



## Resistance Induction by Salicylic Acid Formulation in Cassava Plant against *Fusarium solani*

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**Fusarium root rot caused by the soil-borne fungus *Fusarium solani* is one of the most important fungal diseases of cassava in Thailand, resulting in high yield losses of more than 80%. This study aimed to investigate if the exogenous application of salicylic acid formulations (Zacha) can induce resistance in cassava against *Fusarium* root rot and observe the biochemical changes in induced cassava leaf tissues through synchrotron radiation based on Fourier-transform infrared (SR-FTIR) microspectroscopy. We demonstrated that the application of Zacha11 prototype formulations could induce resistance against *Fusarium* root rot in cassava. The *in vitro* experimental results showed that Zacha11 prototype formulations inhibited the growth of *F. solani* at approximately 34.83%. Furthermore, a significant reduction in the disease severity of *Fusarium* root rot disease at 60 days after challenge inoculation was observed in cassava plants treated with Zacha11 at**

**a concentration of 500 ppm (9.0%). Population densities of *F. solani* were determined at 7 days after inoculation. Treatment of the Zacha11 at a concentration of 500 ppm resulted in reduced populations compared with the distilled water control and differences among treatment means at each assay date. Moreover, the SR-FTIR spectral changes of Zacha11-treated epidermal tissues of leaves had higher integral areas of lipids, lignins, and pectins (1,770-1,700/cm), amide I (1,700-1,600/cm), amide II (1,600-1,500/cm), hemicellulose, lignin (1,300-1,200/cm), and cellulose (1,155/cm). Therefore, alteration in defensive carbohydrates, lipids, and proteins contributed to generate barriers against *Fusarium* invasion in cassava roots, leading to lower the root rot disease severity.**

**Keywords :** cassava, *Fusarium* root rot disease, induced resistance, salicylic acid, SR-FTIR

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Cassava (*Manihot esculenta* Crantz) is considered one of the most important food and economic crops in Thailand. Cassava production annually provides starch and energy source (Buensanteai et al., 2012; Verdier et al., 2004). In the last few years, due to its high starch content, cassava has become an industrial crop because of its application in many industrial processes as a crucial raw material. These increasing demands for cassava products have brought about an expansion of its cultivation in many areas. In 2018, 275 million tonnes of cassava were produced globally, and Africa was the most important cassava-producing

region. Nigeria was the world's biggest producer, at 21.6% of global output, followed by Thailand (10.7%). In Thailand, the production of cassava has been steadily increasing and the planting area of cassava was approximately 1,392,000 hectares, yielding approximately 31 million tonnes/year. The demand for products is growing, leading to increase cultivated areas steadily every year (Jusakulvijit et al., 2021; Sangpueak et al., 2018; Sowcharoensuk, 2020). However, cassava yield is significantly reduced due to attacks by insects and diseases (Buensanteai et al., 2012), threatening the sustainability of cassava production and its profitability. Cassava root rot (CRR) disease is gradually becoming important in the major cassava-producing regions with high yield losses (Charaensatapon et al., 2014; Duchanee, 2015). *Fusarium* sp. is the critical pathogen of CRR disease (Bodah, 2017; de Oliveira et al., 2013). *Fusarium* root rot caused by the soil-borne fungus *Fusarium solani* is one of the most devastating diseases of cassava, as it destroys the plant by limiting the function of the xylem to translocate water and nutrients. In the northern provinces of Thailand, *Fusarium* root rot disease occurred with a severe incidence of approximately 80% (Hohenfeld et al., 2018; Piyachomkwan and Tanticharoen, 2011). Classical practice like the use of fungicides and resistant varieties has largely been ineffective in controlling the disease because of the soil-borne nature of the pathogen and emergence of new strains of the pathogen. Furthermore, due to public concern about residues in food and harmful effects on the environment and human health, there are increasingly more restrictions on the application of fungicides. Hence, a focus on the development of alternate, safe, and practically effective methods to control the disease is required (Saengchan et al., 2022). Induction of resistance to the pathogen is a promising approach for controlling plant diseases. Presently, resistance elicitors have been widely assessed to prevent plant diseases (Buensanteai et al., 2009; Le Thanh et al., 2017; Prakongkha et al., 2013; Zainuddin et al., 2018). Induced resistance is involved in the plant's innate immune system which confers long-lasting resistance to a wide range of plant diseases. The systemic acquired resistance (SAR) requires salicylic acid (SA) as a systemic signaling molecule associated with the accumulation of defense-related enzymes and pathogenesis-related proteins (Métraux, 2001). The induction of systemic resistance against root rot disease was carried in many kinds of plants (Buensanteai et al., 2009, 2012; Métraux, 2001; War et al., 2011). The objective of the present work was to investigate if the exogenous application of SA formulations (Zacha) can induce resistance in cassava against *Fusarium* root rot. and observe the biochemical changes in induced cassava leaf tissues

through SR-FTIR microspectroscopy.

