Received: 17 October 2025 | Revised: 5 February 2026 | Accepted: 25 February 2026

DOI: 10.1002/jsf2.70049

RESEARCH ARTICLE

JSFA Reports

Botanical treatment enhances biochemical responses in enset (*Ensete ventricosum* (Welw.) Cheesman) clones

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Abstract

Background: Bacterial wilt of enset (*Ensete ventricosum* (Welw.) Cheesman) is a severe disease caused by *Xanthomonas campestris* pv. *musacearum*, resulting in complete crop failure and considerable damage. This study assessed the biochemical responses of two enset clones (one resistant and one susceptible) when inoculated with the pathogen and treated with a crude leaf extract of *Agarista salicifolia*. Enset leaf samples were collected at various days post inoculation (DPI) from plants grown in the field, and the analyses included photosynthetic pigments, total nitrogen and protein, total soluble sugar, and total phenolics.

Results: Notable biochemical differences between treatments and clones diminished over time. At 120 DPI, the susceptible clone experienced a 31.17% decrease in chlorophyll a and over 35% losses in nitrogen and protein levels. In contrast, the resistant clone only showed minor declines, with less than 4% loss in nitrogen and protein content. At 270 DPI, the susceptible clone exhibited reductions in photosynthetic pigments of 20%–23%. The treated groups maintained higher levels of soluble sugars compared to the control throughout the sampling periods, but experienced a sharp decline after 5 DPI. Total phenolic content increased significantly at the first DPI, especially in the resistant clone, reaching levels twice that of the susceptible clone by the second and third DPI, peaking at 120 DPI.

Conclusions: The results offer critical insights into enset's defense mechanisms and stress responses. Further research to identify marker genes associated with resistance is crucial for improving disease management and breeding strategies.

KEYWORDS

Agarista salicifolia, bacterial wilt, enset clone, induction, pigments, total phenolic content and total soluble sugar

INTRODUCTION

Throughout their growth and development, plants encounter a diverse array of microorganisms, both harmful and beneficial.[1] However, plants are resistant to most microorganisms using preformed and induced defense mechanisms.[2] Preformed physical and chemical barriers include cuticles, cell walls and constitutively produced antimicrobial compounds that protect the plant against most attempted invasions. Nevertheless, when a virulent pathogen breaches these barriers and is recognized by the plant, it triggers a series of biochemical responses to prevent the pathogen from developing and causing infection.[3] Both biotic and abiotic stress factors induce significant physiological and biochemical changes in plants, including alterations in growth, enzyme activities, and the accumulation of osmoprotectants such as proline, which play a crucial role in stress tolerance and adaptation mechanisms.[4] *Xanthomonas campestris* pv. *musacearum* is a phytopathogenic bacterium that causes a destructive disease in enset (*Ensete ventricosum*) and banana.[5,6] Enset also known as false banana,

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is a multipurpose root crop that has been cultivated for thousands of years only in Ethiopia. It serves as a staple/co-staple food supporting the livelihoods of approximately 20 million people in the south and southwestern parts of the country.[7] The disease causes up to 100% yield loss,[8] which severely affects food security and livelihoods in enset-based farming households.[5,6]

Infection alters plant primary metabolism due to the onset of cost intensive defense responses that require a large amount of assimilates in the plant, and the pathogen manipulates the plant's carbohydrate metabolism for its own needs. The pathogen withdraws nutrients from the host plant, which further increases the demand for assimilates. This consequently leads to the development of chlorotic and necrotic areas that decrease assimilate production.[2–4,9–11] Similar metabolic and biochemical disturbances have been reported in common bean genotypes infected with bacterial pathogens, where susceptible plants exhibited pronounced reductions in photosynthetic pigments and altered biochemical profiles compared with resistant genotypes.[12] Evidently, a significant decrease in photosynthetic pigments has been reported in eggplant (*Solanum melongena*),[13] and *Citrus aurantifolia*,[14] and *Phaseolus vulgaris*[15] plants infected with different pathogenic fungi, *Xanthomonas citri subsp. citri* and *Colletotrichum lindemuthianum*, respectively. Similarly, inoculation with leaf extract, pathogen, elicitors and other treatments has been shown to cause significant metabolic changes in plants. In this regard,[16] have reported a substantial increase in total phenolic content (TPC) in *Cucurbita pepo* plants treated with *Eucalyptus camaldulensis* leaf extract. Moreover, significant increases in total phenols, total proteins and total sugars have been reported in infected resistant and susceptible cotton varieties,[17] and in wheat leaf rust disease treated with silica and orange oil nanoparticles.[18] These changes in metabolism during plant–pathogen and other interactions are relevant. Because the sugar status affects the plant's defense and general metabolism, sugars are both nutrients and signals for the plant and pathogen, further influencing the pathogen's spread and regulating its gene expression. Thirdly, specific pathogens possess extracellular sucrolytic enzymes that alter hexose and sucrose levels in the host apoplast.[2,12] It may be plausible to assume that the low productivity of infected plants is partly attributed to physiological stress associated with the low photosynthetic rate of chlorotic leaves.[10] Biochemical data from an infected plant are essential for identifying desirable traits, which will ultimately help develop evidence-based and effective disease control strategies. Therefore, the present work examined the biochemical responses of resistant and susceptible enset clones inoculated with a pathogenic bacterium and with *Agarista salicifolia* leaf extract.

MATERIALS AND METHODS

Description of the study area

Department of Plant Biology and Biodiversity Management at Addis Ababa University granted the permission to conduct this research. The field study was conducted at Wondo Genet College of Forestry and Natural Resources, located 263 kilometers from Ethiopia's capital, Addis Ababa. The area lies between 7°06′ N and 7°11′ N latitudes and 38°05′ E to 38°07′ E longitudes, southeast of Shashemene town (Figure 1). The elevation ranges from 1700 to 2100 meters above sea level. The area is characterized by mean annual temperatures ranging from 17°C to 19°C, and an average yearly rainfall of about 1200 mm, distributed bimodally. The primary rainy season is from June to September, while a shorter rainy period occurs from March to April. The soils are well-drained loam to sandy loam, shallow on steep convex slopes and deeper at lower elevations.

