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Acibenzolar-S-methyl induces resistance against cassava mosaic geminiviruses in *Nicotiana benthamiana* and their vector *Bemisia tabaci* in cassava (*Manihot esculenta*)

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ABSTRACT

Cassava mosaic disease (CMD), caused by cassava mosaic geminiviruses (CMGs), is a major constraint to the cassava crop in Africa and southeastern Asia. Here, we investigated the ability of acibenzolar-S-methyl (ASM), a functional analog of salicylic acid (SA), to trigger systemic acquired resistance (SAR) against two CMGs, namely African cassava mosaic virus (ACMV) and East African cassava mosaic Cameroon virus (EACMCV) in *Nicotiana benthamiana*. ASM treatment delayed the time to first viral symptoms appearance, reduced virus infection rate, and attenuated symptoms. Furthermore, ASM caused an enhanced recovery from symptoms of both viruses and inhibited plant death observed in *N. benthamiana* plants infected by EACMCV. This study further showed that ASM induced resistance to the whitefly *Bemisia tabaci* (Gennadius), the vector of CMGs, in cassava. Notably, we observed that ASM treatment affected adult whitefly feeding preference and oviposition in cassava under both choice and no-choice conditions. A significant reduction in whitefly adult, egg, and nymph populations was observed irrespective of ASM treatment. The results of this study show that ASM has the potential to control both CMGs and their whitefly vector which is an important first step toward managing whitefly and cassava viruses.

1. Introduction

Cassava mosaic disease (CMD), caused by cassava mosaic geminiviruses (CMGs) is one of the most important challenges to cassava production in all cassava growing regions of sub-Saharan Africa and the Indian sub-continent (Legg et al., 2014a). These viruses are transmitted by the whitefly *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae). In addition to their ability to transmit CMGs, whiteflies also cause important damages to cassava through plant feeding which causes chlorotic mottling and twisting and curling of colonized leaves, leading to reduced plant growth (Bellotti and Arias, 2001; Legg et al., 2014b).

Current CMD control approaches are based on the use of phytosanitation and/or resistant/tolerant cassava varieties obtained through conventional breeding. Conventional cassava breeding programs are challenged, however, by intense inbreeding depression and heterozygosity, and lack of full understanding of the molecular mechanisms underlying CMD resistance in cassava (Bart and Taylor, 2017). Moreover, resistance/tolerance of some of the improved varieties appears to be declining, likely due to increasingly high disease pressure in many locations, the appearance of new CMGs species and strains, and mixed infections of two or more viruses (Fondong et al., 2000; Harimalala et al., 2015; Chikoti et al., 2019).

High whitefly populations contribute to heightened virus inoculum pressure and increased spread of CMD (Colvin et al., 2004; Njoroge et al., 2017). Unfortunately, the use of insecticides to manage whitefly infestation is ineffective, not environmentally friendly (Horowitz et al.,

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2011), and adds to the cost of cassava production. There is, therefore, a need to develop alternative strategies that rely on plant inducible defenses in an integrated pest management (IPM) system. Induction of systematic acquired resistance (SAR) has been shown to have the potential to induce basal resistance signaling, effector-triggered immunity (ETI), phytohormone pathways and thus is a promising approach to control viral diseases (Palukaitis et al., 2017). Furthermore, there is evidence of an overlap between RNA-silencing pathways and salicylic acid (SA)-mediated defense, which can enhance plant resistance (Xie et al., 2001; Alamillo et al., 2006). SAR mechanisms are induced by SA, accompanied by the expression of pathogenesis-related (*PR*) genes and hypersensitive response (HR) (Faoro and Gozzo, 2015). Mechanistically, application of SA or acibenzolar-S-methyl (ASM), a SA functional analog, can activate SAR in some plants (Zhou and Wang, 2018; Tripathi et al., 2019).