## Materials and Methods

***In vitro* assessment of antifungal activity of Zacha formulations against *Fusarium solani*.** *In vitro* assessment of antifungal activity of Zacha formulations against *F. solani* was carried out in an experiment using completely randomized design (CRD) with four replicates and four treatments including Zacha 11, Mancozeb (80% Mancozeb as the active ingredient), and a distilled water control. The Zacha formulations are products of Bioactive Agro-Industry Company Limited, Nakhon Ratchasima, Thailand. *F. solani* isolate SHRD1 was obtained from the stock culture of Plant Molecular Biology Laboratory, Suranaree University of Technology, Thailand.

The direct effect of Zacha formulations, in comparison with antifungal compound Mancozeb and distilled water control, was tested on the growth of *F. solani* grown on potato dextrose agar (PDA) medium. Five mm diameter PDA discs from actively growing colonies of *F. solani* were placed in the center of the Petri plate containing PDA medium. Sterile Whatman filter paper discs of 6 mm diameter impregnated with 500 ppm Zacha formulations are placed on the surface of the PDA medium near the edge of the Petri plate. Mancozeb at a concentration of 2 mg/ml was used as a positive control. A negative control was performed the same as above by replacing formulation with distilled water. All the Petri dishes were incubated at room temperature for 7 days and an increase in fungal growth was measured in diameter (mm) around the fungal discs. The experiment was performed in duplicates three times.

**Induced resistance against *Fusarium* root rot disease in cassava under greenhouse conditions.** The experiment was conducted in a randomized complete block design in four replicates, with four treatments. The experiment was repeated three times in biologically independent tests with similar outcomes. Cassava stalks were selected by cuttings from the lower or mid-section woody part of the stem of plants at least 10 months old and cut about 15 cm long, from Soeng Sang district of Nakhon Ratchasima province. Then, cassava cuttings (cassava cv. Rayong 72) were disinfected on the surface with 1% NaOCl (2 min), then washed in with distilled water three times and dried for 5 min at room temperature. The cassava cuttings were then soaked for 10 min before planting with the solution of Zacha at a concentration of 500 ppm. After planting, the cassava plants were inoculated with *Fusarium* suspension of  $1 \times 10^6$  spores/ml, such suspension was estimated for

the root rot general symptoms after 60 days and mixing infested soil at the rate of 100 ml per pot and plants (de Oliveira et al., 2013). Subsequently, at 15, 30, and 45 days after planting, cassava plants were sprayed with the solution of each elicitor.

Disease severity scores of *Fusarium* root rot were reported at seven days after inoculation (DAI), and disease score according to the scale modified by Sompong et al. (2013):

- 1 = no symptoms
- 2 = stem, and root area affected by less than 25%
- 3 = stem and root area affected by 25-50%
- 4 = stem and root area affected by 51-75%
- 5 = stem and root area affected by more than 75%

After that calculated percentage of disease severity (DS) from formula using by Le Thanh et al. (2017). The reduction in DS for treated cassava was calculated using the formula modified slightly by Thinh and Kunasakdakul, 2013):

Reduced severity of the disease = [(DS of the control group – DS of elicitor group)/DS of control group] × 100%.

Then, investigated the change in the density of *F. solani* by the elicitor treatments at 7 DAI (modified by Bowers and Locke, 2000, 2004).