Isolation of *Xanthomonas campestris* pv. *musacearum* (Xcm)

Diseased pseudo stems of enset were collected from a homegarden at Wondo Genet College of Forestry and Natural Resources. The samples were cut into small pieces using sterile blades and immediately stored in plastic bags in an icebox as described.[19] In the laboratory, the small pieces were disinfected with 5% sodium hypochlorite for 1 min and then rinsed three times in distilled water to eliminate any remaining disinfectant. The small pieces were placed in a beaker with 5 mL of distilled water and left for 5 min to allow bacterial diffusion. Serial dilutions of the suspension were streaked onto a semi-selective agar medium composed of yeast extract (10 g/L), peptone (10 g/L), sucrose (10 g/L), agar (15 g/L), cephalexin (50 mg/L), and amphotericin (150 mg/L).[20] Cephalexin inhibited non-target bacterial microorganisms, while amphotericin suppressed fungal growth. The Plates were incubated at 28°C for 72 h. Isolated pure bacterial colonies were re-streaked onto fresh media. We repeated this sub-culturing process until we obtained uniform, yellow, mucoid colonies indicative of Xcm. These cultures were stored at 4°C and reactivated at 28°C before use. Identification of Xcm was based on colony morphology and confirmed via pathogenicity tests.

Description of Enset clones

Two enset clones, *Genticha* and *Midasho*, were selected based on prior studies on their susceptibility to Xcm and local farmers' preferences for traits such as yield and product quality (Bulla, Kocho, and Amicho). *Genticha* is known to be resistant, whereas *Midasho* is susceptible.[21] The clones and the Xcm isolate were obtained from the Sidama Zone. Suckers from each clone were collected from a single corm to ensure consistent environmental exposure and minimize variability caused by soil or weather conditions. This zone was chosen due to the high disease incidence and the highly virulent nature of the Xcm strains.[21,22] Resistance refers to a plant's ability to limit pathogen proliferation, though such traits can evolve with the pathogen.[23]

Field preparation and planting conditions

The study site, which covered 1224 m^2, was prepared using a tractor and hand-finely harrowed, as described by.[19] The site was adequately

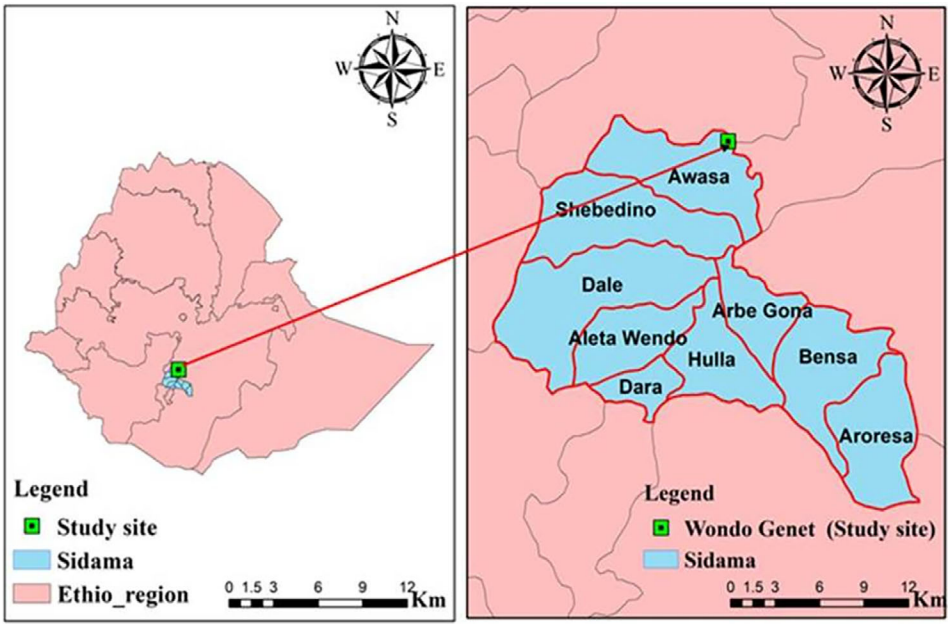

FIGURE 1 Map of the study site.

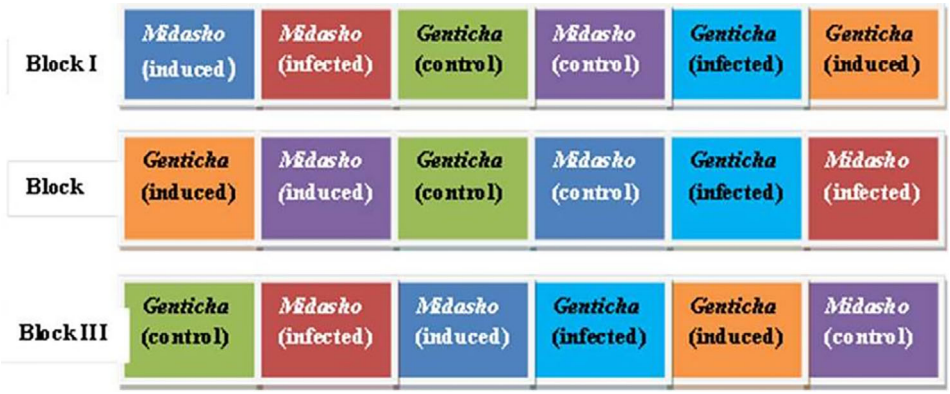

FIGURE 2 Layout of the field experiment showing the random distribution of treatments. *Genticha* and *Midasho* are resistant and susceptible enset clones, respectively.

irrigated before plot layout. The site was divided into three blocks, each containing six plots of 6 m × 6 m (36 m^2) (Figure 2). The blocks and plots were separated by 2 m and 3 m, respectively. Each plot contained nine planting holes, 33 cm deep and 33 cm wide, spaced 2 m far apart. The planting holes were placed 0.5 m away from the edges to reduce edge effects. One year-old and uniform-sized enset suckers (*Genticha* and *Midasho*) were purchased from farmers. The clones were randomly assigned to plots using a completely randomized block design. Two weeks after planting, equal amounts of fresh cattle manure were applied. Standard enset cultivation practices, such as tillage and weeding, were used throughout the experiment.

Agarista salicifolia

The antibacterial activity of several medicinal plants was assessed in vitro, and *Agarista salicifolia* leaf extract showed the greatest potency.[24] The assessed plants were selected based on their antimicrobial activity reports and traditional use against diseases.[25] Fresh leaves were collected from Amaro District, southern Ethiopia, where the species grows at altitudes of 2050–2900 m, especially in Juniperus-Erica scrublands and evergreen forest remnants. Leaves were harvested from a tree at 2131 m elevation.[26] Traditionally, the leaves of *A. salicifolia* are used to treat gastrointestinal disorders such as diarrhea; the bark for skin infections, the root for toothache; and the shoots for bovine Babesiosis.[27,28] The plant was taxonomically identified and a voucher specimen was deposited at the National Herbarium (ETH), Addis Ababa University.

