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Susceptibility of Cassava Varieties to Disease Caused by Sri Lankan Cassava Mosaic Virus and Impacts on Yield by Use of Asymptomatic and Virus-Free Planting Material

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Abstract: Cassava (*Manihot esculenta* Crantz) is a rainfed, smallholder-produced crop in mainland Southeast Asia, and is currently facing a serious challenge posed by the introduction of cassava mosaic disease (CMD). This study assessed the susceptibility of popular Asian varieties to CMD, yield penalties associated with the disease, and the efficacy of selecting clean or asymptomatic plants as seed for the following season. Field experiments evaluated agronomic management practices (i.e., fertilizer application, use of symptomatic and asymptomatic seed stakes) in Cambodia with six to nine popular varieties over three seasons under natural disease pressure. Popular cassava varieties KU50 and Huaybong60 showed superior CMD tolerance, with consistently fewer symptomatic plants, lower disease progress measures, and higher yields. Plants demonstrating symptoms at early stages of development, i.e., 60 days after planting, yielded significantly less than those developing symptoms later (i.e., 270 DAP) or not at all. Plants grown from clean stems yielded on average 20% to 2.7-fold higher than those grown from symptomatic planting material. A yield decline of ~50% was recorded with symptomatic planting materials of susceptible varieties (e.g., SC8, ~25 t ha^{-1}) over successive years. The findings emphasize that farmers could use positive selection by choosing asymptomatic plants to significantly reduce yield losses.

Keywords: cassava; mosaic disease; virus; yield

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1. Introduction

Cassava (*Manihot esculenta* Crantz) is a rainfed crop grown widely in tropical and subtropical countries of Latin America, Africa, and Asia. It grows well on soils with relatively low fertility and is also relatively drought and acid tolerant, making it an ideal crop for smallholder farmers in unfavorable upland environments [1,2]. Cassava production in mainland Southeast Asia has expanded rapidly in the past decade to meet the growing demand from a range of food and industrial applications [1]. The productivity and competitiveness of Southeast Asia's cassava sector has evolved during a period of limited disease pressure, but Southeast Asian farmers increasingly face serious challenges from pests and diseases [3].

Cassava mosaic disease (CMD) is caused by several species of cassava mosaic geminiviruses. In 2016, Sri Lankan cassava mosaic virus (SLCMV; family Geminiviridae, genus Begomovirus) was reported for the first time in Southeast Asia from a single commercial plantation with symptomatic plants in Ratanakiri province, eastern Cambodia, observed in May 2015 [4]. Since then, the virus has spread throughout most of the main production regions of Cambodia, southern Vietnam [5], China [6], Thailand [7], and southern Laos [8] through the movement of infected stem cuttings by value chain actors [9] and the whitefly

vector *Bemisia tabaci*, whose regional biotype Asia II 1 has been reported to be an efficient vector of SLCMV [10,11]. In addition to rapid spread across Cambodia's >500,000 ha of cassava fields [12], the authors have also detected SLCMV in plants of the genus Centrosema occurring in Cambodia (data not published), a known alternative host for cassava-infecting geminiviruses reported in Africa [13].

Developing sustainable solutions for CMD in Asia will require both new resistant varieties, and the enhancement of seed systems to maintain productivity [14]. There are currently no SLCMV-resistant commercial varietal releases in Southeast Asia, although resistance genes have been introduced into several national breeding programs. Varieties developed for African strains of the virus have also been introduced to the region for evaluation of resistance to SLCMV and general agronomic performance [15]. Field trial data is urgently required to inform stakeholders of the yield impacts of CMD on popular varieties and their relationship with disease infection rates.

Without commercially competitive CMD-resistant varieties and in the absence of more formal seed systems, one practical management strategy for farmers is in breaking the chain of self-infection perpetuated through contaminated planting materials. Positive selection (i.e., selecting symptomless mother plants to obtain stems for the following season's planting) may help to maintain profitable cassava production in the following season, and has been promoted as a key practice in maintaining low levels of cassava viral diseases including CMD and cassava brown streak disease [16,17]. The potential of this strategy depends on the ability to obtain virus-free planting material from mother plants within the field and on the yield impact of crops established with stem-cuttings of differing phytosanitary status.

We assessed (a) disease incidence in cassava plots planted with popular industrial varieties, (b) the effect of fertilizer treatments on disease development, (c) the presence of SLCMV in symptomatic and asymptomatic plants using polymerase chain reaction (PCR), and (d) yield impacts between treatments using planting materials from virus free, positive selected (i.e., asymptomatic at harvest time) plants, and symptomatic mother plants. The efficacy of positive selection was evaluated during and at the end of the season through the monitoring of symptom development and PCR-based confirmation of the presence of SLCMV in the remaining asymptomatic plants.

2. Materials and Methods

Six popular Southeast Asian cassava varieties (Table 1) were evaluated for tolerance to SLCMV over three consecutive seasons (2018–2019, 2019–2020, and 2020–2021), totaling five experiments conducted at Chamkar Leu Upland Seed farm of the General Directorate of Agriculture (GDA) in Kampong Cham Province, Cambodia (12°12′15″ N; 105°19′12″ E; 120 m.a.s.l). The beginning of the 2018–2019 season marked the first appearance of CMD on the GDA farm and was hence considered as the epidemic front of the spread of SLCMV in the study area. Natural inoculum of SLCMV originated from whitefly vectoring from surrounding contaminated fields.

Table 1. Name, country of origin, genetic background/pedigree, and source of clean/disease free planting stakes of cassava varieties used in experiments during three seasons.

Variety (Long Name)	Variety (Code)	Origin	Genetic Background/ Pedigree *	Source of Planting Material		
				2018–2019	2019–2020	2020–2021
Kasetsart University 50	KU50	Thailand	R1 × R 90	HBRST		
Rayong 11	R11	Thailand	R5 × OMR 29-20-118	HBRST		
South China 8	SC8	China	CMR38-120-10	CLSKC		
Huay Bong 60	HB60	Thailand	R5 × KU50	CLSKC		
KM98-1(6)	KM98-1	Vietnam	R1 × R5	CLSKC		
Rayong 5	R5	Thailand	22-27-10 XX × R3	CLSKC		
Tropical Manihot Esculenta 3	TME3	West Africa	Landrace	X	TTDI	TTDI
Huay Bong 80	HB80	Thailand	R5 × KU50	X	TTDI	TTDI
Rayong 72	R72	Thailand	R1 × R5	X	X	TTDI

* See Ocampo et al. (2021). HBRST = Huay Bong Research Station, Thailand; CLSKC = Chamkar Leu Station, Kampong Cham, Cambodia; TTDI = Thai Tapioca Development Institute, Thailand.

Sources of planting materials for each season were chosen according to treatment (Table 1). To confirm that the planting materials were disease-free (i.e., clean planting material), copies were planted in a region where no CMD was reported, and PCR tests were conducted on the first sprouts. Positive selected planting materials were chosen by visual observation according to farmers' practice (i.e., no visual symptoms at the end of the season).

2.1. Experimental Design

During the 2018–2019 season, field experiments were established at sites 1 and 2 in May 2018 and harvested following farmer practice after 10 months in March 2019. The two sites were approximately 500 m apart. On site 1, prior to the current experiment, cassava has been continuously grown since 2009, and NPK 15-15-15 was applied at a rate of 200 kg/ha. At site 2, coffee (Coffea sp.) had been grown until 2016, followed by radish (*Raphanus sativus* L.) and maize (*Zea mays* L.), thereafter. Land was prepared by two passes with a six-disk tractor. Approximately 15–20 cm long cassava stakes were hand planted vertically at 13,888 plants ha^{-1} (i.e., planting distance 90 × 90 cm^2). Hand weeding was carried out 4–5 weeks interval. Three sides of site 1 were surrounded by leguminous plants, while site 2 was surrounded by cassava crops of local farmers. Neighboring cassava fields were the primary inoculum source, although at the time of the experiment's inception, these fields were asymptomatic. Higher numbers of whiteflies were anecdotally observed at site 2 compared to Site 1 (visual observation) during the experimental period; however, incidence rates were not directly measured in this experiment.