**Characterizations of biochemical changes of cassava leaf tissues using Synchrotron-based FTIR microspectroscopy.** The experiment was carried out in CRD, three treatments including the most effective Zacha, water control, and fungicide control with four replications. Cassava leaf samples were collected at 7 DAI, embedded in OCT compound, then rapidly frozen in liquid nitrogen. Next, the leaf samples were moved to a –80°C freezer for the cryo-sectioning process. Then, a cryostat was used with each frozen sample which was transversely cut at a thickness of approximately seven µm and put on a barium fluoride win-

dow (13 × 2 mm) for analysis. Preparation for SR-FTIR microspectroscopy data analysis was recorded at the beam-line of BL4.1 IR Spectroscopy and Imaging at Synchrotron Light Research Institute (Public Organization), Thailand (Le Thanh et al., 2017; Thumanu et al., 2017). The spectra were collected in the mid-IR range of 1,800-900/cm at a resolution of 4/cm. The spectra were corrected for background spectrum, displayed in the absorbance mode, and analyzed using OPUS software (Cooperative Library Network Berlin-Brandenburg) and Unscrambler ×10.1 software (CAMO Software AS, Oslo, Norway). The individual spectrum from each group was analyzed using Principal Component Analysis (PCA) to distinguish different biochemical components of the samples by the Unscrambler X 10.1 software (CAMO). Second derivative spectra were calculated in 9 smoothing points of 3rd polynomial mode by the Savitzky-Golay algorithm. Chemical functional groups of carbohydrates, proteins, and lipids were identified according to previously published reports. The experiment was repeated four times in biologically independent tests with similar outcomes.

**Data analysis.** Similar results were obtained in all repeats of each experiment. Statistical analysis was performed using one-way analysis of variance (one-way ANOVA) with SPSS software version 16 for Window (SPSS Inc., Chicago, IL, USA). The multiple comparisons of means using Duncan multiple range test at ( $P = 0.05$ ) was done to show the significant differences among treatments.

## Results

***In vitro* antifungal activity of Zacha formulations against *F. solani*.** The results showed that the mycelial growth of *F. solani* was significantly affected by Zacha11 prototype formulations which inhibited the mycelial growth of *F. solani* after 3 days of incubation (Table 1)

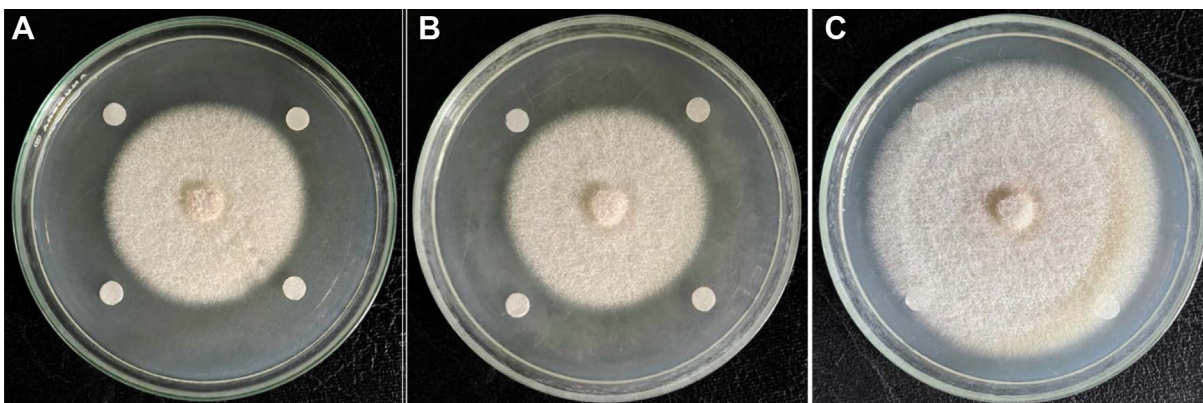
**Table 1.** Antifungal activity of salicylic acid (Zacha formulations) against *Fusarium solani* using a dual culture test

Treatment	Growth inhibition (%) <sup>a</sup>			
	1 DAPFS	3 DAPFS	5 DAPFS	7 DAPFS
Zacha 11 (500 ppm)	3.82 ± 1.10 a	34.83 ± 0.29 a	18.90 ± 0.17 b	11.04 ± 0.13 b
Mancozeb	3.82 ± 1.10 a	34.67 ± 0.29 a	30.90 ± 0.17 a	15.48 ± 0.13 a
Distilled water control	0.00 ± 0.00 b	0.00 ± 0.00 b	0.00 ± 0.00 c	0.00 ± 0.00 c
F-test	**	**	**	**
CV (%)	2.41	0.07	0.06	0.35

DAPFS, days after putting fungal slices; CV, coefficient of variation.