Treatment application

There were three treatments, namely, (1) infected/positive control, where plants were inoculated with 10 mL of Xcm

suspension (1.5×10^8 CFU/mL); (2) induced, plants treated with 10 mL of 12.5 mg/mL *A. salicifolia* leaf extract dissolved in methanol via midrib injection before pathogen inoculation. The first set of plants in this treatment were inoculated with Xcm suspension 30 days after injection, and (3) control/negative control that consisted of plants injected with 10 mL of methanol only. Treatments were applied 1 year after planting. All plants within a plot received the same treatment: Separate 5 mL hypodermic syringes were used for each treatment. Injections were made at the base of the midrib of the youngest expanding leaf. Tools were sterilized and kept separate for each treatment to avoid cross-contamination.

Leaf sample collection

Leaf samples (10 cm × 10 cm) from the enset clones were collected at 4, 9, and 12 months post treatment. The samples were collected from the middle portion of the third leaf (excluding the midrib). Three to five randomly selected plants from each plot were sampled. The discs were stored in liquid nitrogen and transported to Addis Ababa University. The samples were sorted by clone, block, and treatment, then cut into smaller pieces, homogenized per block, and used for biochemical assays. All tests were replicated independently at least twice. Consistent results were observed across replicates.

Chlorophyll and carotenoid content

The leaf samples were dried, ground to a fine powder, and sieved through a 0.6 mm mesh. A 20 mg sample from each treatment was moistened with 0.1 mL distilled water and left for 10 min. Then, 8 mL of 96% ethanol was added to the samples. The mixed samples were incubated overnight in the dark. The next day, the samples were centrifuged at 4000 rpm for 10 min. Absorbance was recorded at 470, 649, and 664 nm using a spectrophotometer (NV202, Sunny). Ethanol was used as the blank. Chlorophyll and carotenoid contents were calculated following the method described by,[29] with slight modifications as follows.

$$C_a = (\underline{13.36A_{664}} \underline{-5.19A_{649}}) * \underline{8.1/\mathrm{DW}} \left[\mathrm{mg\,g^{-1}\,DW}\right]$$

$$C_b = (\underline{27.43A_{649}} \underline{-8.12A_{664}}) * \underline{8.1/\mathrm{DW}} \left[\mathrm{mg\,g^{-1}\,DW}\right]$$

$$C_{a\pm b} = (\underline{5.24A_{664}} \underline{+22.24A_{649}}) * \underline{8.1/\mathrm{DW}} \left[\mathrm{mg\,g^{-1}\,DW}\right]$$

$$C_{x\pm c} = (\underline{4.785A_{470}} \underline{+3.657A_{664}} \underline{-12.76A_{649}}) * \underline{8.1/\mathrm{DW}} \left[\mathrm{mg\,g^{-1}\,DW}\right],$$

where: A649 = Absorbance at 649 nm; A664 = Absorbance at 664 nm; A470 = Absorbance at 470 nm; DW = dry weight of plant tissue extracted (mg), and 8.1 is the volume of the solvent.

Total nitrogen estimation

The total nitrogen content was measured using the Kjeldahl method as described.[30,31] Approximately 0.3 g of powdered sample was digested with 2 g of a catalyst mixture (K_2SO_4, $CuSO_4$, selenium), 7 mL of concentrated H_2SO_4, and 3 mL of 30% H_2O_2. Digestion lasted about 2 h. The digest was distilled with 40% NaOH and collected into a solution containing 2% boric acid and bromocresol green-methyl red indicator. The distillate was titrated with 0.1 N HCl until the green color turned pink. The nitrogen percentage was calculated using the formula below:

Where T = Titration volume of the sample,

$$\mathrm{Total}\,\%\,\mathrm{N} = \underline{(\mathrm{T}-\mathrm{B}) \times \mathrm{N} \times 0.014 \times} \,100,$$

Weight of sample in grams,

B = Titration volume of the blank,
N = Normality of the titrating acid.

Total protein estimation

The total protein was calculated by multiplying the percent nitrogen content by 6.25, a standard conversion factor for plant material.

$$\mathrm{Total\ protein} = \mathrm{percent\ nitrogen}^{*} 6.25.$$

6.25 is a conversion factor for most vegetables.

Total soluble sugar estimation

The total soluble sugars were measured using the phenol–sulfuric acid method.[32] A 0.5 g freeze-dried leaf sample was homogenized with 10 mL of 80% ethanol as described in.[33] The extract was filtered and centrifuged at 2000 rpm for 20 min. To 0.1 mL of the alcoholic extract, 1 mL of 2% phenol was added. Addition of 5 mL of concentrated sulfuric acid followed this. The mixture was gently agitated and incubated at 30°C for 20 min. The 2% phenol concentration was chosen because it yielded the smallest difference in absorbance readings among the three sugars (glucose, fructose and galactose). Moreover, measuring absorbance at 490 nm with 2% phenol in the sugar assay yields the closest match to the absorption coefficients of the three basic monomers without compromising the assay's sensitivity.[34] The absorbance of the solution was measured at 490 nm. A glucose standard curve was used to quantify sugar concentration, expressed as mg/g of fresh leaf weight.

Total phenolic content

TPC was estimated as described by.[19] Briefly, 1 g of each leaf sample was homogenized in 10 mL of 80% methanol. After

filtration and centrifugation at 10,000 rpm for 10 min, 0.2 mL of the supernatant was diluted to 3 mL with distilled water. Then, 0.25 mL of Folin-Ciocalteau reagent and 1 mL of 20% sodium carbonate were added. The mixture was heated in boiling water for 1 min, cooled, and the absorbance was measured at 650 nm. Catechol was used to prepare the standard calibration curve. Results were expressed as mg catechol equivalents per gram of leaf tissue.[35]

RESULTS

The results of the present study demonstrated significant differences in photosynthetic pigments, total nitrogen, total protein, total soluble sugars, and TPC among enset clones and treatments at the various sampling periods. However, the effect of treatments showed a decreasing trend overtime, with the lowest difference recorded 360 days post inoculation (Table 1).