Two treatments, with and without fertilizer application, were included to determine impacts on the appearance of symptoms and yield losses. Fertilizer NPK (80-20-80) was applied 4 weeks after planting. Nitrogen was applied in the form of urea, P as triple superphosphate (TSP), and K as muriate of potash (KCl). The actual rates (kg ha^{-1}) of elemental N, P and K were as follows: N 80, P 8.7, and K 66.5.

Experiments were laid out in a split-plot design with 3 blocks, and variety in main plots (Table 1), and fertilizer in subplots. Each plot size was 54 m^2 (6 m × 9 m), and plant spacing was 90 cm × 90 cm. One half of the main plot was without fertilizer and other half was fertilized to represent subplots. Twelve plants were sampled from each plot, omitting the border rows, to measure starch content and fresh root yield.

During the 2019–2020 season, an experiment was conducted to assess the relative yield penalty of planting stakes from symptomatic, positive selected (asymptomatic), and clean mother plants. Planting material was obtained from three sources: (1) positive selected planting material from 2018–2019 multiplication block (i.e., from visually healthy plants), (2) symptomatic planting material from the 2018–2019 experiment and (3) clean planting material from the Thai Tapioca Development Institute (TTDI), which at that time was unaffected by CMD. Due to the high CMD susceptibility and importation regulations, no clean planting materials were available for SC8 and KM98-1, which were replaced with HB80 and TME3, a breeding line containing the CMD2 resistance gene, obtained from TTDI.

The fertilizer treatment resulted in no impact on disease symptoms or yield during the 2018–2019 season experiments, only one treatment representing an optimum fertilizer rate (N 80, P 8.7 and K 66.5 kg ha^{-1}) was applied in subsequent years.

Visual scoring of disease symptoms (presence or absence) was recorded at 60 days after planting (DAP); 180 DAP; and 270 DAP. Young leaf samples were collected at 63 DAP for molecular confirmation of SLCMV by PCR. The trial was harvested over 4 days (i.e., one block each day) in March 2020. Data collection at harvest followed the same procedure as in 2018–2019.

During the 2020–2021 season, two experiments were conducted. In experiment 1, six varieties were included consistent with the previous year, to assess the yield penalty of planting symptomatic stakes. Three sources of planting material were tested as described for season 2. Due to their high susceptibility to disease, no clean planting materials were

available for SC8, KM98-1, and positive selection planting material of SC8 for planting during 2020–2021 season. TME3 was received from TTDI and planted in place of SC8 clean planting material, and R72 planted in place of positive selection SC8. HB80 was planted in place of KM98-1 clean planting material.

In experiment 2, the consequences of planting asymptomatic stems from a symptomatic plant were evaluated. Asymptomatic stems from symptomatic plants are the result of uneven whitefly transmission (or incomplete host infection) observed in the field. Three varieties—KU50, R5 and HB60—were included in this experiment. Three different kinds of stakes were planted: 1. Stakes from virus-free plants, 2. Asymptomatic stems collected from symptomatic plants, and 3. Symptomatic stems collected from symptomatic plants.

2.2. Measurements

Presence of CMD symptoms on each plant was recorded 3 times during the season, at approximately 60, 150 and 270 days after planting (DAP) for all experiments. However, for 2020–2021 season experiments, to gain a better understanding of early season dynamics, one extra rating was conducted at 30 DAP. During the harvest, symptomatic and asymptomatic plants were separated, and yield and starch content was recorded per plant. Harvest omitted the border rows and plot yield was calculated by including all plants in the harvest area (i.e., with and without disease symptoms together). In some plots, some levels of termite infestation and root rot damaged plants were found. Plot yield was adjusted as described by Pérez et al. [18] for missing plants.

Starch content (%) was measured following the procedure described by Howeler [19] and Chua et al. [20].

Disease incidence was evaluated as the percentage of symptomatic plants in each plot. To measure quantitative disease pressure for each cultivar over the whole season, area under disease progress curves (AUDPC) were calculated [21] using percent infection and following the general equation of Shaner and Finney [22]:

$$\text{AUDPC} = \sum_{i=1}^{n} \left[\frac{Y_i + Y_{i+1}}{2} \right] (X_{i+1} - X_i) \quad (1)$$

in which Y_i = percentage of plants infected at observation i, X_i = time in days at observation i, and n = total number of observations. To compare AUDPC values between seasons with different observation lengths, relative AUDPC (rAUDPC) values were calculated expressing AUDPC as a relative proportion of the total maximum possible score. AUDPC was used for reporting within-year statistics, while rAUDPC was employed for between-year comparisons.

Data were analyzed by calculating means, standard errors, regression, and analysis of variance (ANOVA), where appropriate using R statistical software version 4.0.3 [23]. Significant differences refer to $p < 0.05$. To find significant differences between means, a Tukey's test was carried out. For 2019–2020 and 2020–2021 season, KU50, R11, HB60 and R5 were included in the statistical analysis. The other three varieties are presented as means ($n = 4$) with standard error as those varieties lacked in one or two treatments due to scarcity of disease-free planning material.

Split plot ANOVA analyses and correlation of disease pressure were conducted using the R package agricolae. A combined split-split plot analysis was conducted on data from both sites in 2018 to evaluate variety effects across locations. For 2019–2020, the four complete variety x seed class combinations were subjected to split-plot ANOVA analyses. In all cases, Tukey's honest significant difference (HSD) was used to separate significant effects among means, with the specification of the appropriate error term from the complete model. Due to the consistent presence of interaction effects between variety and seed class, means in 2019–2020 were separated using the interaction means. These methods were applied for overall AUDPC values, as well as on point disease ratings at all observations.

Linear regression combined with Pearson correlation was employed to evaluate relationships between rAUDPC and yield in all 3 study years.

A total of 943 samples (314 from 2019, 574 from 2020, and 55 from clean planting material), corresponding to the topmost, youngest leaves of asymptomatic plants were collected from the harvest areas of all treatment plots. Leaf samples were dried in silica gel and stored at 4 °C before CTAB extraction [24]. PCR diagnostics were carried out with nucleic acid extracts diluted to a working concentration of 60 ng/µL and 1µL used per PCR reaction in a total reaction volume of 20 µL. We used a ready-to-use PCR mix (2X PCR Master Mix, Promega) and primer, SLCMV-F (5′-ATGTCGAAGCGACCAGCAGATATAAT-3′) and SLCMV-R (5′-TTAATTGCTGACCGAATCGTAGAAG-3′), which target the AV1 gene of the virus [25]. This protocol has been validated to detect SLCMV from field-collected cassava samples [7,8]. The PCR program was as follows: initial denaturation at 94 °C for 5 min followed by 35 cycles of denaturation at 94 °C × 30 s, annealing at 58 °C × 30 s and extension at 72 °C × 1 min. After a final extension at 72 °C × 5 min PCR products were resolved by agarose gel electrophoresis as previously described [24].

3. Results

3.1. Disease Symptoms

The percentage of plants with symptoms increased with time in both sites during the 2018–2019 season (Table 2). Varieties in site 2 had higher infection rates than site 1. SC8 and R11 showed the most symptoms (i.e., 100%), and KU50 the least. There was no effect of fertilizer application on disease development.

