resistance gene to CMV in crossing between Guluk-guluk and Srikandi Kuning 1 is controlled by a single dominant gene *Zrec*.

Key words: Guluk-guluk, Srikandi Kuning 1, CMV, ELISA technique.

Introduction

Maize (*Zea mays* L.) is an important crop grown in the world. In Indonesia, maize becomes a staple food. Therefore its production has been increasing in accordance with the high consumption. Madura island is the largest maize production area in East Java and Indonesia; approximatelly 400.000 hectares of maize were grown annually (Roesmarkam *et al*. 2006). The island has some local maize cultivars that are expected to have excellent traits, such as resistance to diseases and drought, and longer seed storageability. However, there few researches have been done on these aspects.

Guluk-guluk, Talango, and Manding are three local maize cultivars of Madura that were collected and developed by the Assessment Institute for Agricultural Technology Malang, East Java, Indonesia. Guluk-guluk known as a cultivar that has no reports in virus infection. The cultivar also has grain color and taste favored by people. Srikandi Kuning 1 is a maize variety released by the Indonesian Cereals Research Institute, Maros, South Sulawesi, Indonesia. This variety known to have a resistance to rust and leaf blight diseases (Azrai 2004), but has no evidences of virus resistance.

Virus diseases are important on maize, since they cancause yield reduction up to 62.2% (Muis 2002). The viruses commonly found on maize in Indonesia are Maize Dwarf Mosaic Virus (MDMV) (Wakman *et al.* 1998), Sugarcane Mosaic Virus (SCMV) and Cucumber Mosaic Virus (CMV) (Semangun 2004). In Indonesia, CMV was described in Bogor in 1972 and has now been reported widespread (Triharso *et al*. 1998).

CMV is a member of the genus Cucumovirus that has a wide host range (Smith *et al*. 1988). The virus can infect more than 1200 species of over 100 plant families and

transmitted by more than 80 species of aphids in a non-persistent manner (Palukaitis 1992; Sacristan *et al*. 2005). CMV is also one of the most agronomically important viruses in Indonesia as it can cause considerable losses on CMV-infected melon and other plants, including maize (Somowiyarjo *et al.* 1993).

One of the effective methods to obtain disease-resistant plants is crosses between resistant and susceptible cultivars (Semangun 2004). So, the aim of this research was to determine the inheritance of resistance to CMV in crosses between Guluk-guluk, a local maize cultivar of Madura and Srikandi Kuning 1.

Materials and Methods

Maize Genotypes

Seeds of Guluk-guluk cultivar was provided by the Assessment Institute for Agricultural Technology Malang, East Java, and seeds of Srikandi Kuning 1 was provided by the Indonesian Cereals Research Institute Maros, South Sulawesi. Crosses of the two cultivars were made in Sidoarjo, East Java, from July 2007 to February 2008 and from April 2009 to October 2011 at *Kebun Pendidikan, Penelitian, dan Pengembangan Pertanian* (KP4), Gadjah Mada University, Sleman, Daerah Istimewa Yigyakarta.

Preparation of Maize Breeding Material

Plants of both cultivars were grown and used for resiprocal breeding. Seed of Srikandi Kuning 1 was planted earlier than Guluk-guluk in three time series, namely 20 days, 15 days, and 7 days before planting Guluk-guluk. Reciprocal crosses were made between Guluk-guluk as female parent and Srikandi Kuning 1 as male parent to obtain F_1 population. F_1 individuals were then selfed to produce F_2 population. Meanwhile, other F_1 individuals were crossed to Srikandi Kuning 1 to produce testcross population. Maize crossing were conducted by covering male flowers with paper bags in the afternoon.

Mature pollens of the male parent that fall into the bag were then sown on the silk of female flower in the following morning. Plants were maintained properly, and seeds were collected from the harvest. As controls, three non-inoculated plants (healthy controls) from each of the parents, F_1, F_2, and testcross populations were also grown and kept under the same conditions.

Preparation of CMV Inoculum and Plant Inoculation

Source CMV inoculum was provided by the Laboratory of Plant Virology, Department of Pest and Diseases, Faculty of Agriculture, Gadjah Mada University, Yogyakarta. The CMV isolate was propagated on chili pepper plants. CMV inoculum was prepared from young symptomatic chili leaves that were macerated in 0.1 M sodium phosphate buffer, pH 7.2 at a ratio of 1 : 10 in a pre-chilled mortar and pestle. All non-biological materials were sterilized before use. Inoculation of maize plants with the CMV inoculum were conducted in the afternoon.

