Article

Altered Cytostructure and Lignolytic Enzymes of *Ganoderma boninense* in Response to Phenolic Compounds

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Abstract: *Ganoderma boninense* is a white-rot fungus that causes basal stem rot (BSR) disease in the oil palm. Potential natural inhibitors, such as gallic acid, thymol, propolis, and carvacrol, were assessed for their antagonistic effects against *G. boninense*. These naturally occurring phenolic compounds have also been utilised to inhibit hydrolytic and ligninolytic enzymes produced by the pathogen. Mycelial inhibition was dose-dependent in the presence of different concentrations of phenolic compounds, including, for example, in cellulase enzyme inhibition (GA mg/mL = 94%, THY 0.25 mg/mL = 90%, PRO 3.5 mg/mL = 92.5%, and CARV 0.15 mg/mL = 90.3%). A significant difference was observed revealing that gallic acid had the greatest inhibitory effect on the secretion of hydrolytic and ligninolytic enzymes, especially at 40 mM GA (cellulase = 0.337 U/mL, amylase = 0.3314 U/mL, xylanase = 0.211 U/mL, laccase = 0.4885 U/mL, lignin peroxidase = 0.218 U/mL, and manganese peroxidase = 0.386 U/mL). The growth and secretion of enzymes (inhibitory action) are inversely proportional to the concentration of phenolic compounds. Phenolic compounds have a greater potential as inhibitory agents and suppress the production of hydrolytic and ligninolytic enzymes. The selected phenolic compounds were evaluated for their ability to alter the morphology and integrity of *G. boninense* mycelia. The reduction in cell viability of *G. boninense* has been explained by research on morphological disruption, such as branching patterns, hyphal length, and rigidity of fungal cells, which eventually interrupt the secretion of enzymes. These studies highlight the efficacy of phenolic compounds in treating *Ganoderma*. In addition, these findings proved that naturally occurring phenolic compounds could be a substitute for chemical controls and other synthetic fungicides to eradicate the occurrence of BSR in oil palms, thus avoiding a situation that is difficult to overcome.

Keywords: basal stem rot disease; *Ganoderma boninense*; gallic acid; hydrolytic enzymes; lignolytic enzymes; phenolic compounds; degradation of dye; oil palm

1. Introduction

The demand for oil palm has grown annually in the global market. As a remedy, the annual establishment and expansion of oil palm plantation areas has been carried out. However, Malaysian biodiversity is in jeopardy because of habitat loss caused by deforestation and agricultural land expansion. Despite the expanded cultivation area, palm oil output is impeded by pests and diseases, such as upper stem rot and basal stem rot [1]. The major disease that causes OP to collapse is BSR disease, which is caused by the white-rot fungus *Ganoderma boninense*. BSR spreads when healthy oil palm roots come into contact with infected roots because of the interaction between the pathogen and roots and the release of spores [2]. Pathogenesis requires the pathogen to break down polysaccharides concurrently or selectively during conversion [3,4].

The pathophysiology of the disease involves the activity of the pathogen's ligninolytic and hydrolytic enzymes, which break down cellulose and hemicellulose components in the cell walls of the oil palm, leading to the simultaneous degradation and penetration of

cellulose. Selective degradation also breaks down lignin and hemicellulose, resulting in the production of wood that is rich in cellulose and consumed by hydrolytic enzymes, such as cellulase, amylase, and xylanase [5–7]. These enzymes convert cellulose into simple sugars and hydrolyse hemicellulose, leading to damage to xylem tissue, which restricts nutrient and water intake. This stress affects oil palm from its roots to its branches. Furthermore, if the activities of lignin-breaking enzymes, such as laccase (Lac), manganese peroxidase (MnP), and lignin peroxidase (LiP), are suppressed, lignin breakdown is hindered. Lignin breakdown is the primary defense mechanism in plants and helps to prevent the penetration of hydrolytic enzymes released by *G. boninense* PER71 [8,9]. Because pathogens rely on cellulose and hemicellulose as their carbon sources, stopping the activity of hydrolytic and ligninolytic enzymes can be crucial in preventing these organisms from invading.

In the agricultural sector, phenolic compounds are increasingly emerging, with host–pathogen interactions as their primary focus. According to previous studies, phenolic substances with low molecular weights might prevent the presence of *G. boninense* PER71 [2,10]. When a pathogen infects a plant, the metabolic pathways of phenolic compounds are triggered, and they play a role in the manufacturing of lignin and cell walls [11]. This clarifies how plants defend themselves against disease invasion and resistance. When infection occurs in the early stages, phenolic chemicals and their derivatives accumulate [12]. Phenolic compounds work well as alternatives to chemical pesticides and are affordable for extensive cultivation. Therefore, the capacity of the five phenolic compounds to suppress the cellular growth and synthesis of hydrolytic and ligninolytic enzymes of *G. boninense* PER71 was investigated. These compounds include gallic acid, thymol, propolis, and carvacrol.

2. Materials and Methods

2.1. Fungi, Culture Maintenance, and Treatments

The Malaysian Palm Oil Board (MPOB), Bandar Baru Bangi, provided *G. boninense* strain PER71. The culture was maintained by subculturing on Potato Dextrose Agar (PDA, Oxoid Ltd., Cheshire, UK) every seven days and incubating at 28 °C. Various concentrations of gallic acid, thymol, carvacrol, and propolis were used in this study with purity more than 98% (Sigma Life Sciences, Burlington, MA, USA).

2.2. Screening of Phenolic Compounds for the Production of Hydrolytic and Lignolytic Enzymes by *G. boninense per 71*

2.2.1. Hydrolytic Enzymes

Evaluation of the endoglucanase activity was conducted whereby a 5 mm plug cut off from an actively growing *G. boninense* PER71 at edge of the culture (provided by MPOB, well-maintained for further studies; it will be very filamentous and has aerial growth) was cultured in the centre of CMC agar media comprising 20 g of CMC and 15 g of agar L^{-1} amended with different concentrations (based on the preliminary studies) of phenolic compounds GA (5, 6, 7, and 8 mg/mL), THY (0.1, 0.15, 0.2, and 0.25 mg/mL), PRO (2, 2.5, 3, and 3.5 mg/mL), and CARV (0.1, 0.13, and 0.15 mg/mL). Medium that was not amended with phenols served as a control. All plates were incubated in the dark for 10 days. After 10 d, the plates were flooded with Congo red (1 mg L^{-1}) obtained from Sinar Scientific Sdn Bhd, Sri Serdang for 15 min. Then, 1 mM of NaCl (Sinar Scientific Sdn Bhd, Sri Serdang, Selangor, Malaysia) was added to observe the cellulose degradation by cellulase. A clear yellow or red background indicates that the cellulose has been degraded [2].

The medium was prepared using 2 g of starch, 1 g of peptone, 1 g of yeast extract, and 20 g of agar L^{-1} (Sinar Scientific Sdn Bhd, Sri Serdang, Selangor, Malaysia) and amended with different concentrations of each phenolic compound. Media plates were inoculated with a *G. boninense* PER71 plug (5 mm) excised from an actively growing culture and incubated in the dark for 10 days. The plates were then flooded with Lugol's iodine (0.25% wv^{-1}) for a few seconds. A medium containing 1 g of birchwood xylan, 0.1 g of peptone, 0.01 g of yeast extract, and 20 g of agar L^{-1} was used to measure xylanase activity.

The medium was supplemented with different concentrations of each phenolic compound (Bucher et al., 2004). Media plates were inoculated with a *G. boninense* PER71 plug (5 mm Ø) excised from an actively growing culture and incubated in the dark for 10 days. The plates were then flooded with a Lugol's iodine solution (0.25% wv^{-1}) (Sinar Scientific Sdn Bhd). A clear zone around the colony or a purple-black background indicates xylanase production.