\*\* $P < 0.01$  compared to control.

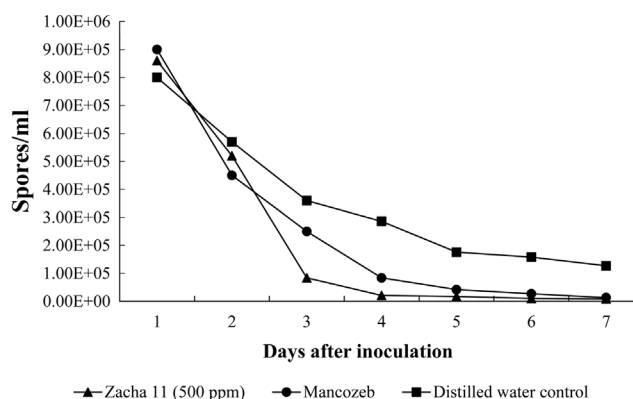
<sup>a</sup>Mean ± standard error followed by the same letter in the column do not differ significantly according to Duncan multiple range test at  $P = 0.05$ .



**Fig. 1.** The inhibition of the mycelial growth of *Fusarium* in Petri dishes with the different treatments at 7 days after putting fungal slices. Sterile Whatman filter paper discs of 6 mm diameter impregnated 500 ppm Zacha11 formulations (A), Mancozeb at a concentration of 2,000  $\mu\text{g/ml}$  was used as a positive control (B) and negative control (C).

with an approximate inhibition percentage of 34.83% was statistically significant to that of water control treatment. Similarly, the fungicide Mancozeb inhibited *Fusarium* growth at approximately 34.67%, significantly higher when compared with the negative control (24.83%). At 5 days after putting fungal slices (DAPFS), the treatments with Zacha 11 could also inhibit mycelial growth compared to the non-treated control at 18.90% (Table 1). Likewise, the percentage of mycelial growth inhibition treated with this elicitor was 11.04% at 7 DAPFS (Fig. 1).

**Efficacy of Zacha formulations in inhibiting *Fusarium* root rot DS and population density under greenhouse conditions.** The greenhouse experiments with the root cassava cuttings soaked in Zacha formulations and foliar spray with a concentration of 500 ppm showed a significant reduction in the severity of *Fusarium* RRD in cassava plants at 7 DAI compared with the negative control, confirming the induction of resistance has occurred. Under greenhouse conditions, Zacha 11 treatment showed the lowest DS (9.0%), followed by Mancozeb treatments when compared



**Fig. 2.** Population densities of *Fusarium solani* infested soil at 7 days after inoculation under greenhouse conditions and with treated Zacha 11, Mancozeb (80% Mancozeb as the active ingredient), and distilled water control.

with distilled water control treatment (13.0% and 37.0%, respectively) (Table 2).

In cassava plants grown on their own roots, *Fusarium* root rot occurred in all treatments. Root rot symp-

**Table 2.** Efficacy of salicylic acid (Zacha formulations) on the severity of *Fusarium* root rot disease caused by *Fusarium solani* under greenhouse conditions at 7 days after inoculation