Table 2. Percentage of symptomatic plants (%) by plot and means for disease reactions of cassava varieties evaluated at 2 sites in 2018–2019 season 60, 150 and 270 days after sowing (DAP) and area under disease progress curve (AUDPC). Different letters indicate statistically different means within a given site x column.

		Symptom Incidence (%)			
Site	**Variety**	**60 DAP**	**150 DAP**	**270 DAP**	**AUDPC**
Site 1	HB60	0 ^{b}	14 ^{b}	14 ^{c}	2353 ^{c}
	KM98-1	0 ^{b}	3b ^{c}	6 ^{c}	736 ^{cd}
	KU50	0 ^{b}	0 ^{c}	2 ^{c}	91 ^{d}
	R11	6 ^{b}	12 ^{b}	47 ^{b}	4347 ^{b}
	R5	0 ^{b}	5b ^{c}	7 ^{c}	868 ^{cd}
	SC8	16 ^{a}	65 ^{a}	94 ^{a}	13201 ^{a}
	HSD ^{c}	8	11	16	1886
Site 2	HB60	20 ^{ab}	44 ^{b}	82 ^{ab}	10442 ^{b}
	KM98-1	19 ^{ab}	35 ^{b}	90 ^{ab}	9938 ^{b}
	KU50	12 ^{b}	26 ^{b}	55 ^{c}	6556 ^{c}
	R11	32 ^{ab}	90 ^{a}	100 ^{a}	16825 ^{a}
	R5	17 ^{ab}	35 ^{b}	71b ^{c}	8695b ^{c}
	SC8	44 ^{a}	97 ^{a}	100 ^{a}	18106 ^{a}
	HSD	28	20	23	10442

Tukey's honest significant difference between means at $p = 0.05$, Means within a column within site followed by a different letter are significantly different ($p = 0.05$).

Analyses of variance indicated significant differences between cassava varieties at all measured periods for the percentage of plants displaying SLCMV symptoms, as well as for overall AUDPC (Table S1). Fertilizer application had no significant effect, although may have incurred interaction effects with the other treatments at some periods, so the factor was left in the model. Site had a significant effect on disease percentages and AUDPC, and variety x site interactions, with significantly higher overall disease pressure at site 2.

Disease incidence (i.e., percentage of plants infected) and AUDPC showed clear differences among varieties, with SC8 and R11 consistently recording the highest values at both sites, and KU50 and R5 consistently ranking the lowest (Table 2). These trends were most pronounced under the heavier disease pressure at site 2.

The percentage of plants with symptoms increased with time in all treatments during 2019–2020 and 2020–2021 season (Table 3). R11 and SC8 approached 100% infection, while KU50 had the least symptomatic plants for all three treatments (i.e., clean, positive selection and diseased planting material) by the end of the season. Unexpectedly, many plants grown from symptomatic planting material did not develop symptoms during the 2019–2020 season. However, during the 2020–2021 season, all plants from symptomatic planting material developed symptoms by the end of the season for all genotypes tested.

Table 3. Percentage of symptomatic plants (%) per plot and area under disease progress curves (AUDPC) of four cassava varieties and three seed classes at four different DAP (days after planting) intervals in the 2019–2020 and 2020–2021 seasons. Group means followed by different letters within a year column (a to f) are significantly different (Tukey's HSD); those not followed by a letter were part of incomplete treatment combinations and were not included in statistical analyses of variance but are reported for completeness.

			Symptom Incidence (%)				
Year	**Variety**	**Seed Class**	**30 DAP**	**60 DAP**	**150 DAP**	**270 DAP**	**AUDPC**
2019	HB60	Clean		7 ^{b}	25 ^{cd}	48 ^{cde}	5804 ^{cd}
		Positive		0 ^{b}	5 ^{d}	8 ^{f}	975 ^{d}
		Symptomatic		14 ^{b}	26 ^{cd}	29 ^{def}	5095 ^{cd}
	KU50	Clean		0 ^{b}	4 ^{d}	17 ^{def}	1438 ^{d}
		Positive		0 ^{b}	8 ^{d}	14 ^{ef}	1652 ^{d}
		Symptomatic		3 ^{b}	5 ^{d}	11 ^{ef}	1255 ^{d}
	R5	Clean		0 ^{b}	18 ^{cd}	29 ^{def}	3565 ^{cd}
		Positive		16 ^{b}	46 ^{bc}	54 ^{bcd}	8775 ^{bc}
		Symptomatic		73 ^{a}	77 ^{ab}	81 ^{abc}	16,250 ^{a}
	R11	Clean		2 ^{b}	68 ^{ab}	94 ^{ab}	12,891 ^{ab}
		Positive		50 ^{a}	100 ^{a}	100 ^{a}	18,749 ^{a}
		Symptomatic		65 ^{a}	96 ^{a}	98 ^{a}	18,841 ^{a}
	KM98-1	Positive		0	25	34	4699
		Symptomatic		60	78	96	16,665
	HB80	Clean		3	31	59	6904
	TME3	Clean		5	65	69	11,195
	SC8	Positive		24	92	100	16,760
		Symptomatic		100	100	100	21,000
	HSD			27	38	40	6569
2020	HB60	Clean	0 ^{c}	14 ^{ef}	57 ^{bcde}	75 ^{abc}	11,352 ^{cde}
		Positive	6 ^{c}	29 ^{def}	56 ^{cde}	60 ^{c}	11,327 ^{cde}
		Symptomatic	18 ^{c}	61 ^{abcde}	93 ^{a}	97 ^{a}	19,546 ^{ab}
	KU50	Clean	0 ^{c}	3 ^{f}	29 ^{e}	53 ^{c}	6388 ^{e}
		Positive	0 ^{c}	16 ^{ef}	39 ^{de}	62 ^{bc}	8787 ^{de}
		Symptomatic	77 ^{a}	84 ^{abc}	93 ^{a}	100 ^{a}	21,924 ^{a}
	R5	Clean	0 ^{c}	36 ^{cdef}	62 ^{bcd}	74 ^{abc}	13,069 ^{cd}
		Positive	0 ^{c}	45 ^{bcdef}	77 ^{abc}	90 ^{ab}	16,185 ^{bc}
		Symptomatic	48 ^{b}	67 ^{abcd}	85 ^{ab}	90 ^{ab}	19,063 ^{ab}
	R11	Clean	0 ^{c}	44 ^{bcdef}	100 ^{a}	100 ^{a}	19,125 ^{ab}
		Positive	6 ^{c}	91 ^{ab}	100 ^{a}	100 ^{a}	23,872 ^{a}
		Symptomatic	91 ^{a}	100 ^{a}	100 ^{a}	100 ^{a}	22,060 ^{a}
	KM98-1	Positive	0	34	79	89	15,693
		Symptomatic	78	85	98	98	22,418
	HB80	Clean	0	55	95	98	19,108
	TME3	Clean	8	58	92	100	19,250
	SC8	Symptomatic	92	100	100	100	23,875
	R72	Positive	2	31	62	70	12,616
	HSD		27	49	29	31	5441

Analyses of variance for the complete variety x seed treatment combinations clearly demonstrated significant effects of both variety and seed selection on the percentage

of plants infected at all evaluation stages and overall AUDPC (Table S2), with a strong interaction effect (except at the 60 DAP evaluation in 2020–2021). Significant differences were present at the earliest periods evaluated.

The progress of infection in 2019–2020 and 2020–2021 varied significantly by variety, but also by seed class (Table 4). Furthermore, plants from clean stakes of susceptible varieties reached very high (90 to 100%) infection rates by 150 DAP.