2.2.2. Ligninolytic Enzymes

The production of ligninolytic enzymes by *G. boninense* PER71 was detected by growing the culture on a medium containing RBBR dye and tannic acid in the presence of phenolic compounds GA (5, 6, 7, and 8 mg/mL), THY (0.1, 0.15, 0.2, and 0.25 mg/mL), PRO (2, 2.5, 3, and 3.5 mg/mL), and CARV (0.1, 0.13, and 0.15 mg/mL). A Petri plate containing 2% MEA supplemented with 0.05% RBBR and different concentrations of each phenolic compound was inoculated with a 5 mm Ø agar plug excised from a seven-day-old *G. boninense* PER71 culture. Petri dishes containing MEA supplemented only with RBBR and inoculated with or without *G. boninense* PER71 were used as control plates. All plates were incubated at room temperature for approximately 10 days until the control plate was fully colonised. Decolourisation of the dye confirms the production of ligninolytic enzymes [13].

G. boninense PER71 cultures were grown on a medium containing 5 g of tannic acid (Sinar Scientific Sdn Bhd, Sri Serdang, Selangor, Malaysia), 15 g of malt extract, and 20 g of agar L^{-1} and amended with four different concentrations, GA (5, 6, 7, and 8 mg/mL), THY (0.1, 0.15, 0.2, and 0.25 mg/mL), PRO (2, 2.5, 3, and 3.5 mg/mL), and CARV (0.1, 0.13, and 0.15 mg/mL), from each phenolic compound. A medium not amended with phenol was used as a control. The prepared media were inoculated with a mycelial disc of 5 mm excised from a 7-day-old culture of *G. boninense* PER71 and incubated in the dark for 10 days. The production of a dark-brown colour under the colony indicated the production of ligninolytic enzymes.

2.3. Quantification of Hydrolytic and Ligninolytic Enzymes Produced by G. boninense per 71

G. boninense PER71 was cultured in a 250 mL Erlenmeyer flask containing 40 mL of sterilised PDB and amended with phenolic compounds to obtain the desired concentrations. Sterilised oil palm wood chips obtained from Malaysian Palm Oil Board, MPOB (0.5 g) were added to the medium to induce the production of hydrolytic enzymes. Each flask was inoculated with five 5 mm mycelial plugs excised from an actively growing 7-day-old culture of *G. boninense* PER71. All of the treated flasks were incubated at 28 °C with constant shaking (200 rev/min^{-1}) (PROTECH, SI 50, TUJUAN CEKAP SDN BHD, Shah Alam, Selangor, Malaysia) for 10 d. *G. boninense* PER71, along with the wood chips, were centrifuged at 1792× *g* for 40 min at 4 °C [14]. The supernatant was transferred to a fresh tube, which served as the enzyme source.

2.3.1. Cellulase Activity

Different concentrations of GA (5, 6, 7, and 8 mg/mL), THY (0.1, 0.15, 0.2, and 0.25 mg/mL), PRO (2, 2.5, 3, and 3.5 mg/mL), and CARV (0.1, 0.13, and 0.15 mg/mL) phenolic compounds were prepared in 100 mL conical flasks containing 50 mL of Sabouraud Dextrose Broth, and 0.5% of carboxymethyl cellulose as a carbon source was added into the conical flask. Flasks containing only culture media without phenolic compounds were used as positive controls. Each conical flask was inoculated with three plugs of 5 mm diameter mycelium excised from the actively growing *G. boninense* PER71 culture [8]. All samples were incubated at 24 °C for 10 days. The contents were then filtered using sterilised cheesecloth and centrifuged at 5000 rpm for 10 min. To measure cellulase activity, the total reducing sugar released from the filtered substrate using Whatman No. 1 filter paper was determined using dinitrosalicylic acid reagent [2]. The reducing sugar content was measured spectrophotometrically at 540 nm using a UV-Vis spectrometer (Model: Multiskan SkyHigh Microplate Spectrophotometer, Thermo Scientific Multiscan Go, Thermo Fisher,

Vantaa, Finland). Enzyme activity was expressed as mg glucose released min^{-1} mg^{-1} fresh weight [2]. Furthermore, 1 U (umol/min) is defined as the amount of the enzyme that catalyses the conversion of one micromole of substrate per minute under the specified conditions of the assay method.

2.3.2. Amylase Activity

An aliquot of the crude enzyme extract (250 µL) from each treatment group was incubated at 50 °C for 30 min with 250 µL of 1% starch in 50 mM of sodium citrate buffer (pH 5). The reaction was stopped by the addition of 500 µL of DNS (3, 5-dinitrosalicylic acid) [15] and submerged in boiling water (100 °C) for 5 min. The mixture was cooled to room temperature, and the reaction was monitored at 540 nm using a UV-Vis spectrophotometer, Model Multiskan SkyHigh Microplate Spectrophotometer, Thermo Scientific Multiscan Go, Thermo Fisher, Vantaa, Finland [15].

2.3.3. Xylanase Activity

An aliquot of crude enzyme extract (250 µL) from each treatment group was incubated at 50 °C for 30 min with 250 µL of 1% xylan from birchwood in 50 mM of sodium citrate buffer (pH 5). The reaction was stopped by the addition of 500 µL of 3, 5-dinitrosalicylic acid (DNS) [15] and submerged in boiling water (100 °C) for 5 min. The mixture was cooled to room temperature, and the reaction was monitored at 540 nm using a UV-Vis spectrophotometer, Model Multiskan SkyHigh Microplate Spectrophotometer, Thermo Scientific Multiscan Go, Thermo Fisher, Finland [15]. The negative control PDB without phenolic compounds served as a reference [2,16].

2.3.4. Laccase Activity

Laccase activity was measured according to the level of oxidation of 2, 2-azino-bis (ABTS) substrate converted to a more stable cation radical, and it was spectrophotometrically determined at 420 nm. The assay mixture consists of 0.5 mM of ABTS in 0.1 M of sodium acetate at a pH of 4.5. The reaction mixture was initialised by adding 100 µL of crude enzyme extract to the existing 2.8 mL of ABTS solution [2].

2.3.5. Lignin Peroxidase Activity

Lignin peroxidase activity was quantified as the amount of veratryl alcohol oxidised to veratryl aldehyde [2]. The reaction mixture contained 1 mL of 125 mM sodium tartrate buffer (pH 3.0), 10 mM of veratryl alcohol, and 0.5 mL of crude enzyme extract (0.5 mL). The reaction was activated through the addition of 500 uL of 0.2 mM hydrogen peroxide solution, and the mixture was read spectrophotometrically (Model: Multiskan SkyHigh Microplate Spectrophotometer, Thermo Scientific Multiscan Go, Thermo Fisher, Vantaa, Finland) at 310 nm.

2.3.6. Manganese Peroxidase Activity

Manganese peroxidase activity was measured based on the oxidation of guaiacol. The reaction mixture contained 0.5 mL each of 100 mM of sodium tartrate buffer (pH 5), 100 mM of guaiacol, 50 mM of hydrogen peroxide, and 0.1 mL of crude enzyme extract. All laccase, lignin peroxidase, and manganese peroxidase activities are expressed in units per millilitre (U/mL) [2].

2.4. Scanning Electron Microscopy

Scanning Electron Microscopy (SEM) was used to investigate the effect of phenolic compounds on the mycelial morphology of *G. boninense* PER71 (SEM). SEM images were obtained by peeling off the surface of each *G. boninense* PER71 culture grown on PDA plates containing various concentrations of phenolic compound. One centimetre (five per sample) of the excised culture was preserved in 4% glutaraldehyde for 12–24 h. Following a 0.1 M sodium cacodylate buffer wash, the cultures were fixed with osmium tetroxide fixed for

two hours. The samples were dehydrated in a sequence of alcohol concentrations (30, 40, 50, 60, 70, 80, 90, and 100%) for 10 min. The prepared samples were immersed thrice for 15 min in a 100% acetone solution. The samples were then coated with gold–palladium and dried to the critical point using liquid CO_2. The coated mycelial cultures were observed under an SEM (JEOL, JSM 6400, Tokyo, Japan) operating at 15 kV [8].