Photosynthetic pigments

All the treated plants showed a significant reduction in chlorophyll a, chlorophyll b, chlorophyll a + b, total nitrogen, and protein at 120 days post inoculation (DPI) except for infected plants of the resistant enset clone (Table 1a). At 120 DPI, control plants of the susceptible enset clone (*Midasho*) had the highest levels of photosynthetic pigments except carotenoids, and total nitrogen and protein contents. Induced and infected plants of the exact clone showed reductions of 18.19% and 31.17% in chlorophyll a content, respectively, compared to the control. On average, the content of chlorophyll b, chlorophyll a + b, total carotenoids, total nitrogen, and protein decreased by 28.11, 26.35, 14, 35.78, and 35.73%, respectively in the treated groups of the susceptible clone (Table 1a). In the resistant enset clone (*Genticha*), the induced group (treated with plant extract) had significantly lower ($p < 0.05$) photosynthetic pigments, total nitrogen, and protein than the control and infected groups. It showed decrease of 13.89, 19.15, 17.44, 3.85, 3.31, and 3.26% in chlorophyll a, b, a + b, total

TABLE 1 Biochemical responses of two enset clones 120 (A), 270 (B) and 360 (C) days post inoculation with methanol, leaf extract, and X cm under field conditions.

A

Clones	Treatments	Chl. a (mg/g)	Chl. b (mg/g)	Chl. a + b (mg/g)	TC (mg/g)	TN (%)	TP
Resistant	Control	7.20 ± 0.00b	14.10 ± 0.00c	8.60 ± 0.00d	2.6 ± 0.0a	8.15 ± 0.09a	50.93 ± 0.54b
	Induced	6.20 ± 0.00c	11.40 ± 0.10d	7.10 ± 0.00b	2.5 ± 0.0a	7.88 ± 0.27a	49.27 ± 1.71b
	Infected	7.50 ± 0.20ab	14.50 ± 0.20c	8.90 ± 0.10ad	2.7 ± 0.1a	8.95 ± 0.70a	55.93 ± 4.37b
Susceptible	Control	7.70 ± 0.10a	15.30 ± 0.20a	9.30 ± 0.10a	2.5 ± 0.1a	12.30 ± 0.76b	76.97 ± 4.87a
	Induced	6.30 ± 0.00c	12.10 ± 0.20b	7.50 ± 0.10b	2.20 ± 0.00b	8.10 ± 0.46a	50.73 ± 2.89b
	Infected	5.30 ± 0.10d	9.90 ± 0.10c	6.20 ± 0.10c	2.10 ± 0.10b	7.70 ± 0.21a	48.22 ± 1.37b

B

Clones	Treatments	Chl. a (mg/g)	Chl. b (mg/g)	Chl. a + b (mg/g)	TC (mg/g)	TN (%)	TP
Resistant	Control	8.11 ± 0.42ab	15.58 ± 0.87ab	9.60 ± 0.52ab	2.98 ± 0.06a	10.40 ± 1.39a	65.01 ± 8.66a
	Induced	7.84 ± 0.18b	15.67 ± 0.56ab	9.51 ± 0.28ab	3.01 ± 0.07a	9.73 ± 0.90a	60.85 ± 5.63a
	Infected	8.07 ± 0.11ab	16.13 ± 0.50a	9.79 ± 0.22ab	3.06 ± 0.04a	8.87 ± 0.22a	55.43 ± 1.35a
Susceptible	Control	9.36 ± 0.43a	17.33 ± 0.84a	10.85 ± 0.51a	3.35 ± 0.14a	10.67 ± 1.86a	66.68 ± 11.60a
	Induced	7.58 ± 0.39b	13.60 ± 0.80b	8.62 ± 0.48b	2.94 ± 0.20a	9.67 ± 0.35a	60.43 ± 2.18a
	Infected	7.42 ± 0.26b	12.95 ± 0.36b	8.31 ± 0.25b	3.05 ± 0.10a	11.07 ± 1.52a	69.18 ± 9.53a

C

Clones	Treatments	Chl. a (mg/g)	Chl. b (mg/g)	Chl. a + b (mg/g)	TC (mg/g)	TN (%)	TP
Resistant	Control	6.71 ± 0.08c	17.45 ± 0.41b	9.60 ± 0.19b	2.73 ± 0.04a	7.67 ± 0.44a	48.12 ± 2.75a
	Induced	5.40 ± 0.12d	12.86 ± 0.14d	7.30 ± 0.07d	2.23 ± 0.09b	7.13 ± 0.52a	44.79 ± 3.23a
	Infected	6.28 ± 0.10c	15.35 ± 0.09d	8.62 ± 0.04c	2.68 ± 0.10a	6.47 ± 0.15a	40.62 ± 0.91a
Susceptible	Control	7.83 ± 0.08ab	17.95 ± 0.08ab	10.33 ± 0.06a	2.60 ± 0.07a	6.53 ± 0.93a	41.04 ± 5.83a
	Induced	7.58 ± 0.13a	16.92 ± 0.17bc	9.85 ± 0.07b	2.48 ± 0.08ab	7.00 ± 0.10a	43.96 ± 0.62a
	Infected	8.05 ± 0.12b	18.59 ± 0.25a	10.67 ± 0.08a	2.63 ± 0.10a	6.30 ± 0.10a	39.58 ± 0.63a

Note: Means ± SE ($n = 5$). Chl. b = chlorophyll b, Chl. a + b = chlorophyll a + b. Mean values within a column that do not share similar letters are significantly different at $p < 0.05$.
Abbreviations: TC, total carotenoids; TN, total nitrogen; TP, total protein.