Seeds of each maize breeding material were grown. Completely expanded 5 to 6 day-old maize seedlings were lightly dusted with carborundum (600 mesh) and rub-inoculated with the CMV inoculum sap using sponge plugs, and maintained to grow for 30 days. All maize plant material were inoculated, except the healthy controls. Symptom of the disease on each plant was observed weekly until 4 weeks after inoculation.

Serological Detection of CMV

CMV detection was done using the ELISA technique in the Laboratory of Plant Virology, Department of Pest and Diseases, Faculty of Agriculture UGM. Maize leaf samples were collected from young leaves of inoculated and healthy plants and used for virus detection. The ELISA testing for the presence of CMV in the maize leaf samples followed the standard non precoated-indirect ELISA technique as described by Clark and Adams (1977). All samples were tested at the same time in a single assay. From each of the leaf samples, 0.2 g leaf sample was macerated in 2 ml carbonate buffer (50 mM, pH 9,6) and used as an antigen. Each of the antigen were then placed into each well of a microtitre plate (100 µl per well). The antigen containing plates were then incubated for 18 hours at 4^{o}C.

The following day, the antigen was discarded from the plates and the plates were incubated with 0.7% Bovine Serum Albumine (150 µl per well) for 2 hours at room temperature. The plates were added with CMV specific antibody (10^{-1} dilution, 150 µl per well) and incubated for 18 hours at 4^{o}C. The plates were then incubated with enzyme-labeled conjugate (antirabbit IgG, 150 µl per well) for 2 hours at 37^{o}C and continued with incubation by adding 1 mg/ml *p*-nitrophenyl phosphate in 10% diethanolamine buffer. Three times of washing were done between each of the assays. The washing solution was 0.02 M PBS, pH 7.4, containing 0.05% Tween-20 and 0.5%

polyvinyl pyrrolidone 40,000 (PBST). The plates were observed using an ELISA Reader at 405 nm absorbance after 45 minutes to 1 hour substrate incubation. To directly compare absorbance values of the samples in plates, at least three or more healthy and CMV infected maize leaf samples were included in each ELISA plate. The average of absorbance values of three wells for each sample was used to evaluate the virus infection. A sample was considered positive for CMV infection when the ELISA absorbance value was two times greater than the average absorbance value of the healthy control plus 3x standard deviations (Grube *et al.* 2000).

Scoring the Disease Symptoms

Symptoms of CMV infection were scored and recorded using symbols, namely 0 = no symptoms; 1 = mild symptoms (mild mosaic and leaf deformation, no stunted plant growth); 2 = intermediate symptoms (intermediate mosaic and leaf deformation, no stunted growth); and 3 = severe symptoms (severe mosaic, shoestring formation, stunted growth or collapsed).

Determination of Genetic Resistance to CMV

The inheritance of CMV resistance on the plant breeding material was analyzed based on data from observation of leaf symptoms observation and ELISA testing. Chi-square analysis was performed to analyze segregation ratios of the F_2 and testcross populations.

Results and Discussion

Detection of Resistance Against CMV by Inoculation

Results of the maize plant inoculations with CMV showed that stunting symptom occurred on Srikandi Kuning 1 but not on Guluk-guluk. Guluk-guluk plants grew taller than the Srikandi Kuning 1 (Figure 1). The CMV infection in Srikandi Kuning 1 plants at one week after inoculation showed severe symptoms as indicated by leaf deformation, twisting the end of the third and fourth leaves, and reduction in size of the leaf laminae (Figure 2.e.). This syndrome is similar to that reported (Madhubala *et al*. 2005) when they inoculated Graminae plants with CMV isolate of vanilla. The infected Graminae plants showed leaf twisting and reducction in size of leaf laminae. (Herison *et al.* 2007) also reported a shoestring appearance as a symptom of CMV infection on red pepper,t similar to the severe symptom on Srikandi Kuning 1.