2.5. High-Resolution Transmission Electron Microscopy (HR-TEM)

As stated in the previous section, mycelial sample surfaces were peeled off and subjected to a 6% glutaraldehyde solution for 24 h at room temperature. The samples were dehydrated in an HPLC graded ethanol series for five minutes, CO_2-dried (SAMDRI 780-B Tousimis, Rockville, MD, USA), and then sputter-coated with gold–palladium in a Nanotech sputter coater after three rinses with 0.02 M of phosphate buffers (BAL-TEC SDC 050, New York, NY, USA). The coated mycelial samples were kept in a desiccator until they were inspected. The samples were embedded in resin (EMS, cat. 14380) and allowed to polymerise, and the samples were maintained. Ultrathin pieces (60 nm thick) were cut using a diamond knife (Ultracut R, Leica, Washington, DC, USA) and collected on Formvar-coated copper grids (EMS cat. FCF200). After contrast in uranyl acetate and lead citrate, they were cleaned and examined using a JEM-2100F field-emission electron microscope [8,17].

2.6. Statistical Analysis

Data were analysed using SAS Statistical Software, version 9.4 English. Statistical significance was set at $p \leq 0.05$. Means were compared using Tukey's Studentized Range (HSD) test. The experiments were conducted at a probability level of 95% ($\alpha = 0.05$). The results are expressed as the mean $\pm$ standard error obtained from four replicates.

3. Results

3.1. Qualitative and Quantitative Analyses of Hydrolytic Enzymes
Cellulase, Amylase, and Xylanase Activity

Figure 1A shows the cellulase enzyme produced by *G. boninense* PER71 under the influence of specific concentrations (mg/mL) of phenolic compounds GA-5, 6, 7, and 8, THY-0.1, 0.15, 0.2, and 0.25, PRO-2, 2.5, 3, and 3.5, and CARV-0.1, 0.13, and 0.15. Based on this observation, mycelial growth was not as prominent in the media treated with GA. Yet, a deeply coloured brown zone was noticeable as a background of the mycelial plug with the PIRG = 94% in GA 8 mg/mL plate, 90% in THY 0.25 mg/mL, 92.5% in PRO 3.5 mg/mL, and 90.3% in CARV 0.15 mg/mL treated plates. Most CMC plates treated with phenolic compounds appear to have very fine and thin mycelial structures.

Figure 1B represents the degradation of starch due to amylase enzyme produced by the *G. boninense* PER71 at different concentrations (mg/mL) of phenolic compounds GA-5, 6, 7, and 8, THY-0.1, 0.15, 0.2, and 0.25, PRO-2, 2.5, 3, and 3.5 and CARV-0.1, 0.13, and 0.15. All plates with specific treatments inhibited the growth of fungi, which corresponded to decreased production of amylase. Each phenolic compound exhibited a *p*-value of <0.05, which is $p = 0.0001$, as referred to in the measurement of mycelial growth diameter. The plates treated with GA8 showed the best result in the degradation of starch by exhibiting the least amount of clear zone and a higher length of purple-black background (Figure 1B); the PIRG values are GA 8 mg/mL = 96%, THY 0.25 mg/mL = 4.6%, PRO 3.5 mg/mL = 8.7%, and CARV 0.15 mg/mL = 3.2%.

Figure 1C, shows the degradation of xylan due to the xylanase enzyme produced by the *G. boninense* PER71 and when treated with specific concentrations (mg/mL) of phenolic compounds GA-5, 6, 7, and 8, THY-0.1, 0.15, 0.2, and 0.25, PRO-2, 2.5, 3, and 3.5, and CARV-0.1, 0.13, and 0.15. Various ranges of GA concentrations inhibited the growth of fungi, which proved that the production of xylanase was decreasing. GA was significantly different from other phenolic compounds, with a *p*-value of <0.05, which was 0.0001, referring to the measurement of the diameter of the mycelial growth (PIRG = 94%).

Other than that, the degradation of dye observed in THY 0.25 mg/mL was 3%, PRO 3.5 mg/mL = 2.7%, and CARV 0.15 mg/mL = 1.8%, respectively. Fully colonised Petri dishes containing *G. boninense* PER71 were used as a reference.
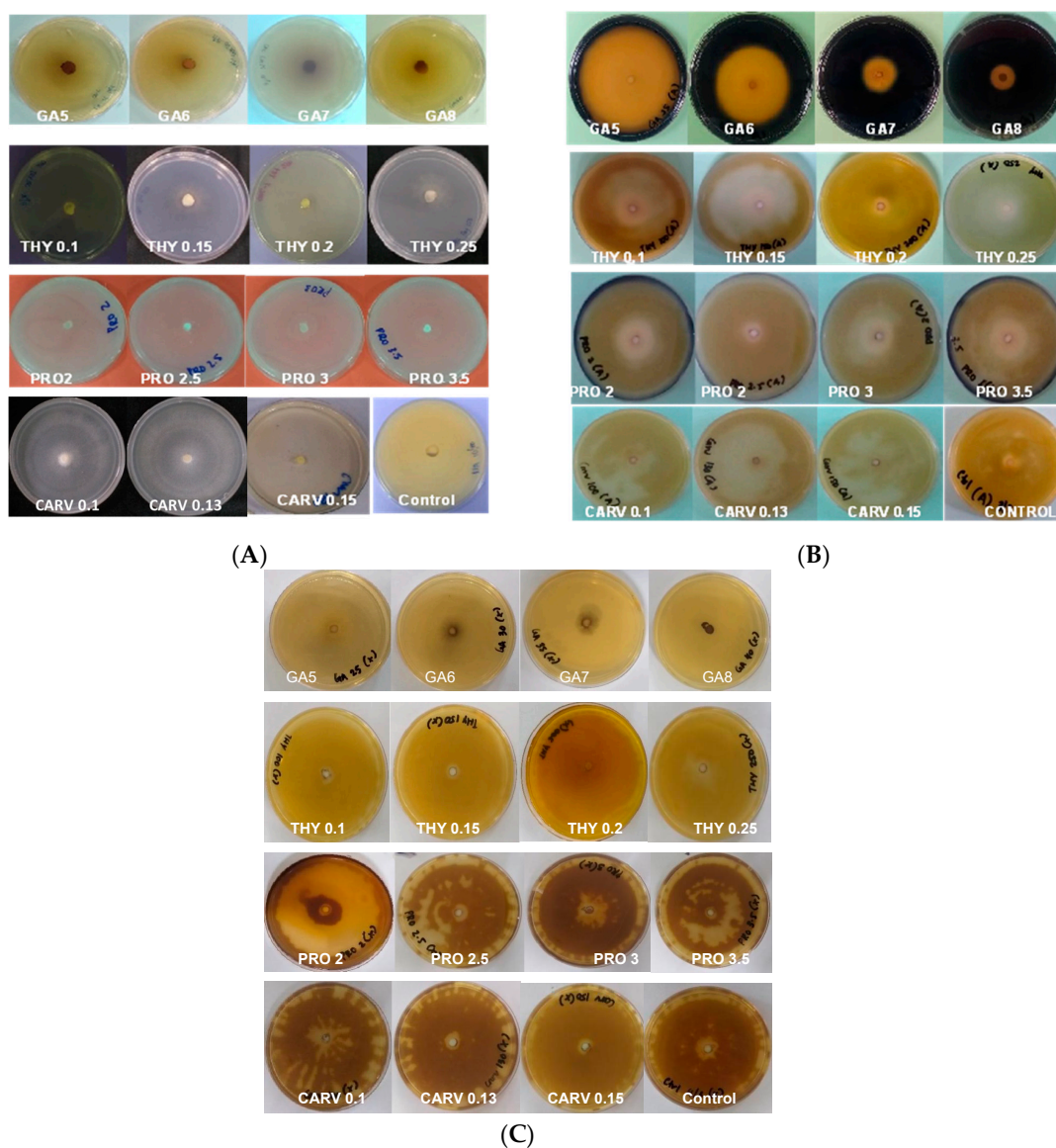

(**A**)

(**B**)

(**C**)

Figure 1. (**A**) Poison Food test of *G. boninense* PER71 in the presence of different concentrations of phenolic acids and degradation of (**A**) cellulose, (**B**) starch, and (**C**) xylan. GA—Gallic acid; THY—Thymol; PRO—Propolis; CARV—Carvacrol.