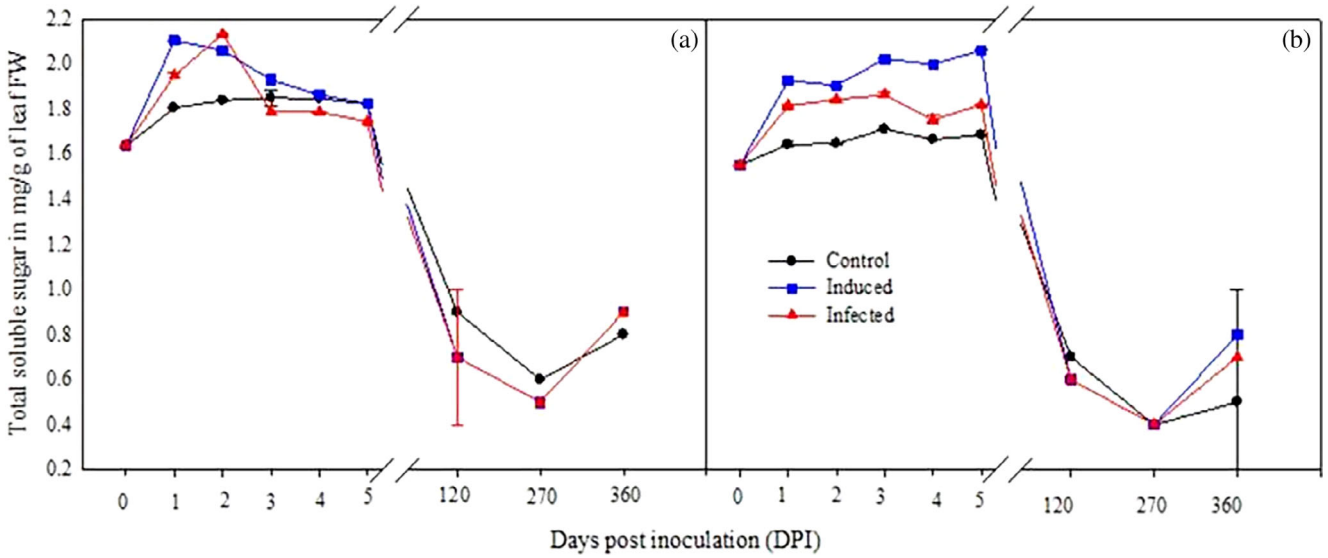

FIGURE 3 Time course changes in total soluble sugar content in leaves of resistant (a) and susceptible (b) enset clones. Values represent means of six replicates. Bars indicate mean ± SE.

carotenoids, total nitrogen and protein, respectively, compared to the control (Table 1a).

The biochemical responses of the two enset clones showed significant ($p < 0.05$) differences among treatments at 270 DPI. In this regard, the content of chlorophyll a, b and a + b was significantly higher in control plants of the susceptible clone as compared to induced and infected plants (Table 1b). The treated groups showed an average reduction of 20.22%, 23.44% and 21.76% compared to control. Nonetheless, the difference between treatments in the resistant clone was nonsignificant. Moreover, no significant differences in total carotenoids were observed between treatments in either clone at 270 DPI. Increased total nitrogen and protein content were recorded in infected control plants of the susceptible clone (Table 1b). On the other hand, chlorophyll a, b, a + b, and total carotenoid contents in infected control plants of the susceptible clone were significantly higher than induced plants at 360 DPI (Table 1c). The measured biochemical responses in the resistant clone's control plants were higher than those infected control plants (Table 1c). However, the differences in total nitrogen and protein content among treatments were nonsignificant.

Total soluble sugar

The analysis of total soluble sugars data revealed a statistically significant difference ($p < 0.05$) between resistant and susceptible enset clones and among treatments (Figure 3). The control plants of the resistant clone exhibited significantly lower ($p < 0.01$) lower total soluble sugar content on the 1st, 2nd, and 3rd DPI. In contrast, the control plants of the susceptible clone showed significantly lower levels at the 1st, 2nd, 3rd, 4th, and 5th DPI, compared to the other treatments (Figure 3a,b). Control plants of the resistant clone had significantly higher total soluble sugar content than that of the susceptible clone at 2, 3, 4, 5, 120, and 270 DPI, respectively (Figure 3a,b). Infected plants of the resistant clone had lower total soluble sugar content than the other treatment groups on 3rd, 4th, 5th, 120th, and 270th DPI (Figure 3a,b). On the other hand, induced plants of the susceptible clone had significantly higher total soluble sugar content than the other treatments at 3rd, 4th and 5th DPI (Figure 3a,b). On average, the treatment groups of the resistant and susceptible clones showed reductions of 23.10% and 14.86% in total soluble sugar content compared to the control groups, respectively, at 120 DPI. In contrast to the susceptible clone, the difference in total soluble sugars between induced and infected plants of the resistant clone was statistically significant at $p < 0.05$ (Figure 3a,b). The total soluble sugar content increased sharply after the 5th DPI. At 270th DPI, a significant difference was recorded between enset clones and treatments (Figure 3a,b).

In contrast to 120 and 270 DPI, the total soluble sugar content in the control plants decreased substantially in both clones at 360 DPI (Figure 3a,b). Induced and infected plants of both clones had significantly ($p < 0.05$) higher total soluble sugar content compared to controls (Figure 3). In general, the resistant clone had higher TSS than the susceptible clone at most sampling periods (Figure 3a,b).

Total phenolic content

The analysis of variance showed that the TPC of enset clones was significantly ($p < 0.05$) higher in the treatment groups than in the controls (Figure 4a,b). However, the difference in TPC between the control groups of both clones was nonsignificant. Statistically significant ($p < 0.05$) differences in TPC were observed across sampling periods within treatments in both clones. In both clones, the highest