Depending on the plant-virus pathosystem, SA can inhibit replication, intercellular trafficking, and/or systemic spread of viruses in plants tissues (Carr et al., 2010). In *Nicotiana benthamiana*, application of ASM was observed to induce the expression of the *PR1* defense genes, as well as reduce infection by iris yellow spot tospovirus (Tripathi and Pappu, 2015), plantago asiatica mosaic virus, potato virus X, and turnip mosaic virus (Matsuo et al., 2019). This observation has been recorded in several other viruses (Momol et al., 2004; Mandal et al., 2008; Takeshita et al., 2013). The broad-spectrum nature of ASM makes it a potent approach to control viruses. In this study, we show that ASM confers resistance to African cassava mosaic virus (ACMV) and East African cassava mosaic Cameroon virus (EACMCV) in *N. benthamiana*, as well as resistance to whitefly in cassava.

2. Materials and methods

2.1. ASM-mediated induction of SAR in N. benthamiana and virus challenge inoculations

N. benthamiana seeds were raised in potting soil in plastic pots. Leaves of ASM-treated plants were sprayed until run-off with a water solution of 0.2 g/L ASM (trademark Bion, Syngenta) plus 0.05% (v/v) Tween-20 using a handheld garden sprayer. Control plants were sprayed with distilled water solution containing 0.05% (v/v) Tween-20. Plants were placed in a growth room with a temperature of 26 °C, 70% relative humidity and under a 16-h photoperiod (200 μE m^{-2} s^{-1}).

Cultures of *Agrobacterium tumefaciens* strain GV3101 harboring disarmed Ti binary plasmids containing tandemly repeated inserts of ACMV or EACMCV DNA-A and DNA-B (Reddy et al., 2012) were inoculated as described by Fondong and Chen (2011). Using this inoculation method, Reddy et al. (2012) and Patil and Fauquet (2015) reported 100% infection rate in *N. benthamiana* plants. Briefly, *N. benthamiana* plants were agroinfiltrated in the abaxial surface of the two oldest leaves with mixtures of equal proportions of appropriate *A. tumefaciens* cultures using a 1-ml syringe without the needle. Plants were either inoculated prior to- or after ASM treatment. For plants inoculated after ASM treatment, inoculations were conducted one-week post-treatment while pre-inoculated plants were treated one-day post-inoculation (dpi). Infiltrated plants were misted with water and placed in a growth chamber. The randomized block design was used with one experimental unit consisting of six plants, with four replications. Symptom development was monitored up to 60 dpi using a symptom severity scale of 0 (no symptoms) to 5 (necrosis/death of the plant) (Fondong et al., 2000; Patil and Fauquet, 2015). Infectivity was determined as the percentage of plants that developed CMD symptoms, 16 dpi for plants inoculated with ACMV, and 21 dpi for plants inoculated with EACMCV. Reversion and death of *N. benthamiana* plants were recorded from 21 dpi until 60 dpi when the experiment was terminated.

2.2. ASM treatment and cassava and whitefly infestation

The cassava varieties TME 693, TMS 95/0211, and TMS 96/0023 were used in this experiment. Cuttings were planted in volcanic soil. In a preliminary assessment, 2 doses (0.2 and 0.4 g/L) of ASM were tested and 0.2 g/L was found to be less phytotoxic and thus used in the experiment. Three-week-old cassava plants were treated by foliar spraying until run-off with a solution containing ASM at 0.05% (v/v) and Tween-20 at 48 h prior to whitefly infestation. Control plants were sprayed with distilled water containing 0.05% (v/v) Tween-20. Plants were placed in insect-proof cages in a greenhouse under natural light at Ekona Regional Research Centre of the Institute of Agricultural Research for Development (IRAD), Cameroon. The greenhouse temperatures during the experiment ranged from 23 °C (night) to 32 °C (day) with 90% average humidity.

In the choice experiment, 1000 unsexed adult whiteflies were released to 10 ASM-treated and 10 untreated control plants of each variety at 5 to 8 expanded leaf stage in an insect-proof cage (190 × 74 × 98 cm) at 48 h after ASM application. After 24 h of oviposition, the number of whitefly adults on each plant was recorded. The number of eggs on the 5 youngest leaves was also recorded using a 10x magnification head lens. Plants were then transferred to separate cages and the number of 4th instar nymphs recorded during the next 20 days. The experiment was repeated 4 times at monthly intervals.