The tested phenolic compounds exhibited significant inhibitory effects on the radial expansion of *G. boninense* mycelium for enzyme degradation at varying concentrations, as illustrated in Figure 2. The minimum concentration required for each phenolic compound to impede the growth of *G. boninense* mycelia range varies depending on the specific phenolic compound in question. For instance, degradation of enzyme and mycelial growth was suppressed on plates supplemented with 8 mg/mL of gallic acid, 3.5 mg/mL of propolis, or 0.25 mg/mL of thymol. Notably, phenolic compounds with high concentrations inhibited mycelial growth compared to growth observed on control plates. In general, gallic acid, propolis, and thymol were the most effective among the phenolic compounds, exhibiting significant inhibitory effects when compared to the other phenols investigated. The presence of phenols at varying concentrations in the growth medium had a notable impact on the growth of *G. boninense* mycelium.
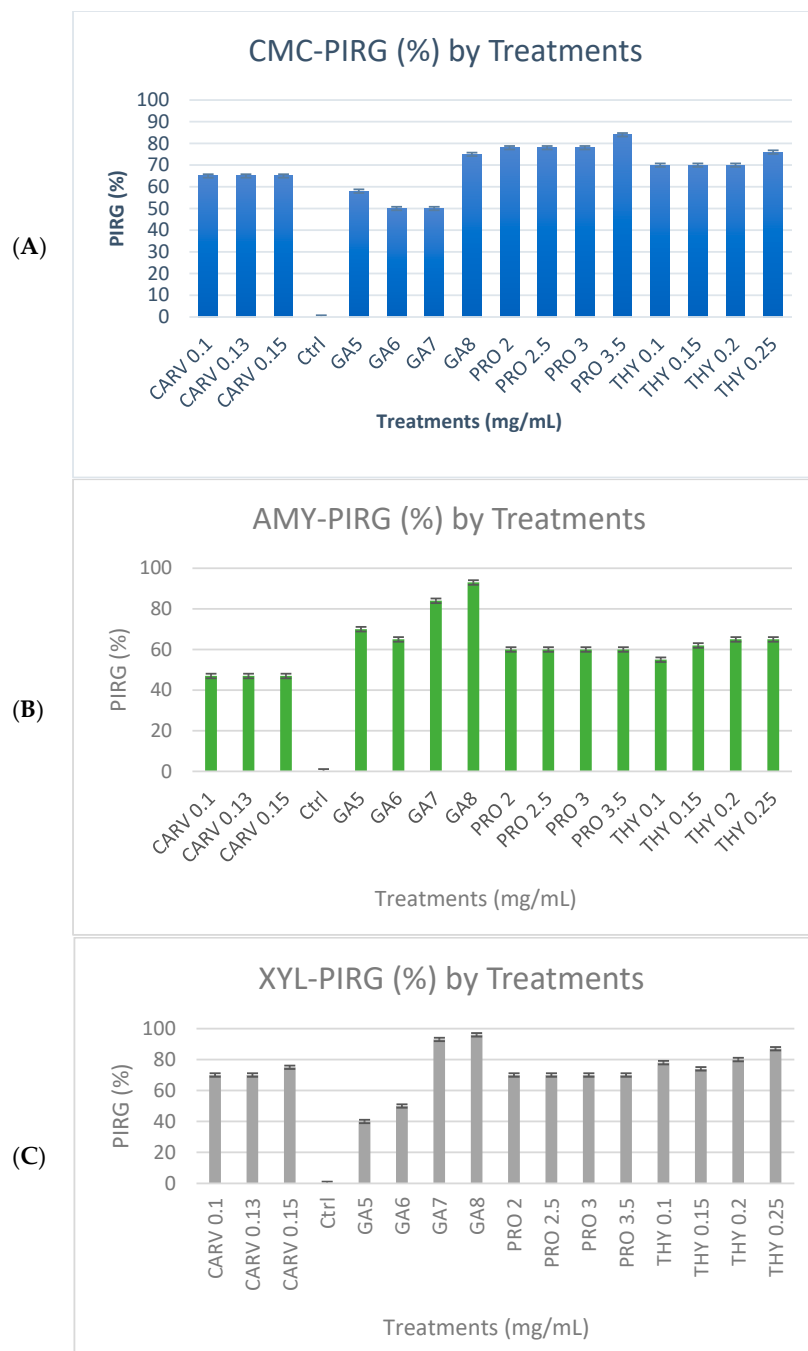
Figure 2. Percentage inhibition of radial growth (PIRG %) of *G. boninense* PER71 treated with different concentrations of phenolic compounds on enzyme production. The graph shows the percentage of enzyme production for (**A**) cellulase, (**B**) amylase, (**C**) xylanase, and the interpretation of data is based on 100% enzyme production. Vertical bars indicate standard error, and each value represents the mean of four replicates.

The effects of the four phenolic acids on hydrolytic enzyme activity of *G. boninense* PER71 are presented in Table 1. The amounts of cellulase, amylase, and xylanase produced by *G. boninese* in the presence of GA were lower than those of the other phenolic compounds. Carvacrol exhibited the highest level of enzyme synthesis among all hydrolytic enzyme activities mentioned below. The amounts of cellulase, amylase, and xylanase produced by the control were 1.43, 1.37, and 1.1147 U/mL, respectively. The production of all of these enzymes by *G. boninense* PER71 was significantly different from that of the control when influenced by the selected phenolic compounds at *p* < 0.05.

Table 1. Effect of phenolic compounds on hydrolytic enzyme activity by *G. boninense* PER71.

	Cellulase	Enzyme Activity (U/mL) Amylase	Xylanase
Control	1.4349 ± 0.68 [a]	1.371 ± 1.3 [a]	1.1147 ± 1.25 [ab]
GA-5	1.0287 ± 1.25 [b]	0.5036 ± 1.2 [b]	1.2503 ± 1.4 [a]
GA-6	0.5266 ± 1.25 [d]	0.4998 ± 1.2 [c]	1.032 ± 1.4 [ab]
GA-7	0.1966 ± 1.25 [e]	0.3389 ± 1.2 [d]	0.746 ± 1.4 [b]
GA-8	0.3387 ± 1.25 [f]	0.3314 ± 1.3 [d]	0.2113 ± 1.4 [b]
THY-0.1	0.8963 ± 1.3 [c]	0.2980 ± 1.7 [d]	0.925 ± 1.8 [b]
THY-0.15	0.8542 ± 1.3 [c]	0.0047 ± 1.7 [e]	0.283 ± 1.8 [c]
THY-0.2	1.0079 ± 1.3 [b]	0.2872 ± 1.7 [c]	0.718 ± 1.8 [b]
THY-0.25	0.2675 ± 1.3 [e]	0.3710 ± 1.7 [c]	0.736 ± 1.8 [b]
PRO-2	0.7163 ± 1.4 [c]	1.1399 ± 1.8 [a]	0.933 ± 1.5 [b]
PRO- 2.5	0.7203 ± 1.4 [c]	0.8949 ± 1.8 [a]	1.075 ± 1.5 [a]
PRO-3	0.3063 ± 1.4 [e]	0.4197 ± 1.8 [c]	1.233 ± 1.5 [a]
PRO-3.5	0.3025 ± 1.4 [e]	0.7082 ± 1.8 [b]	0.038 ± 1.5 [c]
CARV-0.1	1.7525 ± 1.45 [a]	0.6222 ± 1.8 [b]	1.035 ± 1.7 [b]
CARV-0.13	1.2034 ± 1.45 [a]	0.8711 ± 1.8 [a]	0.993 ± 1.7 [b]
CARV-0.15	1.2356 ± 1.45 [a]	0.9519 ± 1.8 [a]	1.057 ± 1.7 [a]

Means with the same letter indicate that there was no significant difference between treatments. Each value represents the mean of four replicates. ± values indicate the standard error.