Mosaic symptom appeared clearly on the twisted Srikandi-kuning 1 leaves and seen transparently yellow. (Daryono *et al.* 2003) reported that CMV infection in melon also showed mosaic symptom, mottling, speckling of leaves, deformed leaves, and plant stunting. On the other hand, the CMV symptoms on cucumber were were shown as vein clearing, mosaic, mottling, malformation and deformation of leaves, and stunting (Zitikaite 2002), while in tomato and *Nicotiana rustica* the plants showed mosaic, filiform leaves and stunting (Kobori *et al.* 2005).

At 30-40 days after inoculation, the infected plants with severe symptoms (Figure 2.e.) had less than 35 cm plant heights compared to that of the healthy controls (Figure 2.a.). At 2 and 3 weeks after inoculations, some of maize plants showed intermediate symptoms of CMV. These symptoms were similar to that at 1 week after inoculation, including mosaic and leaf deformation, but no stunted growth. This might be caused by the ability of plants to supress virus infection until 2–3 weeks after inoculation. According to (Daryono *et al.* 2003), there were plant cultivars that were able to supress virus replications within 15-20 days after inoculation.

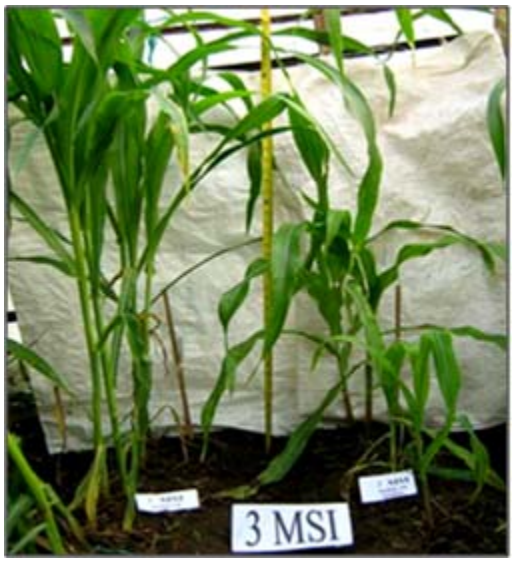

Figure 1. CMV infections in maize cultivars Guluk-guluk and Srikandi Kuning 1. Guluk-guluk plant grew taller (left) than Srikandi Kuning 1 (right) at three weeks after inoculation.

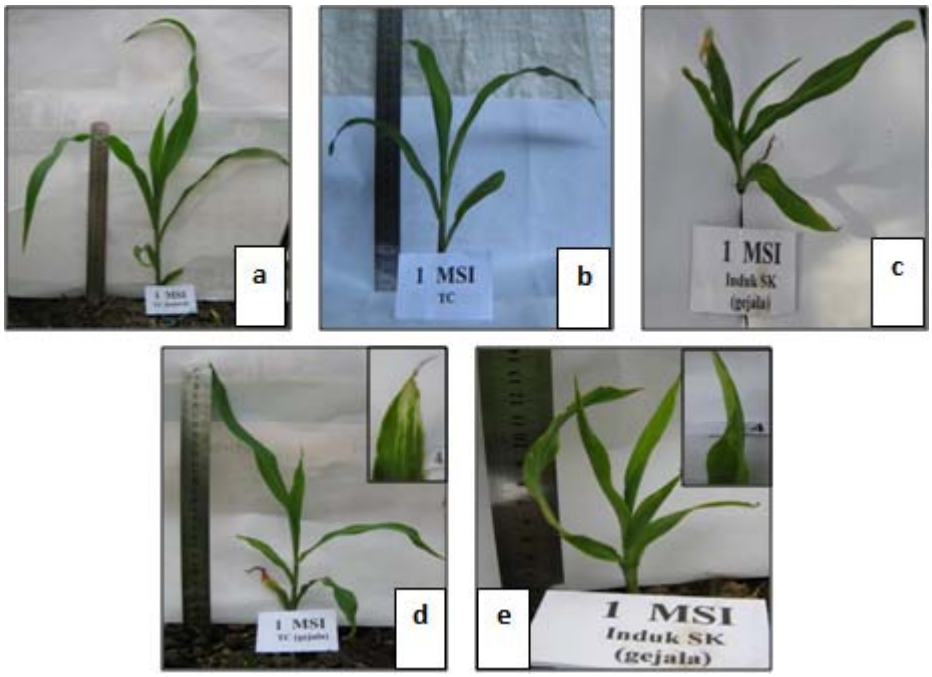

Figure 2. Symptom variations of CMV infection in maize cultivar Srikandi Kuning 1 at one 1 week after inoculation. (a) Healthy plant (control); (b) No symptom; (c) Mild symptom; (d) Intermediate symptom; (e) Severe symptom. Insert: deformed or twisted leaf of the fourth leaf.