Table 4. Percentage of asymptomatic plants testing positive for Sri Lanka cassava mosaic virus (SLCMV) over 2 years. Young leaves from 33.3% of plants without any symptoms from each plot were collected for diagnosis of SLCMV by PCR of 9 varieties of cassava listed. Samples were collected 63 and 270 days after planting (DAP) during 2019–2020 season (year 2) and 21 DAP during 2020–2021 season (year 3).

Varieties	Clean	Positive Selection	Clean	Positive Selection
	2019–2020		2020–2021	
KU50	6.7 ± 0.07	0	6.7 ± 0.07	0
R11	12.5 ± 0.09	66.7 ± 0.13	20 ± 0.20	26.7 ± 0.12
HB60	26.7 ± 0.12	23.5 ± 0.11	0	18.8 ± 0.10
R5	46.7 ± 0.13	35.3 ± 0.12	6.7 ± 0.07	0
SC8	NA	42.9 ± 0.14	NA	NA
TME3 *	30.8 ± 0.13	NA	15.4 ± 0.10	NA
KM98-1	NA	31.3 ± 0.12	NA	18.8 ± 0.10
HB80 **	31.3 ± 0.12	NA	26.7 ± 0.12	NA
R72	-	-	NA	0

* TME3 and ** HB80 were replaced with clean SC8 and KM98-1, respectively, due to unavailability of clean planting material. NA = not available.

During season 3 in experiment 2, plants growing from symptomatic stems from symptomatic plants (SSSP) approached 100% symptom incidence by the end of the experiment for all three genotypes (Figure 1). Asymptomatic stems from symptomatic plants (ASSP) produced similar patterns of symptom expression as positive selected planting material from the previous year. By the end of the season, there was no significant difference in the number of symptomatic plants between ASSP and positive selection planting treatments (Figure 1).

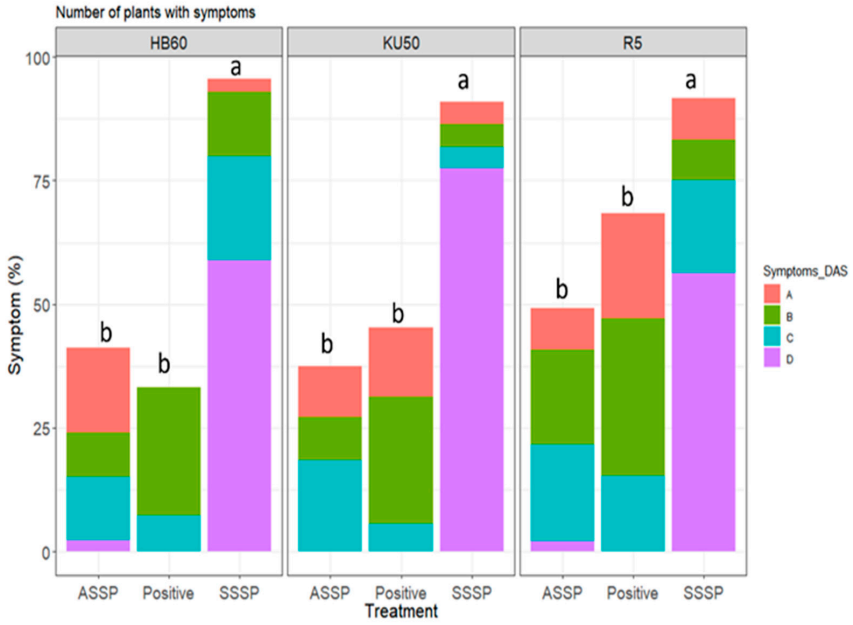

Figure 1. Cumulative number of CMD symptomatic plants (%) per plot 30 (D), 65 (C), 156 (B) and 278 (A) days after planting (DAP) of 3 cassava varieties commonly grown in Southeast Asia. Three kinds of stakes/seeds were planted, positive selection (visually no disease symptoms), Asymptomatic stem from symptomatic plants (ASSP) and symptomatic stems from symptomatic plants (SSSP). Bars with different letters are significantly different ($p < 0.05$).

3.2. Yield and Starch Content

There was no effect of fertilizer application on yield or starch content during the 2018–2019 season (Figure 2a). Fresh root yield (t ha^{-1}) significantly ($p < 0.001$) differed between the varieties. Overall yield at both sites was similar, ranging from 24.1 to 42.9 t ha^{-1} at site 1 and 17.1 to 46.0 t ha^{-1} at site 2. SC8 had the highest yield in both treatments at site 1; however, at site 2, KM98-1 produced the highest. R11 yielded the lowest in both treatments and sites. Starch content (%) significantly ($p < 0.001$) differed between varieties (Figure 2b). Starch content at site 1 was marginally higher (i.e., on average 2.3%) compared to site 2. However, KU50 demonstrated a ~20% increase in starch content in site 1 in response to fertilizer. SC8 had the lowest starch content among tested varieties (26.5% in site 1 and 22.6% in site 2).

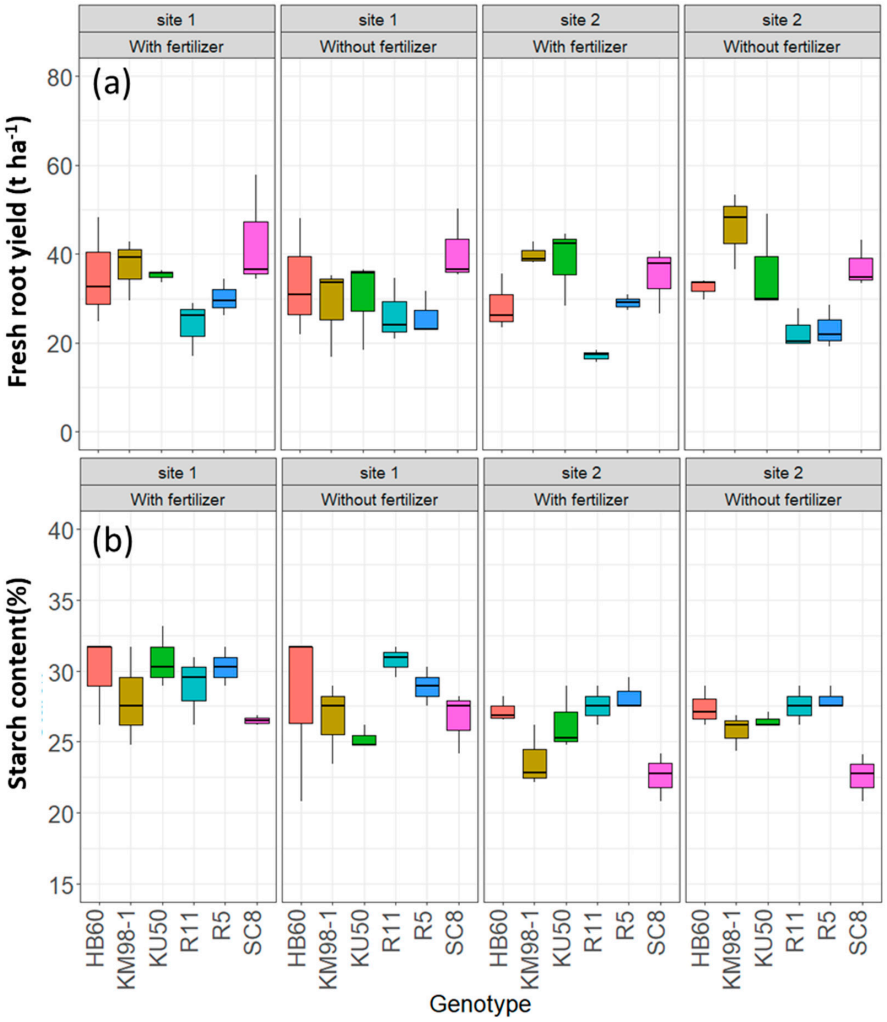

Figure 2. Fresh root yield (t ha^{-1}) (**a**) and starch content (%) (**b**) of six cassava varieties during the 2018–2019 season at two different sites with and without fertilizer. Whiskers indicate the range within a treatment.