3.2. Qualitative and Quantitative Analyses of Ligninolytic Enzymes
Laccase, Lignin Peroxidase, and Manganese Peroxidase Activity

Figure 3A,B represent RBBR and tannic acid degradation by *G. boninese* in the presence of different concentrations of phenolic acids GA-5, 6, 7, and 8, THY-0.1, 0.15, 0.2, and 0.25, PRO-2, 2.5, and 3, and CARV-0.1, 0.13, and 0.15. In GA 8 mg/mL treated plates, the mycelia had dark and light brownish circular patterns with brown exudates, but on THY 0.2 and 0.25 treated plates, orange-coloured exudates were spotted. PRO-treated plates had a circular mycelial structure, and for CARV, an intense dark zone was observed in the background of the plate. The degradation of dye was visible, and the PIRG for plates with the highest concentrations were GA (8 mg/mL = 84%), THY (0.25 mg/mL = 2%), PRO (2.5 mg/mL = 6.5%), and CARV (0.15 mg/mL = 1.8%). Figure 3A presents the laccase produced by *G. boninense* PER71, and GA, THY, and CARV at higher concentrations inhibited the growth of fungi, which corresponded to lower production of enzymes. The PIRG recorded was THY 0.25 mg/mL = 84%, CARV 0.15 mg/mL = 80%, GA 8 mg/mL with 15%, and PRO 3.5 mg/mL = 13.25% at a p-value of <0.05, which is $p = 0.0001$ calculated on the Tukey (HSD) test, which significantly impacts the production of the enzyme (Figure 4). Figure 3B presents the lignin and manganese peroxidase enzyme produced by the *G. boninense* PER71 when treated with different concentrations (mg/mL) of phenolic compounds GA-5, 6, 7, and 8, THY-0.1, 0.15, 0.2, and 0.25, PRO-2, 2.5, 3, and 3.5, and CARV-0.1, 0.13, and 0.15. GA and THY significantly affected the production of MnP and LiP ($p = 0.0055$ and <0.0001, respectively).

G. boninense cultures cultivated on media containing RBBR dye and phenolic treatments exhibited a significantly reduced decolourisation effect ($p \leq 0.005$) when compared to the control group, as shown in Figure 3. Over the observation period, the culture growing on the control plate completely colonised the plate and effectively decolourised the RBBR dye. This decolourisation process indicated the production of ligninolytic enzymes. Conversely, media containing phenols at different concentrations inhibited the growth

of *G. boninense*, preventing the fungus from producing ligninolytic enzymes and, consequently, not decolourising the media. Of note, *G. boninense* growth on media containing 0.25 mg/mL of THY was inhibited at a higher range ($p \leq 0.05$), highlighting this phenol as a particularly potent inhibitor compared to the others. Similarly, *G. boninense* cultures grown on agar media containing tannic acid and phenolic treatments exhibited significantly reduced growth and a notably smaller dark-brown area (8 mg/mL of GA) formed under the colony in comparison to the control plates ($p \leq 0.005$), as depicted in Figure 3. The presence of a dark-brown area beneath the colony indicated the secretion of ligninolytic enzymes.

The influence of the four different treatments on ligninolytic enzyme activity of *G. boninense* PER71 is shown in Table 2. The significant variations in the enzymatic activities of Lac, LiP, and MnP, affected by the four different phenolic compounds, demonstrated that the treatments prevented *G. boninense* PER71 from producing its own enzymes. Higher levels of phenolic acids, particularly gallic acid, have a significant impact on fungal growth and boost the inhibitory function of the enzyme. Between 2.5 and 3.5 mg/mL of propolis, there was a noticeable drop in laccase enzyme activity. Similarly, lignin peroxidase activity modulated by thymol showed a reduction in enzyme activity. MnP activity was significantly reduced by gallic acid.
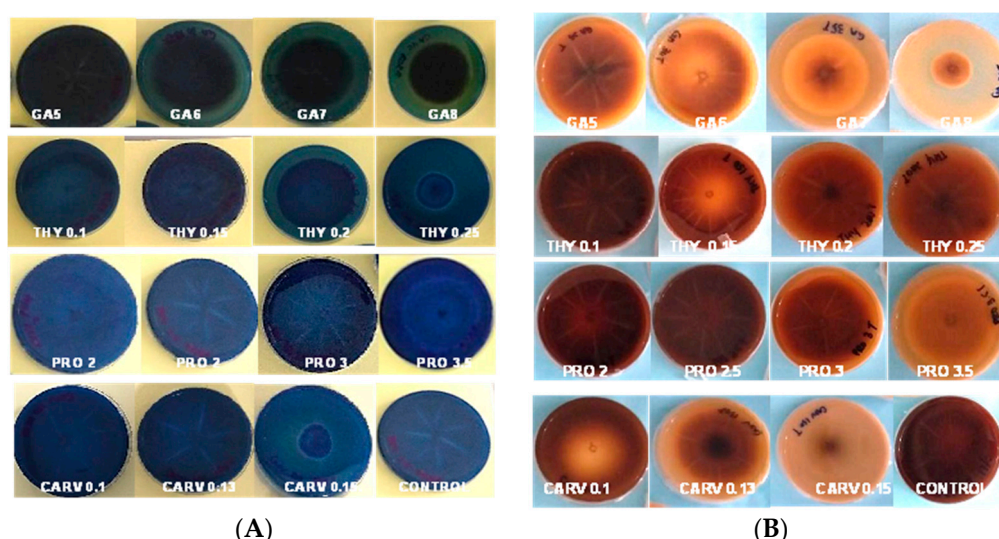

(A)   **(B)**

Figure 3. Poison Food test of *G. boninense* PER71 in the presence of different concentrations of phenolic acids and degradation of (**A**) RBBR dye and (**B**) tannic acid. GA—Gallic acid; THY—Thymol; PRO—Propolis; CARV—Carvacrol.

Table 2. The effect of phenolic compounds on the production of ligninolytic enzyme activity by *G. boninense*.

	Enzyme Activity (U/mL)		
	Laccase	Lignin Peroxidase	Manganese Peroxidase
Control	0.9865 ± 1.85 [a]	1.883 ± 1.5 [a]	0.846 ± 1.7 [a]
GA-5	0.0844 ± 1.3 [c]	1.613 ± 1.8 [a]	0.916 ± 1.8 [a]
GA-6	0.7255 ± 1.3 [a]	1.844 ± 1.8 [a]	0.807 ± 1.8 [a]
GA-7	0.6754 ± 1.3 [b]	1.655 ± 1.8 [a]	0.564 ± 1.8 [b]
GA-8	0.4885 ± 1.3 [b]	0.218 ± 1.8 [c]	0.386 ± 1.8 [b]
THY-0.1	0.1321 ± 1.8 [c]	2.080 ± 1.5 [a]	0.037 ± 1.3 [d]
THY-0.15	0.0878 ± 1.8 [c]	1.700 ± 1.5 [b]	0.056 ± 1.3 [d]
THY-0.2	0.0776 ± 1.8 [c]	1.563 ± 1.5 [b]	0.101 ± 1.3 [c]
THY-0.25	0.1184 ± 1.8 [c]	2.023 ± 1.5 [a]	0.831 ± 1.3 [a]