The same procedure was used in the no-choice experiment, except that 100 unsexed whitefly adults were transferred to each plant in individual insect-proof cages and placed randomly in the greenhouse. The numbers of adult flies and eggs were recorded 24 h post infestation and nymphs were counted at 20 days after infestation. The experiment was also repeated 4 times at monthly intervals.

2.3. Polymerase chain reaction (PCR) detection of viral DNA

PCR analysis was used to detect viral DNA. Total DNA was isolated from systemic *N. benthamiana* leaves using the cetyltrimethylammonium bromide (CTAB) extraction method (Doyle and Doyle, 1987). PCR primers used for the amplification of ACMV and EACMCV coat protein genes were described in Fondong et al. (2000). The PCR conditions were 5 min at 94 °C, and then 30 cycles of 1 min at 94 °C, 1 min at 57 °C and 1 min at 72 °C, followed by a final extension period of 5 min at 72 °C. Amplification products were separated on an ethidium bromide stained 1% agarose electrophoresis gel and amplicons were visualized and photographed using a Gel Doc™ XR Imaging System (Bio-Rad).

2.4. Data analysis

Data analysis was conducted using SPSS (version 25 for Windows, SPSS Inc., Chicago, IL, USA). Whitefly adults, eggs, and nymphs counts were analyzed separately using 2 factor ANOVA with blocking on means of 10 plants within each replication. The factors were ASM treatment (2 levels: treated, control) and cassava variety (3 levels: TME 693, TMS 95/0211, TMS 96/0023). To gain a better understanding of the effects of ASM on whitefly life stages (egg, nymph and adult), the percentage reduction for each replicate of each of the stages was analyzed. Means of treatments and treatment combinations, as well as interactions were compared using Tukey's honestly significant difference (HSD) test at a significance level of 5%.

3. Results

3.1. ASM induces resistance to CMGs in N. benthamiana

Acibenzolar-S-methyl (ASM), a functional analog of salicylic acid (SA), was investigated for the ability to induce resistance to two cassava geminiviruses. In the experiment, *N. benthamiana* plants treated with ASM reduced infectivity of ACMV and EACMCV compared with

untreated control plants. For ACMV, untreated plants displayed symptoms of local chlorotic spots and mild leaf curling at 5 dpi while ASM-treated plants displayed symptoms at 8 dpi. The incidence and severity of ACMV symptoms varied between control plants and ASM-treated plants. For example, by 8 dpi, all untreated control plants had developed severe leaf curling and reduced growth while only mild leaf curling symptoms were observed in ASM-treated plants (Table 1, Fig. 1A). All plants displayed symptoms by 16 dpi; however, disease severity was lower in ASM-treated plants compared to control plants (Table 1). Furthermore, whereas treated and untreated plants displayed the recovery phenotype as reported previously (Reddy et al., 2012; Patil and Fauquet, 2015), the recovery was stronger in ASM-treated plants (Fig. 1A). PCR analyses showed the presence of ACMV DNA in all inoculated plants (Fig. 2).

As reported by Reddy et al. (2012), EACMCV symptoms appear later than those of ACMV in *N. benthamiana*. In the present study, EACMCV symptoms were first observed on untreated control plants at 7 dpi, in contrast, symptoms first appeared on a proportion of plants treated with ASM at 12 dpi. Strikingly, infectivity and severity of EACMCV symptoms were lower in treated plants compared with control plants (Table 1, Fig. 1B), especially for plants treated prior to virus inoculation. Specifically, at 21 dpi, 20% infection was observed in plants treated before virus inoculation while plants inoculated prior to ASM treatment exhibited 85% infectivity (Table 1). As expected, all control plants displayed severe EACMCV symptoms (Table 1, Fig. 1B) and by 50 dpi, these plants displayed yellowish symptoms resulting in systemic necrosis and plant death (Table 1, Fig. 1B). All ASM-treated *N. benthamiana* displayed a recovery phenotype which could be grouped into two categories: moderate recovery and total recovery (Table 1; Fig. 1B). PCR analyses showed the presence of EACMCV DNA in all control plants, as well as in all treated plants, including those that exhibited a recovery phenotype (Fig. 2).