During the 2019–2020 and 2020–2021 seasons, fresh root yield was significantly ($p < 0.001$) higher for clean planting material over symptomatic planting material (Figure 3a,b). Plot yield was 20% to 2.2-fold higher and 20% to 2.7-fold higher in plants from clean and/or positive selection planting material than those from symptomatic planting material during the 2019–2020 and 2020–2021 seasons, respectively (Figure 3). The smallest yield difference (i.e., 20%) in planting clean material over symptomatic planting material occurred in KU50 and HB60 during the 2019–2020 season. In the 2020–2021 season, the smallest yield difference (i.e., 20%) occurred in HB60, and for KU50 the difference was 80%.

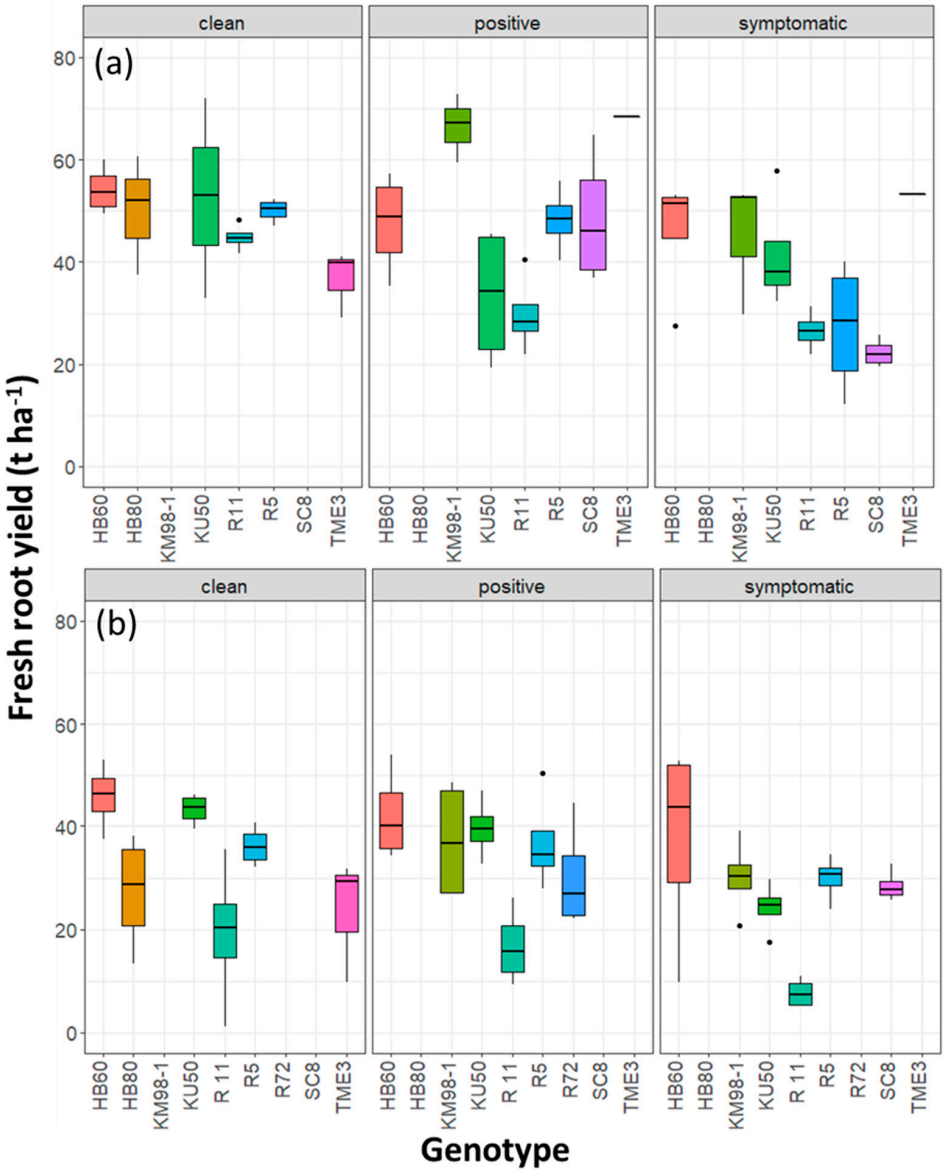

Figure 3. Fresh root yield (t ha^{-1}) of cassava varieties planted using disease-free stakes (clean), positive selected stakes from diseased fields (positive selection) and stakes selected from symptomatic plants (symptomatic) during the 2019–2020 season (**a**) and the 2020–2021 season (**b**).

The most highly susceptible variety, SC8, demonstrated the highest root yield difference between positively selected and symptomatic planting material (2.2-fold, Figure 3a). In season 2020–2021, no asymptomatic SC8 was available, all having become infected in the previous season, and yielded 28.4 t ha^{-1} (Figure 3a). R11 had the highest yield difference during the 2019–2020 and 2020–2021 seasons, 1.7- and 2.5-fold between clean and positive selection planting material over symptomatic planting material, respectively. TME3 had the lowest yields (35 and 23 t ha^{-1}, 2019–2020 and 2020–2021, respectively), only 70% and 40% of the yield of clean KU50 during the 2019–2020 and 2020–2021 seasons, respectively (Figure 3a,b).

TME3 and HB80 were planted as clean planting material due to the scarcity of clean planting material of SC8 and KM98-1, respectively, and R72 was planted in place of positive selection SC8 during the 2020–2021 season. There was no significant difference in root yield between clean and positive selected stakes; however, both were significantly different from symptomatic plants for both seasons (Figure 3b).

Plants from clean or positive selection planting material produced generally higher yields than diseased planting material from all genotypes tested (Figures S1 and S2) in

both seasons. Furthermore, plants showing disease symptoms during early growth (i.e., 26 or 64 DAP) produced lower yields than those showing disease symptoms during later growth (i.e., 270 DAP) or asymptomatic plants (Figures S1–S3). No significant effect was observed of disease on root starch content (%) (Figure S4). KU50 had the highest starch content (29.8%), and SC8 had the lowest (21.7%).

Experiment 2: In all three genotypes, SSSP produced the lowest yields; there were no significant difference between plots from positive selected stems and ASSP (Figure 4a). The starch content in roots was measured at whole plot level and there was no significant effect of disease on starch content from different planting material (Figure 4b).