Table 2. *Cont.*

	Enzyme Activity (U/mL)		
	Laccase	**Lignin Peroxidase**	**Manganese Peroxidase**
PRO-2	0.1424 ± 1.25 [a]	1.660 ± 1.83 [b]	0.132 ± 1.5 [c]
PRO-2.5	0.0978 ± 1.25 [d]	1.607 ± 1.83 [b]	0.163 ± 1.5 [c]
PRO-3	0.0665 ± 1.25 [d]	2.040 ± 1.83 [a]	0.048 ± 1.5 [d]
PRO-3.5	0.0644 ± 1.25 [d]	2.226 ± 1.83 [a]	0.275 ± 1.5 [c]
CARV-0.1	0.0543 ± 1.4 [d]	1.492 ± 1.9 [b]	0.005 ± 1.5 [e]
CARV-0.13	0.1834 ± 1.4 [c]	1.443 ± 1.9 [b]	0.039 ± 1.5 [d]
CARV-0.15	0.1246 ± 1.4 [c]	1.743 ± 1.9 [b]	0.056 ± 1.5 [d]

Means with the same letter indicate that there was no significant difference between treatments. Each value represents the mean of four replicates. ± values indicate the standard error.
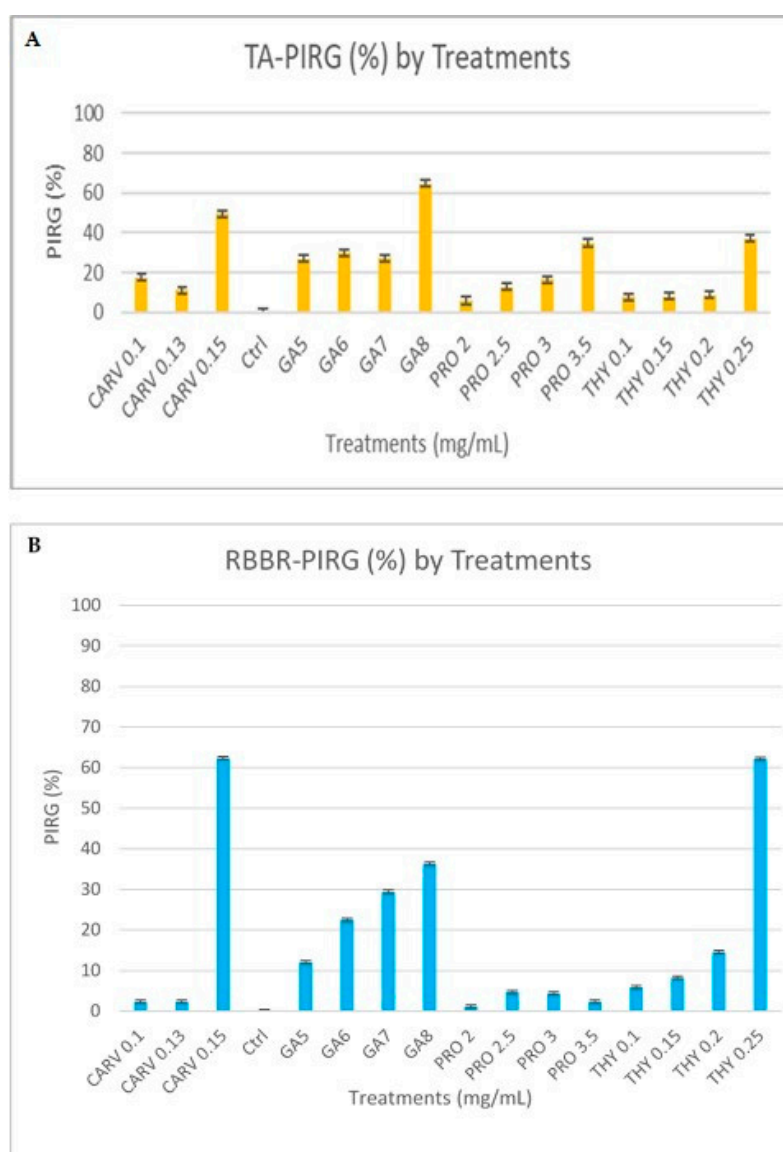

Figure 4. Percentage inhibition of radial growth (PIRG %) of *G. boninense* PER71 treated with different concentrations of phenolic compounds on enzyme production. The graph shows the percentage of enzyme production of (**A**) tannic Acid and (**B**) RBBR. The interpretation of data is based on 100% of enzyme production. Vertical bars indicate standard error, and each value represents the mean of four replicates.

3.3. Scanning Electron Microscopy (SEM)

The mycelium cultivated without the presence of phenolic acids displayed a smooth, dense, and healthy appearance, featuring a diameter of 1.244 mm and clearly visible clamp connections (see Figure 5A). In contrast, the mycelium subjected to gibberellic acid (GA) exhibited reduced density and a shrivelled and fused appearance with a diameter of 2.732 mm, and the hyphae's wrinkled surface displayed perforations (refer to Figure 5B). Thymol-treated mycelium showed signs of distortion (diameter of 2.005 mm), fused openings revealing empty hyphae, shrinkage (1.528 mm diameter), and detachment of the mycelium (see Figure 5D). Conversely, treatment with carvacrol (CARV) did not induce significant alterations in the mycelial structure, resulting only in shrivelled mycelium with a diameter of 0.709 mm (see Figure 5E).
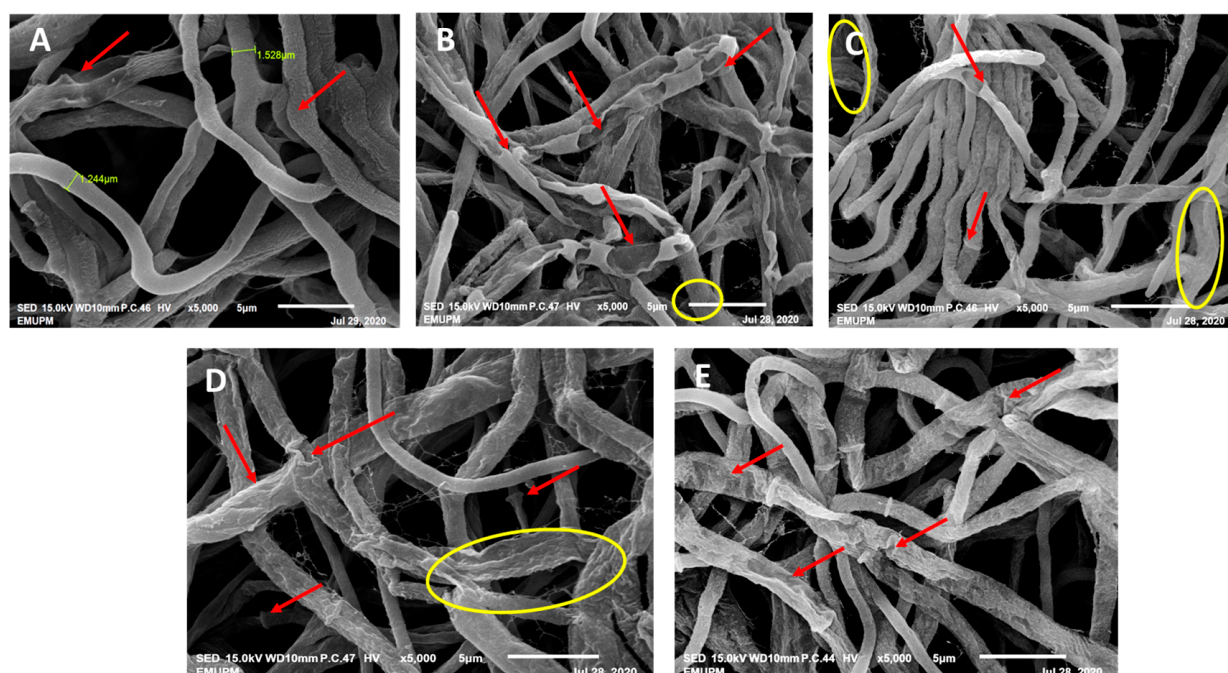

Figure 5. Alterations in mycelial morphology after treatment with phenolic compounds were observed through SEM at 5000× magnification. (**A**) Healthy mycelia of *G. boninense* PER71 excised from the control plate. The arrows indicate clamp connections. (**B**) Mycelium of *G. boninense* PER71 treated with GA. The arrows indicate fused mycelium with holes and shrinkage of the mycelium, and the hyphae are encircled. (**C**) Mycelium of *G. boninense* PER71 treated with thymol. The arrows indicate the holes, shrivelling, and empty hyphae, and the encircled mycelial region indicates the rupture of hyphae. (**D**) Mycelia of *G. boninense* PER71 cells treated with propolis. The arrows indicate shrivelling and collapse, and the encircled region indicates the breaking/detachment of the mycelium connection. (**E**) Mycelium of *G. boninense* PER71 treated with carvacrol showing shrivelling of mycelium (arrow). Note: scale 5 µm.