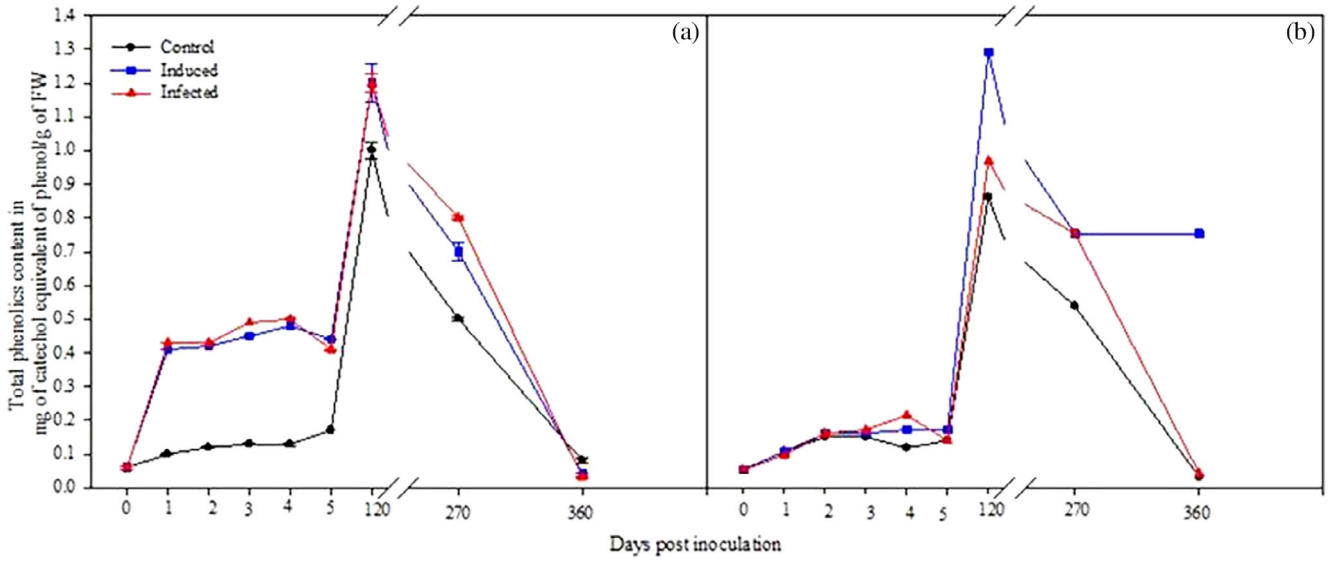

FIGURE 4 Time course changes in total phenolic content of resistant (a) and susceptible (b) enset clones. Values represent means of six replicates. Bars indicate mean ± SE.

TPC was observed at 120th DPI (Figure 4a,b). In terms of treatment effect, higher TPC was observed in infected plants of the resistant clone at the 3rd and 4th DPI, followed by induced plants of the exact clone at the 4th DPI (Figure 4a,b). These values were significantly higher than those of other treatments ($p < 0.05$). Similarly, higher TPC was observed in infected plants of the susceptible clone compared to other treatments within the clone (Figure 4a,b). The TPC was drastically increased at the 1st DPI and then increased slowly in infected and induced plants of the resistant clone (Figure 4a).

On 2nd and 3rd DPI, the TPC in the infected and induced resistant clone was twofold greater than in the susceptible (Figure 4a,b). In the susceptible clone, the difference between treatments was not clear until the 3rd DPI. Despite the decreasing trend in TPC at 120 DPI, induced and infected plants of both clones had significantly higher TPC than their respective controls (Figure 4a,b). The effect of treatments on the TPC of the two enset clones declined at 360 DPI. In this sampling period, the TPC in induced plants of the susceptible clone was significantly greater than that of control and infected control plants. Unlike other sampling periods, induced and infected control plants of the resistant clone had substantially lower TPC than control plants (Figure 4a,b). In most cases, plants of the resistant clone had higher TPC than those of the susceptible one.

According to the two-way analysis of variance (ANOVA), clone, treatment, and their interaction (clone*treatment) had a significant effect on all measured parameters at 120th DPI (Table 2). Similarly, significant differences in chlorophyll a and total chlorophyll content were recorded due to clone, treatment, and their interaction on 270th DPI. The treatments and the interaction effect resulted in substantial variations in chlorophyll b and TPC (Table 2). Likewise, the influence of clone, treatment, and their interaction on chlorophyll a, b, total chlorophylls, total soluble sugars and TPC was statistically significant at ($p < 0.01$) on 360th DPI (Table 2). The effect of the independent variables on total nitrogen percentage and total protein was nonsignificant at the later data collection periods (Table 2). Partial eta squared values indicated that clone, treatment, and their interaction had significant effects on chlorophyll a, total chlorophyll, and total soluble sugars across the consecutive data collection periods. The interaction also had a substantial impact on TPC at 360th DPI (Table 2).

DISCUSSION

The results of the present study demonstrated significant differences in the biochemical responses of resistant and susceptible enset clones across the various treatment groups. The treatment groups caused a substantial decrease in photosynthetic pigments in the susceptible clone, whereas leaf extract induction produced a significant reduction in the resistant clone. This is corroborated by the findings of several studies[14,15] have found significantly lower photosynthetic pigment content in *Citrus aurantifolia* infected with *Xanthomonas citri subsp. citri* and susceptible (Widusa) common bean cultivar infected with *Colletotrichum lindemuthianum*, respectively. Similarly,[13,36] have reported lower photosynthetic pigment content in infected plants of cucumber and eggplant, respectively. According to[9] and,[11] total chlorophyll, chlorophyll a, and b, and total chlorophyll contents significantly decreased in mustard and banana plants infected with turnip mosaic virus (TMV) and banana bunchy top virus (BBTV), respectively. Reduced concentrations of chlorophyll a and b have been found in yellow vein mosaic virus-infected genotypes of *Abelmoschus esculentus* leaves.[37] In tobacco mosaic virus (TMV) infected pepper varieties, reduced chlorophyll content has been observed compared to controls.[38] Infected cotton plants have been found to have lower chlorophyll content than controls.[39] Moreover, 80.28, 100, and 97.30% reduction in chlorophyll a, b, and carotenoid contents, respectively,

TABLE 2 Two-way ANOVA results of biochemical responses of resistant and susceptible enset clones treated with leaf extract and XCM at different sampling periods.