3.2. ASM treatment induces resistance to B. tabaci in cassava

3.2.1. Choice assays

In the choice experiment, ASM treatment affected the number of whitefly adults, eggs and nymphs, independent of variety (Table 2, Fig. 3). Main effects analysis showed that mean numbers of whitefly adults, eggs and nymphs found on ASM-treated plants were significantly lower than those found on the control plants. There was no significant difference in whitefly colonization on cassava varieties. However, a significant effect of variety was found on egg and nymph densities. TMS 95/0211 showed a significantly lower mean numbers of eggs (P = 0.005) and nymphs (P = 0.035) than TME 693.

3.2.2. No choice assays

In the no choice assay, there was a statistically significant interaction between the effects of ASM treatment and cassava variety on whitefly abundance and nymph density (Table 3, Fig. 4). Mean numbers of whitefly adults, eggs and nymphs found on ASM-treated plants were significantly lower than those found on the control plants. However, ASM treatment affected the number of eggs independent of variety. Statistically significant effects of both ASM treatment and variety were

found on egg density. Therefore, mean number of eggs recorded on TMS 96/0023 was significantly higher than the one on TME 693 (P = 0.026) and TMS 95/0211 (P < 0.001). Simple main effects analysis showed that TME 693 exhibited a significantly higher mean number of whitefly adults than TMS 95/0211 and TMS 96/0023 in both control (P < 0.001) and ASM-treated plants (P = 0.001) (Fig. 4). In control plants, TMS 95/0211 exhibited significantly lower mean number of nymphs than TME 693 (P = 0.004) and TMS 96/0023 (P < 0.001). Also, TMS 95/0211 showed a significantly lower mean number of nymphs than TME 693 (P = 0.008) and TMS 96/0023 (P = 0.001) in ASM-treated plants.

3.3. Percentage reduction

ASM treatment affected the reduction of all life stages independent of variety in both choice and no-choice conditions (Tables 4 and 5). Similarly, there was no significant difference in the percentage reduction between the different life stages in both choice and no-choice conditions. No significant effect was also observed on variety in no-choice assays. In contrast, there was a significant varietal effect in choice assays, thus, TMS 96/0023 showed a significantly higher percentage reduction than TME 693 (P = 0.007) and TMS 95/0211 (P = 0.009).

4. Discussion

4.1. Acibenzolar-S-methyl (ASM) induction of a resistance to ACMV and EACMCV

In this study, ASM treatment was shown to delay CMD symptoms, reduce CMD incidence, and attenuate systemic symptoms in *N. benthamiana* plants. This may presumably be explained by the ability of this functional analog of SA to induce host immune response system. The most commonly tested resistance marker genes associated with the SA pathway belong to the family of pathogenesis-related protein (PR) genes, the phenylalanine ammonia-lyase gene (PAL) and the NON-EXPRESSOR OF PATHOGENESIS RELATED GENES 1 (NPR1). In previous studies, foliar ASM application in *N. benthamiana* was observed to induce expression of genes of the acidic PR proteins (NbPR-1a, NbPR-3Q and acidic NbPR-5) as well as NPR1, a bone fide SA receptor, which plays a central role in SAR (Cortes-Barco et al., 2010; Manohar et al., 2015; Tripathi and Pappu, 2015; Matsuo et al., 2019). Thus, although the mechanism by which ASM induces resistance against viruses is yet to be fully elucidated (Matsuo et al., 2019; Murphy et al., 2020), it is likely that this is due to its interaction with members of the NPR1-like protein family in the SA signaling pathway, which is crucial to immune responses against pathogens (Howe and Jander, 2008).