Table 1. Scores of CMV symptoms in plants of maize cultivars Guluk-guluk and Srikandi Kuning 1 as well as their crosses.

Generation	Cultivar	Number of plants	Number of controls	Disease Index[a)]			
				0	1	2	3
Parent	Srikandi	15	3	1	1	7	6
	Guluk	15	3	14	1	0	0
F_1	@& Srikandi	15	3	14	1	0	0
	@& Guluk	15	3	15	0	0	0
F_2	@& Srikandi	50	3	35	2	8	5
	@& Guluk	49	3	37	3	8	1
Testcross	@& Srikandi	10	3	3	1	6	0
	@& Guluk	9	3	4	2	3	0

[a)] Disease index (score): 0 = no symptom; 1 = mild symptom; 2 = intermediate symptom; 3 = severe symptom.

At four weeks after inoculation, the location of leaves were clustered. This may be due to systemic virus infection that affect the stems and nodes growths. Virus infection might affect all stages of plant growths and caused stunting as a results of internodes and petioles shortening, so the leaves were clustered and developed a rosette appearance near the leaf basel (Agrios 1997). Systemic symptoms observed in this study were wilting, stunting and leaf discoloration. Due to a certain condition, these symptoms could dissapeared and called masked symptoms (Semangun 2001). Virus infection in plants was also influenced by ambient temperatures. For example, in *Tetragonia expansa*, a member of the family *Aizoaceae*, that was inoculated with pepo strain of CMV, showed only local infection at 24°C, whereas a systemic infection occured at 36°C (Kobori *et al*. 2003).

The CMV symptoms observation on cultivars Guluk-guluk and Srikandi Kuning 1 as parents and their progenies aimed to find out the mode of inheritance of resistance to CMV in maize. Data on Table 1 indicated the resistance to CMV in the F_1 plants from reciprocal breeding of Srikandi Kuning 1 and Guluk-guluk were not inherited from one of the parent (not maternal inheritance), but both of them contributed the same influences. Hence, the resistance to CMV in the F_1 plants was controlled by chromosomal genes

inside the nucleus. Daryono and Natsuaki (2002) reported on melon that results of reciprocal breeding in the F_1 generation showed DNA bands that were inherited from both parents. According to Agrios (1997) and Hartwell *et al*. (2000), characteristics of the progenies controlled by genes outside the nucleus were of maternal inheritance, where the female parent contibuted higher influences than the male parent; reciprocal breeding results in various progenies and there were no segregation, and specific phenotypic ratio of the inheritance followed the Mendel's law.

Determination of Resistance to CMV Using a Serological Technique

Results of serological detection using the non-precoated indirect ELISA technique showed that symptomic leaf samples that were positively infected with CMV gave no significant differences in absorbance values compared to those of controls (Table 2). It may be because the strain of CMV used as source inoculum to inoculate the maize plants was from chili pepper. The virus probably needs adaptation process to infect a new host plant. Besides, the host ranges of a virus can make a specific consistency in its evolution. The evolution of a pathogen may involved in many ways, but the principal bases are a trade off between virulence and transmission of the pathogen (Escriu *et al*. 2000), and host selection that produce optimum level of virulence for pathogen multiplication and transmission (Sacristan *et al*. 2005).

Chi-square analyses were performed to determine segregation ratios of the F_2 and testcross populations. The results showed that Srikandi Kuning 1 as parent cultivar, showed 13 susceptible reactions of the 15 inoculated plants (Table 3). The percentage of virus infection in Srikandi Kuning 1 was 87% and considered as susceptible. According to Muis (2002), plants with virus infection percentages 61-100% were considered susceptible to th disease. On the other hand, results of inoculation of Guluk-guluk plants with CMV showed that 14 of the 15 inoculated plants did not show disease symptoms, only one plant showed mild symptoms with an ELISA absorbance value (A_{405}) in range of resistant reaction to CMV. The inoculated F_1 plants showed 29 of 30 inoculated plants symptomless and only one plant showed mild symptom with an ELISA absorbance value in the range of resistant reaction.