3.4. High-Resolution Transmission Electron Microscopy (HR-TEM)

Through the use of HR-TEM, it was observed that the hyphal cell walls and interiors treated with phenolic compounds underwent notable ultrastructural modifications. Mycelium retrieved from a solution lacking phenolic compounds exhibited well-defined cytoplasmic organelles, including mitochondria and endoplasmic reticulum, along with a nucleus enclosed by a robust and intact cellular membrane. The cell membrane manifested as a sharp, electron-dense lipid bilayer closely adhering to the inner surface of the fungal cell wall (see Figure 6A). Two prominent alterations induced by the application of phenolic chemicals included the development of electron-dense inclusions within the cell wall and the inner space between the cell membrane and the cell wall.
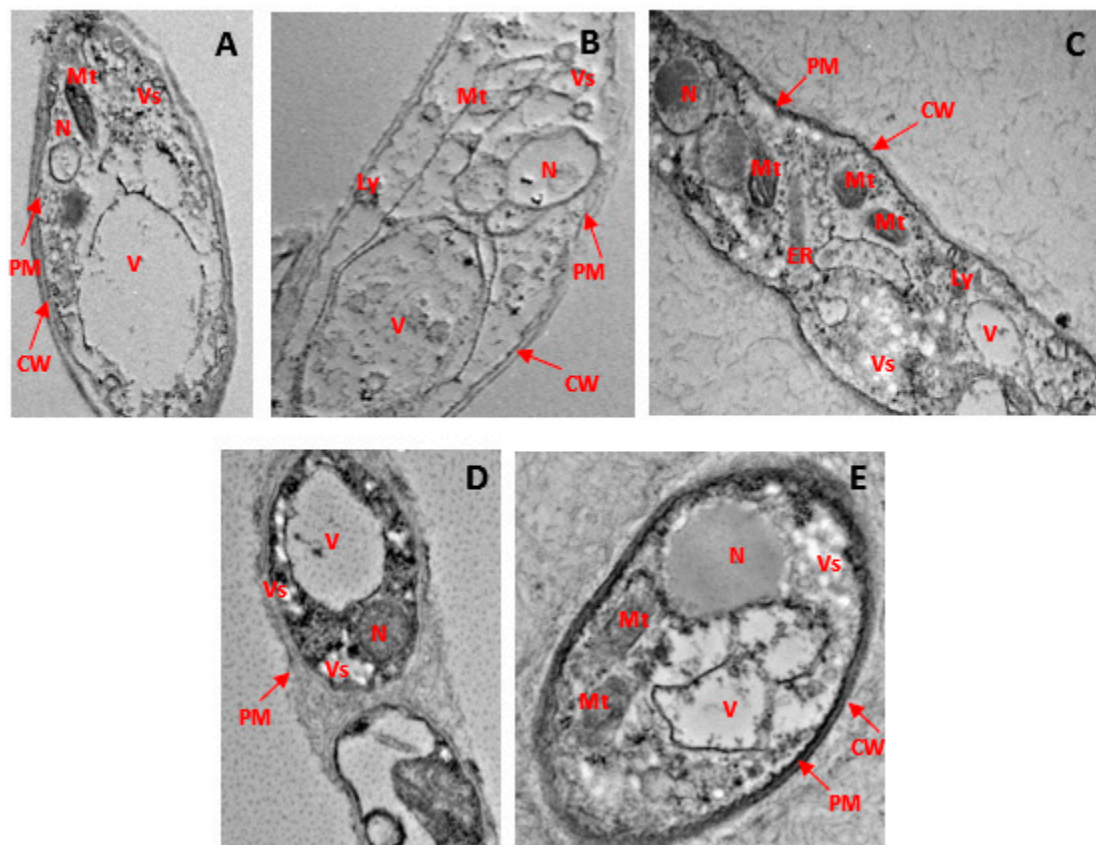
Figure 6. Ultrastructural modifications in the hyphae of *G. boninense* PER71 treated with phenolic compounds as observed through HR-TEM at 6000× magnification. (**A**) Rigid and intact cell wall and plasma membrane, prominent cytoplasmic organelles, such as mitochondria, vesicles, vacuoles, and endoplasmic reticulum, as well as the nucleus in healthy hyphae. (**B**) The arrows indicate disruption of the cell wall and membrane, loss of vesicle with empty regions, condensed nucleus, deformed mitochondria, and disorganisation of cytoplasmic contents in hyphae treated with 8 mg/mL of GA. (**C**) indicates the cell component leakage, disruption of the cell membrane, and disorganisation of cytoplasmic content in hyphae treated with 0.25 mg/mL THY. (**D**) shows the disruption of the cell membrane, disorganisation of cytoplasmic content, and less disintegration of the cell wall and plasma membrane; cytoplasmic organelles are evident in hyphae treated with 3.5 mg/mL of PRO. (**E**) Less damage to fungal hyphae with intact cell wall and irregular but continuous plasma membrane; prominent nucleus and cytoplasmic organelles in hypha treated with 0.15 mg/mL of CARV. Note: scale 1 µm. CW: cell wall, PM: plasma membrane, Mt: mitochondria, ER: endoplasmic reticulum, V: vacuole, N: nucleus, Vs: vesicle.

In the case of mycelium exposed to gibberellic acid (GA), there was an irregular membrane structure, accompanied by cellular membrane disintegration and the fusion and rupture of vacuoles. Numerous electron-dense inclusions of varied sizes and forms were observed (refer to Figure 6B). Hyphae treated with thymol (THY) displayed significant damage to the cell wall and cytoplasmic organelles (see Figure 6C). A substantial portion of the cell membranes and cell walls was either destroyed or barely visible. Organelle disintegration, blurry and damaged walls, along with a detached plasma membrane from the cell wall structure were evident in propolis (PRO)-treated hyphae (see Figure 6D). In hyphae subjected to carvacrol (CARV) treatment, reduced nuclear content was observed, and certain sections of the cell membranes exhibited irregular borders, murky walls, and damaged plasma membranes (see Figure 6E). These substantial morphological or ultrastructural changes were occasionally accompanied by the compromise of membrane structures in the nucleus and cytoplasmic organelles.

4. Discussion

Infections are typically caused by pathogens due to the presence of hydrolytic and ligninolytic enzymes [18]. Ligninolytic enzymes produced by *G. boninense* PER71 play a crucial role in the establishment of BSR. Restricting the proliferation and breakdown of these enzymes can help to reduce BSR infection. The lignolytic and hydrolytic enzyme activity was assessed through spectrometric analysis. Based on the findings, gallic acid and thymol exhibited decreased and unstable synthesis of enzymes, $min^{-1} mg^{-1}$, whereas propolis and carvacrol were proportional to the concentration of the enzymes. Overall, it has been discovered that gallic acid may prevent the development of ligninolytic enzymes, which can help to reduce the oxidation of phenolic compounds [19,20]. An increase in phenolic compound concentration was accompanied by an increase in cell wall breakdown, disintegration, disruption, absence of septa, thin cell walls, electrolyte leakage, accumulation of cellular components, and other abnormalities because of the interaction between the lignocellulolytic enzyme and the cell wall [16].