This study shows that ASM caused a recovery from both viruses in *N. benthamiana* and if confirmed in cassava, would provide another tool in the fight against these viruses. The study confirms that to obtain the best results, ASM treatment must be applied prior to pathogen or pest attack. As noted in this report, both pretreatment and posttreatment of ASM strategies induced resistance to CMGs. Thus, ASM-induced host resistance could be effective in reducing CMD infectivity through the treatment of cassava cutting propagules, as well as in infected planting

Table 1
Effect of ASM on ACMV and EACMCV infectivity, reversion and death in *N. benthamiana* plants.

| Treatment with ASM	Symptom severity		Infectivity (%)		Phenotype		Lethal symptom	
	ACMV	EACMCV	ACMV	EACMCV	ACMV	EACMCV	ACMV	EACMCV
Untreated	Severe	Severe	100	100	R	NR	None	Yes
Pre-treatment	Moderate	None or mild	100	20	R	MR; R	None	None
Post-treatment	Moderate	None or mild	100	85	R	MR; R	None	None

Symptom severity and infectivity on *N. benthamiana* plants were recorded at 16 dpi for ACMV and 21 dpi for EACMCV while reversion and death were recorded from 21 dpi until 60 dpi.
R = Recovery, NR = no recovery, MR = moderate recovery.
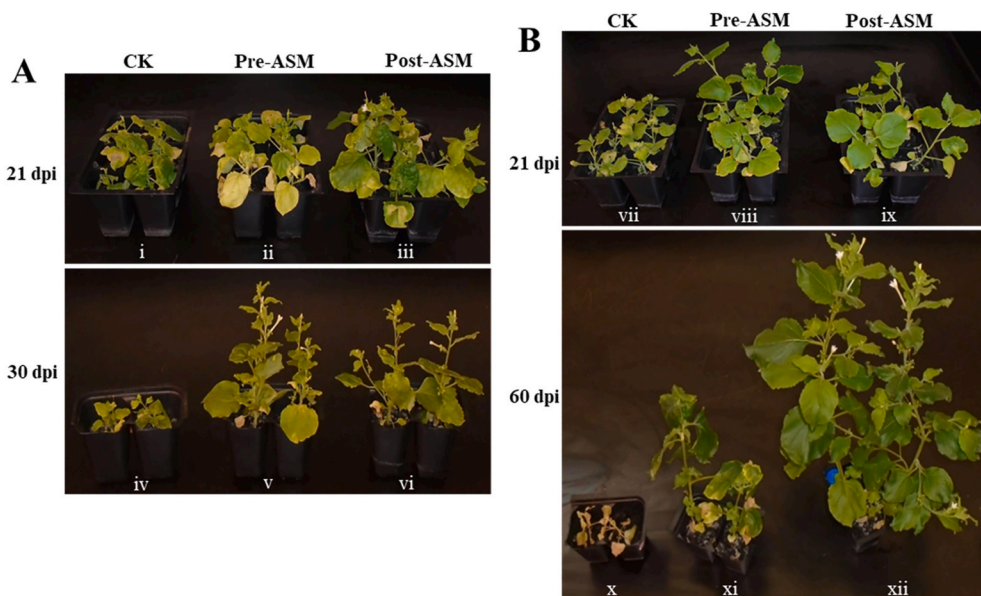
Fig. 1. Effect of ASM on infectivity of ACMV (A) and EACMCV (B). At 21 days post inoculation (dpi), untreated *N. benthamiana* plants displayed severe ACMV symptoms (i), while plants treated with ASM after inoculation (ii) and those inoculated after treatment (iii) displayed milder symptoms. At 30 dpi, the recovery displayed by untreated control plants (iv) was less extensive than that observed in plants inoculated before ASM treatment (v) or plants inoculated after treatment (vi). Correspondingly, at 21 dpi, untreated plants inoculated with EACMCV displayed severe symptoms (vii) compared with plants that were inoculated before ASM treatment (viii) or after treatment (ix). By 60 dpi, untreated plants all died (x) while treated plants displayed either a moderate recovery phenotype (xi) or were fully recovered (xii).
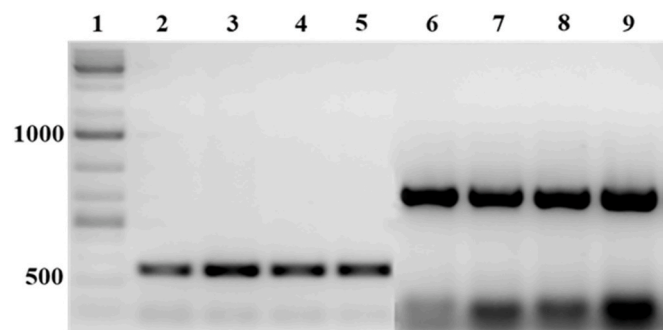
Fig. 2. Agarose gel electrophoresis for detection of CMGs in *N. benthamiana* leaves at 50 dpi. Lanes: 1, size ladder (bp); 2, EACMCV positive control; 3, untreated plant inoculated with EACMCV; 4, ASM-treated plants after EACMCV inoculation; 5, ASM-treated plants before EACMCV inoculation; 6, ACMV positive control; 7, untreated plants inoculated with ACMV; 8, ASM-treated plants after ACMV inoculation; 9, ASM-treated plant before ACMV inoculation.