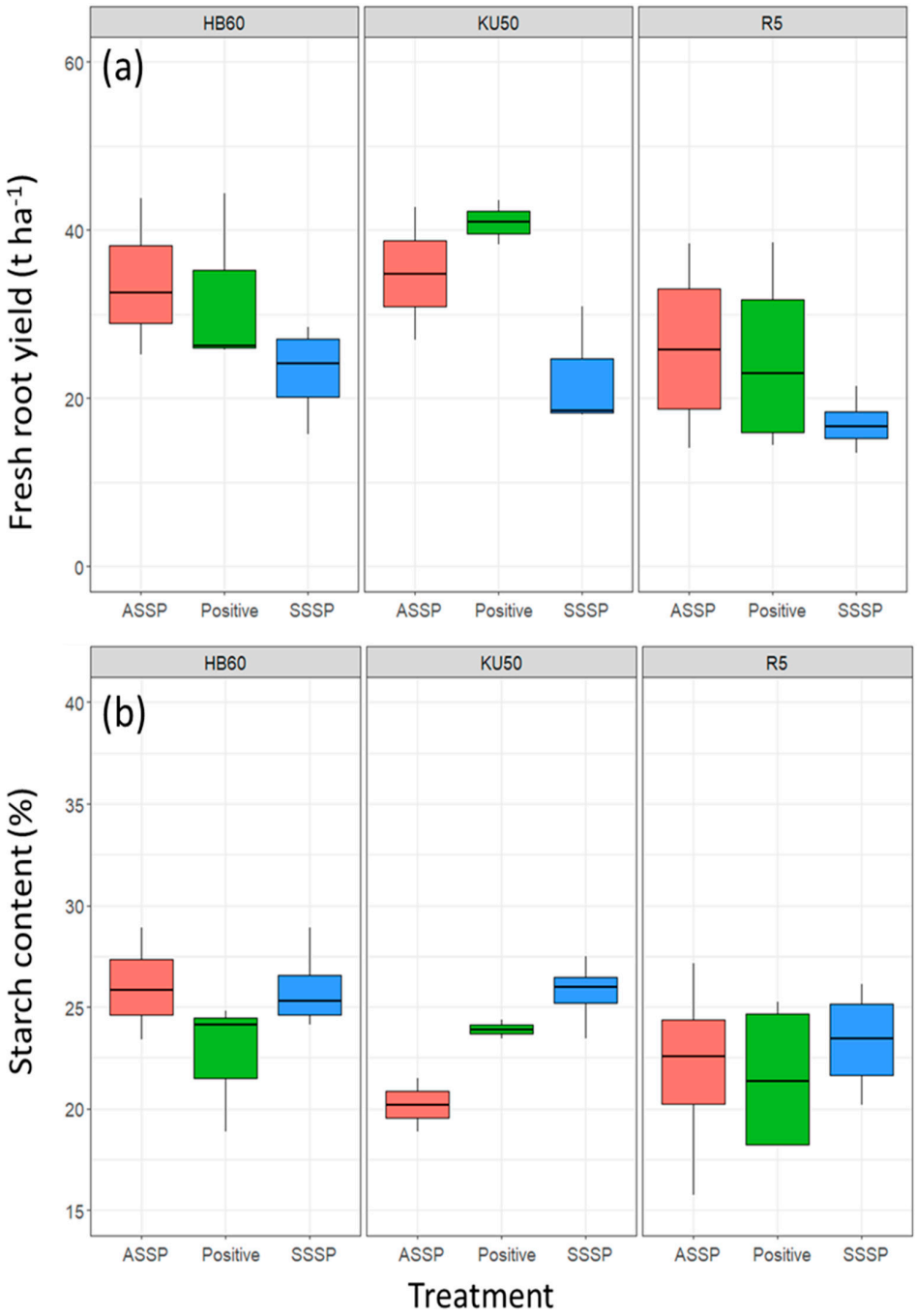

Figure 4. Fresh root yield (t ha^{-1}) (**a**) and starch content (%) (**b**) of three cassava varieties planted with three planting material classes: positive selection, asymptomatic stems from symptomatic plants (ASSP) and symptomatic stems from symptomatic plants (SSSP).

3.2.1. Relationship between Onset of Symptoms and Root Yield

Plants demonstrating symptoms early in development (i.e., 60 DAP) produced less compared to plants with later onset (i.e., 270 DAP) and/or no symptoms; however, the effect was not significant due to a large variation in the number of plants and the time of infection during the 2018–2019 season (Figure S3). At site 1, plants demonstrating symptoms at 60 DAP produced on average 1.5 to 2.2 kg plant^{-1}, whereas at the time of harvest asymptomatic plants produced 2.5 to 3.8 kg plant^{-1}. At site 2, plants demonstrating symptoms at 60 DAP produced on average 1.9 to 2.2 kg plant^{-1}, whereas asymptomatic plants at the time of harvest produced 3.2 to 3.8 kg plant^{-1}.

Plants without disease symptoms and/or with disease symptoms developed at later stages of development (e.g., 270 DAP) had higher (10%) starch content compared to those developing symptoms at early stages of development (60 DAP) (Supplementary Table S3). There was no effect of fertilizer application on starch content.

Pearson correlation of disease and yield did not detect strong relationships between yield and rAUDPC at either site in the first year of infection (Figure 5a). Site 1 incurred many 0 scores, suggesting low inoculum levels. Higher AUDPC scores were registered at site 2, indicative of higher levels of inoculum, with yield depression increasing as infection worsened.

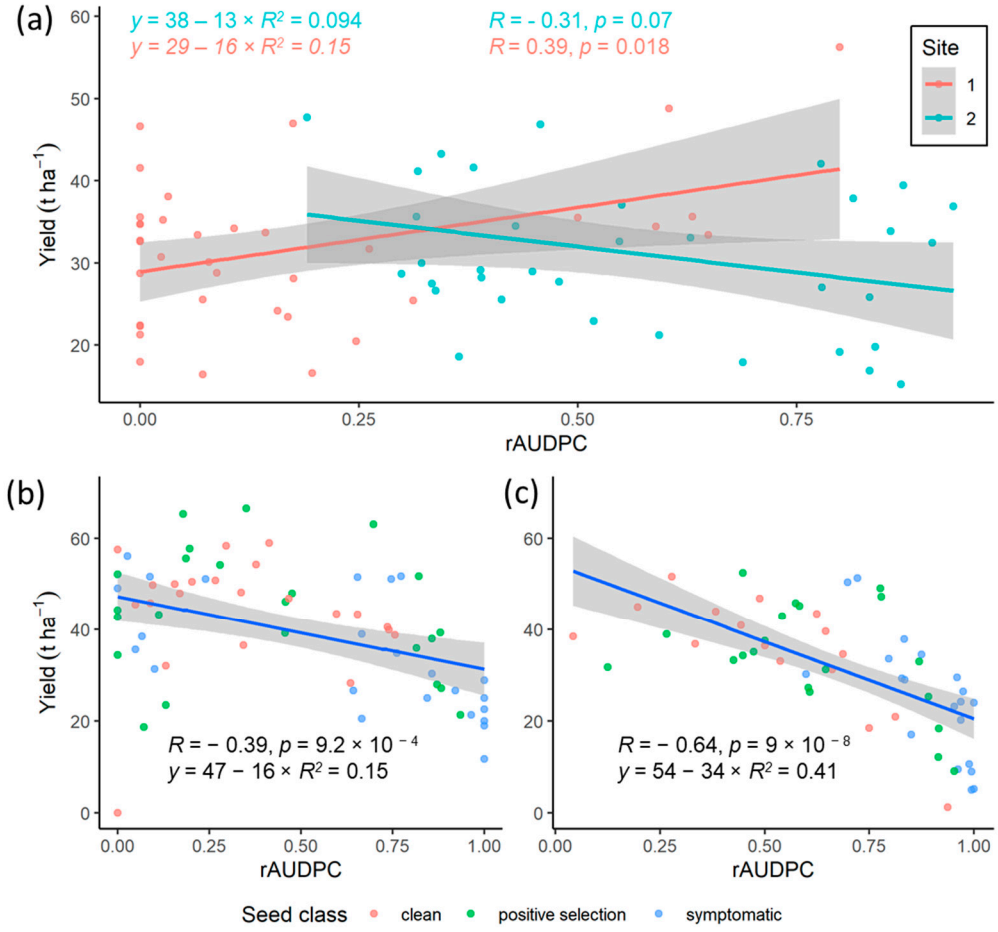

Figure 5. Linear regression with Pearson correlation of relative AUDPC (rAUDPC) and yield of varieties at two sites in season 2018–2019 (**a**) and with three different seed classes, in the 2019–2020 season (**b**) and 2020–2021 season (**c**). Regression line equations are presented in-panel, and grey margins indicate 95% confidence fits.