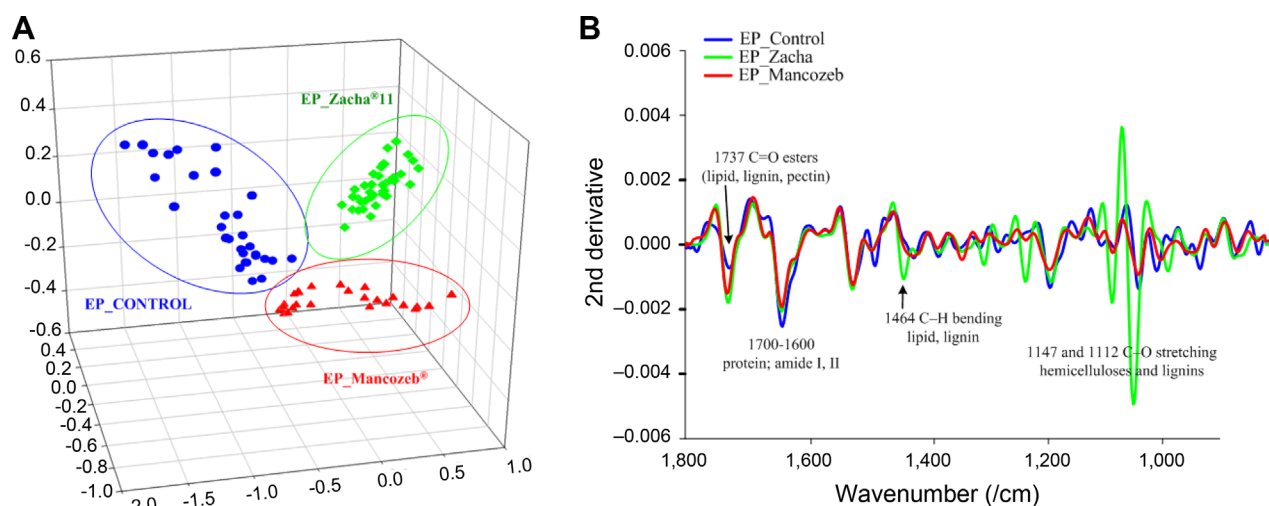
Treatment	Disease severity (%) <sup>a</sup>	Disease severity reduction (%)
Zacha 11 (500 ppm)	9.00 ± 14.75 b	75.68
Mancozeb	13.00 ± 12.55 b	64.86
Distilled water control	37.00 ± 14.83 a	-
F-test	*	
CV (%)	37.80	

CV, coefficient of variation.

\* $P < 0.05$  compared to control.

<sup>a</sup>Mean ± standard error followed by the same letter in the column do not differ significantly according to Duncan multiple range test at  $P = 0.05$ .





**Fig. 3.** Principal component analysis (PCA) and average 2nd derivative spectrum of the epidermis of leaf tissues treated with 500 ppm salicylic acid formulation (Zacha11) and compared with Mancozeb, water, and after challenge inoculation with *Fusarium solani* at 7 DAI. (A) 2D scatter plot of score from PCA analysis. (B) The second derivative in epidermis tissues of cassava leaf.

toms first appeared at 60 to 70 days after transplanting into soils infested with *Fusarium* suspension of  $1 \times 10^6$  spores/ml. Zacha11 at a concentration of 500 ppm significantly reduces the soil populations of *F. solani* (Fig. 2) compared with the distilled water control at 7 DAI (8,450 and  $1.27 \times 10^5$  spores/ml, respectively).

**Biochemical changes in induced cassava leaf tissues using SR-FTIR microspectroscopy.** The SR-FTIR spectra are used for investigating changes in cassava biochemical and cellular compositions after treating with elicitors. Fig. 3A depicts the response of defense mechanisms against root rot disease by using PCA technique to analyze biochemical changes. It was used to statistically evaluate the

relevant spectral data for the Zacha-treated cassava leaves challenged with the *Fusarium* root rot pathogen. The results revealed that a clear difference between the blue points representing negative control with red points of the Mancozeb could be easily distinguished from the green points of the Zacha11. The loading plot was used to identify variables on spectral bands that correlate with the average 2nd derivative spectrum.

The conformational changes of protein amide at the range of wavelength 1,700-1,600/cm indicated detailed information on protein secondary structure like a beta-sheet (peak at 1,635/cm), alpha-helix (peak at 1,653/cm), and beta-turn (peak at 1,685/cm) (Fig. 3B). Results also indicated that the average infrared spectra of epidermis

**Table 3.** Band assignments of FTIR vibration peak (/cm) of plant tissues

Spectral ranges	Peak name	Vibration peak assignments
3,000-2,800	C-H stretching vibration	C-H Asymmetric and symmetric stretching vibration of mainly lipid groups combining to protein
1,740-1,700	C=O esters	Stretching vibration of C=O ester of the bond of lipid, lignin, pectin, or their esters
1,700-1,600	Amide I	Amide I due to C=O stretching of $\alpha$ -helix protein, the contribution from C-N stretching (C=O stretch [80%], C-N stretch [10%], N-H bending [10%])
1,658, 1,607, and 1,571	Amide II	Amide II due to N-H bending and C-N stretching of protein (N-H bend [60%], C-N stretch [40%])
1,470-1,350	C-H bending	C-H bending from CH <sub>2</sub> and CH <sub>3</sub> from mainly lipids and lignin
1,320-1,200	C-O Stretching hemicellulose and lignin	C-C, C-O skeletal
1,147 and 1,112	C-O-C glycoside	C-O-C glycoside ether mainly hemicelluloses

FTIR, Fourier-transform infrared spectromicroscopy.

tissues of cassava treated with Zacha11 showed the biochemical components at the peak of 1,737/cm assigned to C=O ester vibration from pectin. Other vibrational peaks of 1,658, 1,607, and 1,571/cm were assigned to Amide I and Amide II. The cassava plants treated with Mancozeb had differences in the vibrational peaks of 1,464, 1,147, and 1,112/cm, which were assigned to C–H bending and C–O stretching of hemicelluloses and lignins (Table 3).