materials.

4.2. ASM induction of whitefly colonization of cassava

Although CMD is spread mainly through infected cutting propagules, a substantial proportion is transmitted by its whitefly vector. Naturally, cassava grows in the field under conditions that tolerate the presence of whiteflies. It appears that even under high whitefly pressure conditions, treatment with ASM may limit whitefly preference and oviposition as shown in the lower number of whitefly adults, eggs and nymphs on

plants treated with ASM under choice and no-choice conditions of this study. These results agree with those observed by others in cotton (Inbar et al., 2001), tomato (Nombela et al., 2005) and cucumber (Correa et al., 2005), but contrast with results recorded for the silver leaf whitefly (*B. argentifolii*) in tomato. The later study explained the lack of treatment effect on the whitefly by the low density of individuals despite the increasing trend toward the end of the growing cycle which did not allow the detection of any significant difference between treatments and control (Inbar et al., 1998).

The ability of ASM to limit whitefly colonization of cassava as observed here may involve several mechanisms. ASM and pathogens stimulate the SA pathway. *B. tabaci*, which is phloem-feeding is perceived as pathogens and therefore can induce SAR defense (Zarate et al., 2007). Since ASM-induced plant defense was not initially thought to be effective against herbivorous insects (Thaler et al., 1999), our results could be explained by the possibility of a crosstalk or both pathways (Hunter, 2000) in the induction of host response to whitefly infestation.

Together, our results show that ASM has the potential to be employed in the management of CMD, and possibly cassava brown streak disease (CBSD), which is also transmitted by the whitefly coupled with the critical need to develop and deploy varieties with dual resistance to CMD and CBSD (Mukiibi et al., 2019). Additional studies would determine the mechanisms involved in these responses to CMGs and *B. tabaci*. Additionally, because experiments were conducted in greenhouse conditions using the laboratory host *N. benthamiana*, future studies will need to determine whether results obtained here occur in cassava and under field conditions.

Table 2
Linear mixed-effects models to test the effect of ASM treatment and variety on whitefly adult colonization and the number of eggs and nymphs recorded on cassava plants under choice conditions.

Sources of variation	Adults			Eggs			Nymphs		
	Df[a]	*F*-value	P-value	Df[a]	*F*-value	P-value	Df[a]	*F*-value	P-value
Intercept	1	138.4	<0.001	1	1024.1	<0.001	1	545.2	<0.001
ASM	1	12.1	0.003	1	19.3	<0.001	1	14.7	0.001
Variety	2	0.5	0.592	2	6.7	0.006	2	3.8	0.039
ASM x Variety	2	0.7	0.471	2	1.6	0.221	2	1.8	0.189

[a] Error df = 18.