Highly significant relationships between yield and rAUDPC were observed in both the 2019–2020 and 2020–2021 season (Figure 5b,c), with a strengthening negative association

from years 1–3. The stronger negative association in 2020 was largely due to a significant clustering of symptomatic treatment plots at very high rAUDPC values at or approaching 100%, accompanied by significant yield depression. The relationships observed were particularly robust given the inclusion of a wide range of varieties with differing levels of susceptibility (Tables 2 and 3).

3.2.2. Analysis of Asymptomatic Plants

Varietal variation was observed in PCR-based SLCMV detection (Figure S5). KU50 originating from TTDI's clean multiplication field was SLCMV positive in 6.3% of asymptomatic plants tested at the end of the season (Table 4), with a similar result in the 2018–2019 season. TME3 plants grown from planting material of the same origin demonstrated 30.8% positive detection of SLCMV after 9 weeks in the field (Table 4). To verify the clean status of the original planting material (KU50, HB60, R5, HB80 and TME3) received from TTDI in the following season (2019–2020), plants from these materials were grown in isolation. All samples collected of isolated plants at 14 DAP tested negative for SLCMV (data not shown), confirming that infection occurred during the field experiment.

Sequence analysis indicated that the SLCMV isolates occurring in the location of the trials grouped with all other published SLCMV isolates from SEA, specifically isolates that present the short version of the rep gene [7]. The analysis was carried out using random amplification of circular DNA and nanopore sequencing using available protocols [26]. No other species of CMD-associated geminiviruses were detected in the samples analyzed. We maintain an open resource page archiving SLCMV isolates in SEA (https://nextstrain.org/community/pestdisplace/CMDASIA?c=virus&p=grid&r=location) (accessed on 8 July 2021) [27].

4. Discussion

Despite extensive research on cassava breeding and agronomy for yield gain and starch stability in Southeast Asia [1], very little is known about variation in disease resistance. To our knowledge, this is the first field study on varietal susceptibility to cassava mosaic disease (CMD) caused by SLCMV and its impact on yield in Southeast Asia. Of the six popular Southeast Asian industrial cassava varieties evaluated over three years in the natural disease pressure experiment in Cambodia, KU50 and HB60 demonstrated significantly superior tolerance over other varieties. Yield losses were strongly correlated with CMD symptom expression and were more severe in plants with early season symptom development. The use of clean planting material and selection of asymptomatic planting materials resulted in lower disease scores, emphasizing the potential for use of positive selection and clean planting material to minimize field infection levels and associated yield loss.

The early PCR analysis of asymptomatic plants and planting material confirmed the rapid spread of SLCMV within plots, indicating efficient vectoring [11] from viral reservoirs in the surrounding environment. High rates of SLCMV transmission have been reported for *Bemisia tabaci* biotype Asia II 1 whitefly [11], which has been reported in the region [10].

High levels of asymptomatic SLCMV infections in cassava were observed in Southeast Asia during field surveys 3 to 5 months after planting [7,8,28]. Our work shows that most asymptomatic infected plants eventually developed CMD symptoms in all varieties tested, but the rate of symptom development differed among varieties (Table 2). Asymptomatic infections are a challenge for viral disease surveillance and control efforts in cassava [29], and our findings confirm the importance of early-season timing for molecular-based monitoring, and mid to late-season timing for visual symptom-based monitoring (Table 2 and Figure 1). Further research is needed into the relationships between cassava varieties, SLCMV viral titers, symptom development, and pathogen transmissibility.

The AUDPC values observed over the three seasons of our experiment described persistent and rising levels of disease pressure as the CMD epidemic front progressed over the study location (Tables 2 and 3). The first season (i.e., 2018–2019), in which all treatments

were planted with clean materials in the absence of CMD, is representative of an early epidemic invasion scenario. By 60 DAP, differences in disease pressure between the two sites evaluated were apparent in the presence of symptomatic plants at plot level (Table 2)., and significant effects of site, variety, and a variety x site interaction persisted throughout the experiment. The presence of the interaction effect was related to discrepancies in the performance of some of the middle-ranking varieties, while AUDPC scores were consistent at both sites for highest disease scores (SC8 and R11) and lowest scores (KU50) (Tables 2 and 4).

Genetic variation in disease susceptibility among vegetatively propagated crops has been demonstrated in sugarcane [30], potato [31], sweet potato [32] and the current cassava study and [33]. The varieties evaluated in the current experiment were selected for their popularity in Southeast Asia [1], thus generating impactful immediate recommendations from a policy and management perspective. Tolerance to disease quantified through visible appearance of foliar symptoms has been considered a reliable indicator of CMD infection and a direct consequence of viral titer in the host tissue [34]. Our findings suggest that SLCMV in Southeast Asia differs from its better-studied African counterpart in much higher levels of asymptomatic infection, especially early in the season, and that visual assessment of infection status becomes increasingly reliable approaching the end of the growing season (Tables 2 and 3).

Fresh root yield (FRY) and starch content are among the most important traits in industrial cassava production settings, and their relationship to disease expression is a critical point of investigation. Varietal variation in root yield in response to natural disease pressure is an important metric for disease response programs and its quantification is often a priority in emerging epidemics [35,36]. KU50 and HB60 showed superior tolerance by consistently demonstrating fewer symptomatic plants and higher yield across sites with different disease pressures (Table 2, Figure 2a). By contrast, KM98-1 and SC8 demonstrated higher disease incidence; however, similar yield compared to the above mentioned two varieties when established with disease-free stems, supporting the finding that early season infections (<60 DAP) induce greater yield losses (Figure S3). Consequently, higher yields of KU50 and HB60 can be attributed to symptom development at later stages (Table 2) compared to R11. Although disease severity was not recorded in this study due to a need for adjusting existing symptom scales to the SLCMV scenario, plants were observed to continue to grow even when disease symptoms appeared early in the season. Presumably, reduced photosynthetic carbon resulting from leaf deformation and chlorosis was primarily allocated to vegetative growth rather than storage carbohydrate, with both KM98-1 and SC8 producing lower starch content (Figure 2b).

The use and distribution of disease-free planting materials is one of the major strategies for controlling plant disease epidemics in developing countries. Clean seed materials routinely produce higher yields in vegetatively propagated crops such as sweet potato [37,38], cassava [39,40], and banana [41]. Our results indicate that the use of clean or positive selection-derived seed resulted in mean root yields ~45 t ha^{-1}, 20% to 1.8-fold higher than plots originating from diseased (i.e., symptomatic) planting materials (Figure 3) in both seasons across all genotypes tested; although the yield did vary within and between varieties in different planting materials. In some varieties, for example, HB60, the difference between symptomatic and positive selection planting material was only 2 t ha^{-1} in 2019–2020 of the experiment. By contrast, the difference between symptomatic planting material and positive selected planting material was much higher (6 t ha^{-1}) in 2020–2021. The discrepancy may have been due in part to the inadvertent planting of ASSP planting materials as symptomatic stakes. The results of a small experiment conducted in season three suggested that ASSP materials can produce similar yields as positive planting material (Figure 4a).