## Discussion

*F. solani* is the most common root rot pathogen in cassava plants (Bodah, 2017). *Fusarium* is genetically diverse and universally present in the soil, with a frequency of isolation at approximately 45% of root rot-infected cassava fields (Aigbe and Remison, 2010). The application of exogenous SA (Zacha) prototype formulations through cassava cuttings and foliage spray could induce resistance in cassava plants against *Fusarium* RRD. Faoro et al. (2008) has reported that the extent of disease resistance is dependent upon the concentration of formulations using different pathosystems (Faoro et al., 2008). A high induction efficiency was achieved when a 500-ppm concentration of Zacha formulations was used, in comparison with an antifungal compound Mancozeb as positive control and water as a negative control. It was observed that Zacha11 formulation could reduce DS (approximately 35-40%), compared with the negative control. The results are in agreement with few other studies on induced resistance against *Fusarium* disease in banana plants where an induced resistance of 53.1% (Fernández-Falcón et al., 2003), in tomato plants at 5.3-85.8% (Amer et al., 2014) are reported. The possible mode of action to inhibit the pathogen might be that the Zacha11 could directly affect the pathogen in conjunction with the induction of systemic resistance against *F. solani*. Moreover, the reduction in population densities of *F. solani* and increased healthy plant stand in the greenhouse indicates that SA formulation has the potential as environmentally sound alternatives and could have important roles in biologically based management strategies for control of *Fusarium* root rot diseases.

The SR-FTIR microspectroscopy has been applied as a tool to characterize changes in the biochemical traits of tissues at a high resolution and sensitivity, and analysis was used to the spectral differences of epidermal tissues characterized. Moreover, SR-FTIR microspectroscopy identified composition and concentration changes of proteins, polysaccharides, pectins, and lipids of cassava leaves which characterized the roles of plant induction of defense by Zacha11 formulation. The obtained results of FTIR spec-

tra interestingly revealed that cassava leaves treated with Zacha11 and challenge inoculated with *F. solani* exposed an alteration of pectins and lignins, essential components of polysaccharides, and of amide I structure of a protein. There are many results on the importance of their enhanced levels in plant resistance to various diseases caused by phytopathogens. Still, their role in defense responses has been described in recent publications (Le Thanh et al., 2017; Sangpueak et al., 2021; Wang et al., 2015). The lignin and pectin alteration plays a crucial role in disease resistance to the cassava cell-wall reinforcement in cassava leaf tissues (Heil and Bostock, 2002). Also, the conversion of  $\alpha$ -helix structure of amide I protein into the type of  $\beta$ -sheet structure leads to expose the high-affinity binding site for receptors on cell membranes to signal transduction systemically (van't Slot et al., 2003). Besides, increased levels of carbohydrates and proteins were recorded and showed the relationship between elicitors and host plants, including tomato and safflower against the infection and invasion of *Fusarium* root rot (Amer et al., 2014). The results of this research are in line with the previous studies of Thumanu et al. (2017). The authors showed that biotic elicitor induced resistance against anthracnose disease caused by *Colletotrichum acutatum* in chili by inducing the biochemical components changes in the plant tissue that related to defense compounds involved in plant defense mechanisms.

In conclusion, SA content was associated with the expression of SAR genes, correlated with the resistance level to DS of root rot, and can determine the selective activation of defense responses during pathogen infection and invasion that can induce physiological, and biochemical changes through molecular expression in cassava plant. Elicitor formulations (Zacha) could cause the structural changes of the cassava epidermal cells. In a further study, leaf samples will be collected at different harvesting times to understand the interactions of plant disease compared with spectral differences that may appear at the same time as growth, development, or incidence of disease in cassava.

## Conflicts of Interest

No potential conflict of interest relevant to this article was reported.

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