Fig. 3. Mean number of whiteflies adults (A), eggs (B) and nymphs (C) (±standard error) recorded on control and ASM-treated cassava plants under choice conditions.
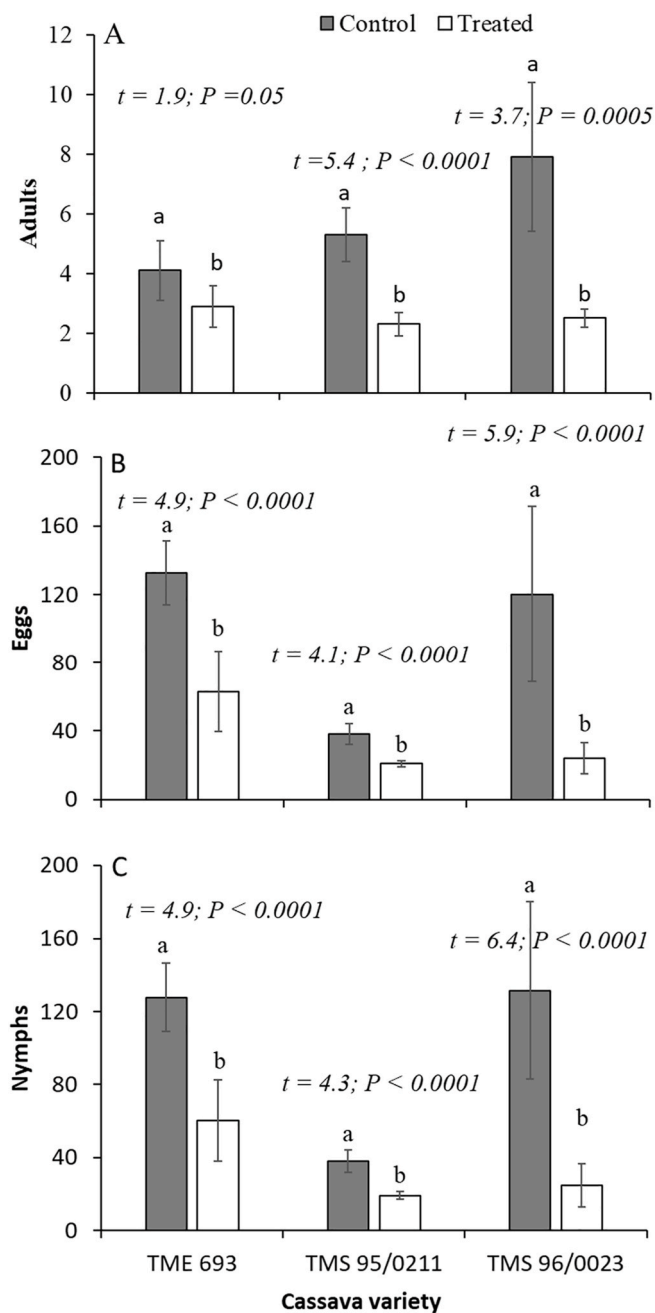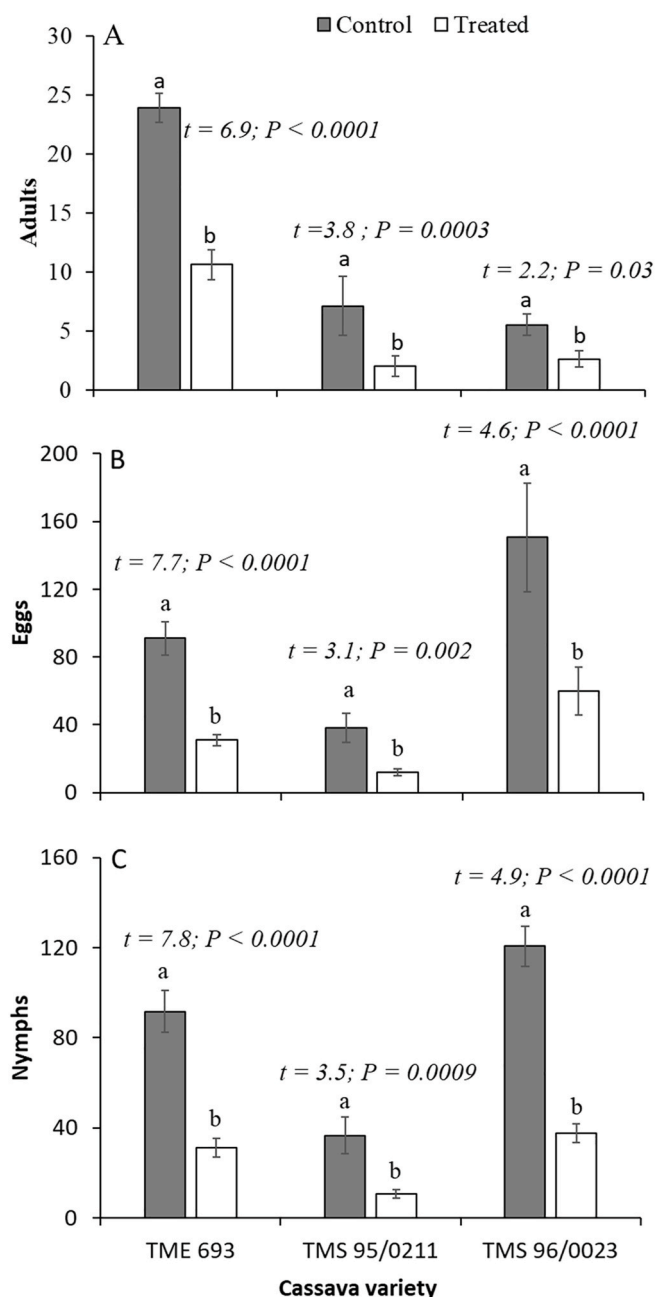