Despite high susceptibility to CMD, SC8 produced high yields when planted with clean (~40 t ha^{-1}, Figure 2a) or positive selected planting material (48 t ha^{-1}, Figure 3a). However, yield declined by ~50% when symptomatic planting materials were used (Figure 3b). Yield reductions of about 20% were recorded over the three years of the experiment in

the less susceptible variety KU50, emphasizes the need for clean sources of planting material to preserve yields, even in tolerant varieties. For the most susceptible varieties, maintaining a clean source of stems through positive selection is likely not feasible, even under intermediate levels of inoculum pressure.

Due to variation in varieties and treatments each year, we chose to evaluate correlations between rAUDPC and yield across all samples for each year. Strong negative relationships were observed between the two variables, with relationships strengthening as inoculum pressure mounted year on year (Figure 5). In year 1, the site with the lowest rAUDPC values was the exception, displaying a weakly positive relationship, primarily caused by the high yield performance of SC8 under relatively weak disease pressure (Figure 5a). A negative relationship was observed increasing in slope in site 2, and years 2 and 3 (Figure 5). Similar negative regression relationships and end-of-season incidences approaching 100% infection have been demonstrated in Africa with CMD caused by local viral strains [35], while multi-country compilations of African yield data also report serious reductions, which, as in our study, were more significant when infections occurred earlier in the season (such as through planting of infected cuttings or early whitefly vectoring) [42].

Highly significant relationships were observed between seed classes in both seasons two and three, with clean and positive selection planting materials clearly resulting in lower disease levels and AUDPC (Table 3). This was tempered in some cases by a variety x seed treatment interaction, particularly in cases such as R11, in which all seed treatments approached 100% symptomatic by the end of the season. PCR testing also supported the interpretation that positive selection resulted in a large proportion of resultant cuttings being free of detectable levels of SLCMV (Table 4).

The phenomenon of obtaining disease-free progenies from cuttings taken from diseased mother plants is termed 'reversion,' [43] and is a documented phenomenon with African CMD strains at reversion levels of up to 70% with high levels of varietal variability [44]. Along with 'recovery' as described by Jennings [45], in which diseased CMD-resistant cultivars become symptomless, reversion is one of the most important factors in determining the potential performance of farm-based strategies for coping with CMD. Our experiment in the third year demonstrated that disease-free planting materials can be obtained from symptomatic plants (Figure 4), suggesting that reversion is also an important factor in the management of SLCMV.

TME3, a landrace from Africa which has been used as a parent in the development of resistant varieties against African Cassava Mosaic Viruses, displayed high levels of symptomatic infection in our experiment, reaching 69% of plants symptomatic by harvest at 270 DAP, a middling value among the other varieties evaluated in the same year. This finding prompted us to check the purity of the planting material used in the current experiments, and DNA finger printing revealed that TME3 was without the CMD2 resistance gene (unpublished data Zhang, Xiaofei and Barrera, Vianey Paola). This is a notable finding as cassava plants propagated through somatic embryogenesis, including TME3, have been reported to permanently lose CMD2-mediated resistance [45], demonstrating the importance of DNA fingerprinting (and database) for identity checking prior to the introduction of resistant varieties from abroad into breeding programs, in addition to field or laboratory confirmation of active resistance.

Our results indicate that varietal recommendations, positive selection, and the use of clean seed can mitigate the impacts of CMD in Southeast Asia. Significant yield losses can be avoided through access to clean planting materials. Planting clean KU50 or HB60 currently remains the best strategy for most farmers to protect their yields at this stage. If the level of disease pressure allows the positive selection of healthy stems from infected fields. it is a viable method for farmers to limit yield impacts for at least several seasons. Asymptomatic stems from infected plants also demonstrated some promise as a seed class of 'last resort' by exploiting reversion, but symptom development in the subsequent season was influenced strongly by variety. This echoes conclusions from several other studies evaluating reversion in whitefly-vectored viral diseases of cassava [44,46,47].

In the absence of resistant varieties, the strategies we outline are promising options farmers can use immediately to reduce the spread and impacts of CMD. In the medium-term, support for intermediate seed system interventions providing virus-free materials will play an important role in maintaining production levels as the disease spreads and intensifies. More research on inoculum levels, the relative importance of planting material vs. insect-vectored spread, and management effectiveness is urgently needed, along with decision-support tools to assist farmers in adopting and switching between strategies. In the longer term, further breeding will be required to introgress sources of resistance into elite breeding lines to maintain productivity and competitiveness of the crop in different market segments.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy12071658/s1, Figure S1: (Season 2019–2020) Fresh root yield (kg/plant) of cassava infected with cassava mosaic disease (CMD) at after 60 (A), 150 (B) and 270 (C) days of planting (DAP) and asymptomatic plants (D) at harvest of popular cassava varieties in Southeast Asia using disease-free stakes (clean), positive selected stakes from diseased fields (positive selection) and stakes selected from symptomatic plants (symptomatic). Bars are standard errors of the mean (n = 3 to 4). Clean TME3 and HB80 were planted as scarcity of clean planting material of SC8 and KM98-1; Figure S2: (Season 2020–2021) Fresh tuber yield (kg/plant) of cassava infected with cassava mosaic disease (CMD) at after 26 (A), 64 (B), 156(C) and 278 (D) days of planting (DAP) and asymptomatic (E) plants at harvest of popular cassava varieties in Southeast Asia using disease-free stakes (clean), positive selected stakes from diseased fields (positive selection) and stakes selected from symptomatic plants (symptomatic). Bars are standard errors of the mean (n = 3 to 4). Clean TME3, positive selected R72 and clean HB80 were planted as scarcity of clean planting material of SC8 and KM98-1; Figure S3: (Season 2018–2019) Fresh root yield (kg plant^{-1}) of six cassava varieties displaying CMD symptoms early, mid, or late (60, 150, or 270 days after planting, respectively) during the 2018–2019 season at site 1 (A) and site 2 (B), without fertilizer application and with fertilizer (80-20-80) application; Figure S4: Starch content (%) cassava varieties in Southeast Asia using disease-free stakes (clean), positive selected stakes from diseased fields (positive selection) and stakes selected from symptomatic plants (symptomatic) of during 2019–2020 season (A) and 2020–2021 season (B). Twelve plants were harvested from each plot. TME3 and Huaybong80 (HB80) were planted as clean planting material due to the scarcity of clean planting material of SC8 and KM98-1, respectively, and Rayong72 (R72) was planted as positive selected plant in place of SC8 during 2020–2021 season. There was no significant difference between positive selected and symptomatic stakes, however, it was significant different from clean plants for 2019–2020 seasons; and there was no significant difference between treatment in 2020–2021 season; Figure S5: PCR detection of SLCMV, experiment 2, season2020–2021 (asymptomaticplants). Top youngest leaves from each plant were collected and analyzed using SLCMV coat protein gene-specific primers, as described in Materials and Methods. HB60, R5, KU50: genotypes evaluated as described in the main text. Ni: not-infected control plant, B: Blank controls, L:1Kb Plus DNA Ladder (Invitrogen), black arrowheads indicated 650 bp; Table S1: Analyses of variance of disease development (i.e., symptoms) after 60, 150 and 270 days after sowing (DAP), area under disease progress curve (AUDPC) and yield of cassava varieties with and without fertilizer at two different sites during 2018–2019 season; Table S2: Analyses of variance of disease development (i.e., symptoms) after 60, 150, and 270 days after sowing (DAP), and area under disease progress curve (AUDPC) during the 2019–2020 and 2020–2021 season; Table S3: Starch content (%) of six varieties grown in two sites with and without fertilizer where plants demonstrated symptoms at different time during growth period (i.e., 60, 150 and 270 days after planting) and did not show any symptoms till the harvest (i.e., asymptomatic)

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Data Availability Statement: The data presented in this study are available on request from the corresponding author.

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