Table 2. Results of CMV detection from leaf samples of maize breeding materials of Guluk-guluk and Srikandi Kuning 1 using the indirect ELISA technique.

Maize generation	Symptom scores[a)]	Number of plants	A_{405} from of ELISA detection [b)]	Number of R/S[c)]		Total
				R	S	
Srikandi	0	1	0.1533 ± 0,0309	2	13	15
	1	1	0.1943 ± 0,0127			
	2	7	0.2413 ± 0,0229			
	3	6	0.2420 ± 0,0342			
Guluk-guluk	0	14	0.1953 ± 0,0095	15	0	15
	1	1	0.1987 ± 0,0376			
	2	0	-			
	3	0	-			
F_1	0	29	0.1320 ± 0,0128	30	0	30
	1	1	0.1563 ± 0,0104			
	2	0	-			
	3	0	-			
F_2	0	72	0.1450 ± 0,0069	72	27	99
	1	5	0.2550 ± 0,0160			
	2	16	0.2443 ± 0,0202			
	3	6	0.2590 ± 0,0226			
Testcross	0	7	0.1613 ± 0,0127	10	9	19
	1	3	0.1543 ± 0,0139			
	2	9	0.2317 ± 0,0447			
	3	0	-			
Healthy controls	0.1563 ± 0,0168		10			
Healthy controls + (3 x SD)	0.2067					

[a)] Disease index: 0 = no symptoms; 1 = mild symptoms; 2 = intermediate symptoms; 3 = severe symptoms.
[b)] A_{405} = Average value of absorbances at 405 nm wavelength (λ)
[c)] S = Susceptible, $A_{405} > A_{405}$ of healthy leaf samples + (3 x SD) (>0.2067); R = Resistant, A_{405} <0,2067. SD = standard deviation.

Table 3. Resistance segregation in progenies from crosses between maize cultivars Guluk-guluk and Srikandi Kuning 1.

Generation	Number of plants[a)]			X^2		
	Total	R	S	Ratio	Value	Probability
P_1 = Srikandi	15	2	13			
P_2 = Guluk-guluk	15	15	0			
F_1	30	30	0			
F_2	99	72	27	3:1	0,275	0,50-0,70
Testcross	19	10	9	1:1	0,060	0,70-0,90

[a)] R = Resistance; S = Susceptible

Seventy two individual plants of the F_2 progenies showed resistance to CMV, while 27 plants were susceptible. Ten individual plants of testcross were resistant, while 9 plants were susceptible. Based on results of the chi-square analysis, the F_2 ratio of resistant and susceptible individuals was 72 : 27 (3 : 1) and the ratio of resistant and susceptible individual of testcross plants was 10 : 9 (1 : 1) following the Mendel's law. These results revealed that the inheritance of resistance to CMV in maize crosses between cultivars Guluk-guluk and Srikandi Kuning 1 is controlled by a single dominant gene and inherited in accordance with the Mendel's law. Daryono *et al.* (2003) crossed melon cultivar *Yamatouri,* resistant to strain CMV-B2, with a susceptible cultivar *Vakharman* and selected for resistance to the virus. The results revealed that in F_1, F_2 and reciprocal backcross populations were resistant to CMV-B2, and the resistance was controlled by a single dominant gene assigned as *Creb-2*.

There are various resistance mechanisms in plants. Daryono *et al.* (2005) explained that these mechanisms could involve the inhibition of virus movement from inoculated leaves to upper leaves. This type of resistance would affect long-distance movement of viral particles. The resistance mechanisms may also influenced by condition of the virus, such as mutation. A mild strain of CMV that combined with CMV-RNA satellite could be used as a "vaccine" or preinoculation treatment and has been applied for the prevention of severe strain CMV infection in pepper and melon plants (Montasser *et al*. 1998).

Conclusions

The results of the study revealed that the inheritance of resistance gene to CMV in crossing between Madura's local maize (*Zea mays* L. cv. Guluk-guluk) and Srikandi's maize (*Zea mays* L. cv. Srikandi Kuning 1) is controlled by a single dominant gene (*Zrec*). Guluk-guluk could be used as a donor of resistance to CMV for breeding program in order to obtain resistant maize cultivars.

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