Fig. 4. Mean number of whiteflies adults (A), eggs (B) and nymphs (C) (±standard error) recorded on control and ASM-treated cassava plants under no-choice conditions.

Table 3

Linear mixed-effects models to test the effect of ASM treatment and variety on whitefly adult colonization and the number of eggs and nymphs on cassava plants under no-choice conditions.

Sources of variation	Adults			Eggs			Nymphs		
	Df[a]	F-value	P-value	Df[a]	F-value	P-value	Df[a]	F-value	P-value
Intercept	1	220.8	<0.001	1	102.7	<0.001	1	393.0	<0.001
ASM	1	37.0	<0.001	1	21.9	<0.001	1	104.2	<0.001
Variety	2	55.5	<0.001	2	13.5	<0.001	2	35.1	<0.001
ASM x Variety	2	7.4	0.004	2	2.2	0.139	2	9.0	0.002

[a] Error df = 18.

Table 4

Linear mixed-effects models to test the effect of life stage and variety and the percentage reduction (%) in cassava plants under choice and no-choice conditions.

Sources of variation	Percentage reduction (choice)			Percentage reduction (no-choice)		
	Df	F-value	P-value	Df	F-value	P-value
Intercept	1	274.8	<0.001	1	252.2	<0.001
Life stage	2	2.0	0.147	2	0.1	0.881
Variety	2	7.1	0.003	2	0.02	0.980
Life stage x Variety	4	1.3	0.294	4	0.5	0.701
Error	27			27		

Table 5

Percentage reduction* (mean ± SE) of whitefly adults, eggs and nymphs on cassava varieties under choice and no-choice conditions.

Variety	Percentage reduction (choice)			Percentage reduction (no-choice)		
	Adults	Eggs	Nymphs	Adults	Eggs	Nymphs
TME 693	27.8 ± 13.0a	55.5 ± 11.9 ab	55.9 ± 11.6 ab	55.6 ± 5.1a	64.6 ± 6.1a	64.9 ± 6.1a
TMS 95/0211	53.2 ± 13.3a	42.3 ± 8.4b	46.8 ± 7.3b	71.7 ± 7.1a	55.3 ± 20.9a	58.1 ± 20.3a
TMS 96/0023	58.4 ± 11.1a	79.0 ± 4.6a	84.7 ± 5.4a	53.6 ± 11.4a	58.0 ± 7.7a	68.5 ± 3.0a

* Percentage reduction within columns (comparisons between varieties) followed by the same letter are not significantly different by Tukey's HSD test (P > 0.05). Percent differences in reduction within a variety were not significantly different by Tukey's HSD test (P > 0.05).

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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