		4 months post treatment application						9 months post treatment application						12 months post treatment application					
Source	Dependent variable	Type III sum of squares	df	Mean square	*F*	Sig.	Partial eta squared	Type III sum of squares	df	Mean square	*F*	Sig.	Partial eta squared	Type III sum of squares	df	Mean square	*F*	Sig.	Partial eta squared
Clone	Chlorophyll a	1.42	1	1.42	244.04	0.00	0.95	4.93	1	4.93	109.00	0.00	0.90	11.23	1	11.23	1258.30	0.00	0.99
	Chlorophyll b	4.30	1	4.30	198.56	0.00	0.94	0.12	1	0.12	3.70	0.08	0.24	31.00	1	31.00	138.68	0.00	0.92
	Total chl.	1.79	1	1.79	285.26	0.00	0.96	1.45	1	1.45	69.42	0.00	0.85	13.29	1	13.28	618.56	0.00	0.98
	Carotenoids	0.49	1	0.49	359.53	0.00	0.97	0.55	1	0.55	13.02	0.00	0.52	0.01	1	0.01	0.37	0.56	0.03
	Total nitrogen	5.01	1	5.01	6.94	0.02	0.37	2.88	1	2.88	0.67	0.43	0.05	1.03	1	1.03	1.50	0.25	0.11
	Total protein	195.82	1	195.82	6.96	0.02	0.37	112.40	1	112.40	0.66	0.43	0.05	40.08	1	40.08	1.49	0.245	0.11
	TSS	0.05	1	0.05	208.78	0.00	0.95	0.05	1	0.05	27.70	0.00	0.70	0.12	1	0.12	246.68	0.00	0.95
	TPC	0.23	1	0.23	24.04	0.00	0.67	0.00	1	0.00	0.40	0.54	0.03	0.00	1	0.00	7.69	0.02	0.39
Treatment	Chlorophyll a	4.86	2	2.43	418.17	0.00	0.99	1.44	2	0.72	15.93	0.00	0.73	2.94	2	1.47	164.82	0.00	0.97
	Chlorophyll b	26.74	2	13.37	617.02	0.00	0.99	2.73	2	1.36	40.89	0.00	0.87	27.67	2	13.84	61.91	0.00	0.91
	Total chl.	8.70	2	4.35	695.09	0.00	0.99	1.35	2	0.67	32.38	0.00	0.84	7.57	2	3.78	176.20	0.00	0.97
	Carotenoids	0.14	2	0.07	51.38	0.00	0.90	0.03	2	0.01	0.31	0.74	0.05	0.45	2	0.22	16.36	0.00	0.73
	Total nitrogen	17.42	2	8.71	12.06	0.00	0.67	2.17	2	1.09	0.25	0.78	0.04	1.96	2	0.98	1.43	0.28	0.19
	Total protein	680.28	2	340.14	12.09	0.00	0.67	84.84	2	42.42	0.25	0.78	0.04	76.70	2	38.35	1.43	0.28	0.19
	TSS	0.10	2	0.05	200.70	0.00	0.97	0.03	2	0.01	7.91	0.01	0.57	0.12	2	0.06	118.14	0.00	0.95
	TPC	0.25	2	0.13	12.97	0.00	0.68	0.12	2	0.06	39.21	0.00	0.87	0.01	2	0.01	25.00	0.00	0.81
Clone * Treatment	Chlorophyll a	5.41	2	2.70	465.62	0.00	0.99	10.02	2	5.01	110.75	0.00	0.95	1.10	2	0.55	61.84	0.00	0.91
	Chlorophyll b	26.73	2	13.36	616.81	0.00	0.99	67.92	2	33.96	1018.81	0.00	0.99	13.27	2	6.64	29.69	0.00	0.83
	Total chl.	9.06	2	4.53	723.52	0.00	0.99	20.58	2	10.29	494.38	0.00	0.99	3.35	2	1.68	78.13	0.00	0.93
	Carotenoids	0.11	2	0.06	39.96	0.00	0.87	0.27	2	0.13	3.20	0.08	0.35	0.18	2	0.09	6.67	0.01	0.55
	Total nitrogen	23.39	2	11.70	16.19	0.00	0.73	4.49	2	2.25	0.52	0.61	0.08	0.97	2	0.48	0.70	0.51	0.11
	Total protein	913.66	2	456.83	16.24	0.00	0.73	175.49	2	87.74	0.52	0.61	0.08	37.77	2	18.87	0.70	0.51	0.11
	TSS	0.01	2	0.01	19.39	0.00	0.76	0.01	2	0.01	2.80	0.10	0.32	0.01	2	0.01	11.74	0.00	0.66
	TPC	0.06	2	0.03	3.28	0.07	0.35	0.02	2	0.01	5.65	0.02	0.49	0.01	2	0.01	55.46	0.00	0.90

have been observed in *Alternaria tenuissima*-infected tomato plants.[40] The contrasting biochemical responses observed between resistant and susceptible enset clones in the present study are consistent with findings in dry bean, where resistant cultivars exhibited lower disease severity, slower disease progression, and greater physiological stability under common bacterial blight infection.[41]

In the present study, the effect of infection on chlorophyll a content was more pronounced than on chlorophyll b, which agrees with the findings of,[42] who reported lower chlorophyll a in Thilarani sesame cultivar infected with *Alternaria sesami*. This might be due to higher degradation of chlorophyll a to b since chlorophyll b is synthesized from chlorophyll a and is catabolized after it is reconverted to chlorophyll a.[43] According to[44] and,[45] decreases in chlorophyll content are caused by increased chlorophyllase activity that participates in shaping the plant defense response by causing a rise in reactive oxygen species that result in programmed cell death.[40,45] The low chlorophyll content in infected plants might also be due to inhibition of chlorophyll synthesis.[45]

On the other hand, induced plants of the resistant clone didn't increase in chlorophyll and carotenoid content through time as compared to control and infected plants. In contrast, induced plants of the susceptible clone showed a progressive increment in photosynthetic pigments content compared to infected plants. In this regard,[16,40,46–48] have reported increased levels of chlorophyll and carotenoid in plants treated with various bioagents and elicitors before infection. Treating *Fusarium oxysporum* inoculated tomato plants with arbuscular mycorrhizal fungi, salicylic acid (SA), or Jasmonic acid (JA) alone or in combination significantly decreases the effect of infection on the content of total chlorophyll.[46] Similarly, foliar application of 100 mM SA before pathogen inoculation restored the metabolism of infected faba bean leaves to levels comparable to those of healthy controls.[49] Comparable improvements in chlorophyll contents are reported in *Alternaria solani*-infected tomato plants treated with SA[48] and Headline (Pyraclostrobin 20% WG) treated groundnut plants grown on *Sclerotium rolfsii*-infested soil.[47] Moreover, inoculations of *Cucurbita pepo* with *Trichoderma viride* + potassium silicate and *E. camaldulensis* leaf extract have increased all photosynthetic pigments.[16] Likewise, an increased chlorophyll a, b, total chlorophyll and carotenoid content has been noticed in wheat plants treated with silica and orange oil nanoparticles.[18] The high chlorophyll content in elicitor and bioagent treated plants could be due to their stimulatory effect on Rubisco activity and absence of toxic products from the pathogen due to post and prior-pathogen inoculation.[40]

Overall, plants of both clones in the treatment group had lower total nitrogen and protein contents, which align with the finding of,[17] who reported lower total protein content in infected resistant and susceptible cotton varieties compared to healthy control plants. In support of this,[13] found a significant reduction in total soluble protein content in eggplants infected with different pathogenic fungi as infection progressed, compared to the control. Correspondingly,[50,51] reported lower total nitrogen content in rice plants infected with *Pyricularia oryzae* and in chickpea infected with rust fungi, respectively. In contrast,[52,53] have reported increased total nitrogen content in infected alfalfa, and turmeric plants, respectively, compared to healthy plants. The increased total nitrogen and protein content might be attributed to increased catabolic reactions, decreased in proteolytic degradation or both.[52] Comparable infected-induced alterations in nitrogen and protein metabolism have also been reported in common bean genotypes, where bacterial pathogens caused pronounced biochemical shifts, particularly in susceptible cultivars, reflecting genotype-dependent metabolic sensitivity to disease pressure.[54]

Similar to the results of the present study, application of inducers such as SA, Agrileen (AG), Headline, and leaf extracts enhanced total protein content.[40,47] In the same way,[18] have found higher protein content in wheat plants treated with silica and orange oil nanoparticles. The disparities in reports might be due to differences in the study plants, the pathogens, and the analytical methods.