In general, phenolic chemicals interfered with intra- and intermolecular hydrogen bonding, which had an adverse effect on the synthesis of enzymes and accelerated the degradation of those involved in pathogenic pathways. As phenolic compound concentrations increased, the effect on enzyme production grew. In particular, gallic acid demonstrates a significant rise in oxidative properties that may support ligninolytic enzyme activity. Gallic acid displayed the strongest antifungal efficacy and a sizable impact on the suppression of *G. boninense* PER71 mycelia [21].

Lignin and polysaccharides are broken down by the hydrolytic and ligninolytic enzymes produced by *G. boninense* PER71. Hydrolytic enzymes are necessary to break down holocellulose (cellulose and hemicelluloses) [22]. Lignin is broken down and degraded by ligninolytic enzymes [2]. Regarding qualitative analysis, various colour intensities were observed during the experiment, which provided a quick indication of the enzyme production. The degradation of the dye, colour shifts, and emergence of a strongly coloured zone serve as indicators of extracellular oxidase and secreted enzymes. Many morphological traits, including fine, thin mycelium, fluffy, thicker, and thinner outer layers, as well as an uneven distribution, were observed. This might have resulted from the interaction of phenolic compounds with the physical barriers of *G. boninense* PER71.

Gallic acid and thymol, at the highest tested concentrations, effectively inhibited the growth of *G. boninense* PER71 as well as the activity of hydrolytic and ligninolytic enzymes. Fungal hyphae directly absorb the available nutrients as a result of these extracellular enzymes' action on the nutrients nearby and enable the nutrients to enter the cell. The existence of substrates and the stability of enzymes are closely related to enzyme synthesis. Additionally, strain type and substrate dependence have an impact on enzyme manufacturing efficiency [23]. According to [24], the lag phase is associated with the antifungal efficacy of phenolic acids. The growth of the mycelia increased in a medium rich in nutrients (control plate devoid of phenolic compounds), but the opposite was true in media deficient in nutrients and rich in phenolic compounds. The presence of more phenolic compounds and fewer nutrients caused a prolonged lag phase in mycelia formation. As a result, enzyme secretion decreased, and resistance to pathogen attack could not develop.

Syringaldehyde and vanillin phenol comprise approximately 69% of the phenolic monomers found in oil palms. These phenols are prevalent in oil palm wood, and when the phenol level is low, ligninolytic enzymes can oxidise phenolic compounds and destroy lignin [25]. The combination of phenolic chemicals can inhibit these lipid redox peroxidase processes, which aids in the reduction of BSR [14]. As a result, a sufficient quantity of phenolic compounds helps reduce *G. boninense* PER71. Increased GA concentration exhibited a stronger inhibitory effect on the synthesis of enzymes, notably when amylase, xylanase, laccase, and extracellular haeme protein peroxidases were qualitatively analysed (LiP and MnP). According to [2], both ligninolytic and hydrolytic enzymes are degraded simultaneously. In lignocellulosic materials, xylanase (a hemicellulolytic enzyme) starts the hydrolysis of $-1, 4$-xylans, and when the GA concentration increases, less xylanase,

amylase, laccase, LiP, and MnP are produced. Thymol significantly inhibited the synthesis of the enzymes Lac, LiP, and MnP. According to one study, when there are enough lignin-modifying enzymes (LME), the amount of ligninolytic enzyme production may change, indicating that there is less of a need for it [16]. Lac levels decreased, demonstrating THY's excellent potential to reduce *G. boninense* PER71. Propolis' antifungal qualities have been shown to be a successful strategy for inhibiting enzymes, particularly xylanase and lignin peroxidase. This could be due to preventing invasion of the host cell and obstructing proteins and enzymes [26]. As propolis contains a variety of bioactive chemicals, differing propolis concentrations can disrupt cellular organelles and components [27,28]. Peroxidase enzymes are widely used in the polymerisation of cell wall components and aid in creating barriers that prevent pathogens from invading the oil palm basal zone [29]. The defense system works on the basis that it is activated whenever a plant pathogen infects the host. In contrast, the defense mechanism will eventually stop functioning if *G. boninense* PER71 is unable to initiate and repress the resistance reaction. This results in apparent infection [30]. The inner cell wall of OP is protected by lignin, which makes it difficult for fungal pathogens to take hold. It is crucial to research how enzymes are triggered by the presence of phenolic substances [31]. Plant cells, called lignocelluloses, contain lignin, which is linked to cellulose and hemicellulose. The capacity to prevent the fungal pathogen from spreading the illness is promising, and the efficiency of ligninolytic enzymes demonstrates that the first-line defense is secured. As a result, there is less opportunity for hydrolytic enzymes to spread and cause BSR in OP plants [29]. When an enzyme attack occurs, the polymeric lignin structure chemically binds to hemicellulose and cellulose to protect against it [32]. In general, the amount of phenolic compounds present is directly correlated with the inhibition of *G. boninense*-produced hydrolytic and ligninolytic enzymes (PER 71).

The structural alterations found through SEM and HR-TEM demonstrated that phenolic compounds significantly changed the structural morphology, external surface, internal organelles, and cytoplasmic components of the mycelium. The mycelium was most significantly affected by GA and THY, which led to holes, a reduction in the size of the mycelium, shrivelling of the outer membrane, and severe disruption of internal cytoplasmic elements, such as the nucleus, mitochondria, endoplasmic reticulum, vacuoles, and vesicles. These essentially describe the severity of the biological and chemical processes of the mycelium [33]. GA and THY significantly affected mycelia compared to PRO and CARV. The integrity of the cell wall was weakened as a result of THY and GA targeting the cell membrane [34]. Cell division requires the integrity of the cell wall, but in this study, the mycelium was severely disrupted by phenolic compounds, which prevented it from surviving in an environment that normally controls the creation of biological activities and enzymes [34,35]. Interactions with cellular sulfhydryl compounds have been connected to propolis separation from the fungal cell wall and interruption of division. [31] discussed how phenolic compounds obstruct yeast proliferation and germ tube pathways. They continued by claiming that plasmalemma invasion through phenolic compound therapy alters the dynamic interaction between ergosterol and chitin. Additional SEM and HR-TEM data revealed that transformation of the morphological and ultra-cellular structure of mycelia was associated with the inhibition of *G. boninense*. The integrity of the mycelial membrane and polarity of the cell membrane are lost because of the antifungal activity of benzoic acid. Fungal survival is critically dependent on the integrity of the plasma membrane, and membrane injury can result in loss of intracellular components [36].

Central to antifungal activity is the capacity to alter the function of cell lipoprotein membranes, disrupt cellular ionic homeostasis, acidify vacuolar and cytosolic pH, and compromise structural cellular integrity [35,37]. Changes in cell permeability may be responsible for modifications in the shape and size of internal organelles, such as mitochondria and vacuoles, as observed in recent studies [38]. The membrane potential is frequently disrupted by phenolic compounds, allowing both internal and external elements to enter and exit cells [39]. It has been proposed that the hydroxyl, amide, and sulfonic groups

of phenolic substances can interact with the cell membranes [31]. In addition, improved formulations of GA and THY could make them suitable treatments and preventatives for basal stem rot. The development of optimal concentrations for industrial use as a replacement for synthetic fungicides has also been suggested.

5. Conclusions

In conclusion, this study contributes to the investigation of the effects of different phenolic compounds at various concentrations on *G. boninense* PER71 growth and suppression, as well as on the synthesis of hydrolytic and ligninolytic enzymes. Phenolic chemicals, particularly GA and PRO, were found to suppress the enzymes in this in vitro study. Phenolic compounds are natural barriers that protect substrates from enzyme degradation and prevent this wood-degrading WRF from commencing.

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Abbreviations

GA	Gallic acid
THY	Thymol
PRO	Propolis
CARV	Carvacrol
BSR	Basal stem rot
Lac	Laccase
MnP	Manganese peroxidase
LiP	Lignin peroxidase
SEM	Scanning Electron Microscopy
HR-TEM	High-resolution transmission electron microscopy
LME	Lignin-modifying enzymes
OP	Oil palm

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