The results of this study demonstrated a significantly higher total soluble sugar content in the induced and infected treatment groups of both resistant and susceptible enset clones up to 120 DPI, followed by a sharp decline thereafter. In agreement with this,[15,17,55,56] have reported higher total soluble sugar content under infection both in the susceptible and tolerant clones. Sunflower plants infected with sunflower chlorotic mottle virus showed an increase in total soluble sugars.[55] The same was observed in tobacco plants infected with tobacco mosaic virus.[55] According to,[15] both the susceptible and resistant common bean cultivars showed an increase in total soluble carbohydrate content in the pathogen-infected plants. In addition, higher levels of total sugars have been recorded in resistant and susceptible cotton varieties infected with Alternaria and Corynespora fungi.[17] Similar increases in total soluble sugar content under pathogen pressure have been documented in resistant and susceptible bean genotypes, indicating that sugar accumulation represents a common metabolic adjustment associated with both defense activation and pathogen exploitation of host resources.[4,12] Equally, treating enset clones with leaf extract increased the total soluble sugar content, which aligns with the findings of.[46,57,58] Pretreating faba bean plants with shikimic acid, salicylic acid alone, or in combination could minimize the adverse effects of *Botrytis fabae* by increasing total soluble sugar content, thereby improving osmotic pressure and relative water content in the leaves of infected plants.[58] At the later stages of bacterial wilt development (270 and 360 DPI), the total soluble sugar content decreased and reached a similar level to the control under all treatments. This indicates that the pathogen's adverse effect decreased over time. The elevated content of total soluble sugar might be due to increased invertase expression and microbial sucrolytic activity, which degrade sucrose to hexoses in the cells. These changes consequently activate defense and down regulate photosynthesis by delaying photosynthetic gene expression.[2] Moreover, the accumulation of total soluble sugar in treated plants signifies the relationship between sugar regulation and activation of systemic resistance.[46] It is evident from several research reports that soluble sugars are involved in the responses of plants to abiotic and biotic stresses, and act as nutrient and metabolite signaling molecules that activate specific or hormonal-crosstalk transduction pathways, resulting in essential modifications of gene expression.[59]

The TPC in resistant and susceptible enset clone leaf samples, collected at different time points after inoculation with leaf extract and *Xanthomonas campestris* pv. *musacearum* exhibited notable variation (Figure 4a,b) yet, the TPC of the resistant clone (*Genticha*) was significantly higher than that of the susceptible one (*Midasho*). In line with this finding,[60–62] have reported higher TPC in the resistant than the susceptible pearl millet, potato, rice and cotton varieties, respectively.[63] sesame plants treated with *Azadirachta indica* leaf extract and[62] rice leaves inoculated with *Cnaphalocrocis medinalis* have exhibited significantly high TPC. In the same way, the application of infected wheat with silica and orange oil nanoparticles,[18] zucchini plants treated with *E. camaldulensis* leaf extract + *T. viride* suspension,[16] and eggplants inoculated with pathogenic fungi[13] have produced significantly higher TPC. Inoculation of induced plants with the target pathogen yielded a similar pattern of increased TPC to that observed with the first challenge inoculation. In both cases, the phenolic content was significantly higher in treated plants than in controls. In relation to this,[64] have reported greater accumulation of phenolic compounds in induced and infected *Lupinus albus* seedlings upon pathogen infection compared to healthy plants. Moreover,[65] have reported an increase in the level of phenols in rice leaves treated with *Datura metel* leaf extract and inoculated with *Rhizoctonia solani* or *Xanthomonas oryzae pv. oryzae*. This evidence suggests that TPC plays a key role in the development of disease resistance. Thus, the accumulation of phenolic compounds after challenge inoculation has been correlated with the restriction of pathogen development, as these compounds are toxic to pathogens.[62,66] Also, phenolic compounds may impede pathogen infection by increasing the mechanical strength of the host cell wall.[66]

In this study, both clone and treatment, and their interaction, had a significant impact on nearly all measured parameters across various data collection periods. In support of this,[18] have found significant effect of treatments such as infection, silica, and nanoparticles-stimulated orange oil on chlorophylls, carotenoids and TPC in wheat. Likewise,[67] have reported a marked influence of genotype on total protein content, with resistant rice lines having significantly high concentration than the susceptible ones. This suggests that the variations among the clones, the applied treatments, and their combined effects played a crucial role in shaping the outcomes observed throughout the study. The influence of these factors was evident in the data collected at different periods, indicating that the interactions between clone types and treatments were important in determining the overall results.

CONCLUSION

The results revealed significant differences in biochemical responses between the resistant (*Genticha*) and susceptible (*Midasho*) clones. At 120 DPI, treatment groups exhibited a notable decrease in chlorophyll a, chlorophyll b, total chlorophyll (a + b), total nitrogen, and protein levels, except for the infected plants of the resistant clone, which showed no significant reduction compared to controls. In contrast, the treatment groups had higher total soluble sugars and phenolic contents than the controls. Notably, the resistant clone (*Genticha*) exhibited significantly higher TPC than the susceptible clone (*Midasho*) when treated with the pathogen and leaf extract. This suggests that leaf extract treatment activates the plant's defense system and may enhance resistance in both clones. Further research on identifying marker genes for resistance is recommended.

ACKNOWLEDGMENTS

The authors are grateful to Addis Ababa University for the financial support.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

All datasets on which conclusions of the study have been drawn are presented in the main manuscript.

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How to cite this article: Yemata G, Fetene M. Botanical treatment enhances biochemical responses in enset (*Ensete ventricosum* (Welw.) Cheesman) clones. JSFA Reports. 2026; 6(6):236–47. https://doi.org/10.1002/jsf2.70049