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Oxidative damage in *Echinochloa crus-galli* seeds exposed to *Diaporthe* sp. (Diaporthales, Ascomycota) fungal extract during germination

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This study investigates the inhibitory effects of *Diaporthe* sp. isolate EC010 extract on barnyardgrass (*Echinochloa crus-galli*) seed germination and growth. Application of sequential extraction techniques to *Diaporthe* sp. mycelium resulted in partial separation of the phytotoxic compounds. The ethyl acetate (EtOAc) fraction most greatly reduced seed germination (81.01%), root length (89.18%), and shoot length (84.74%) compared to the control. Chemical characterization using gas chromatography-mass spectrometry revealed major constituents of linoleic acid, butyl ester (9.69%), hexadecanoic acid (7.99%), and 14-pentadecenoic acid (7.86%). With regard to physiological and biochemical indexes, treated seeds exhibited lower imbibition, significantly decreased α -amylase (EC 3.2.1.1) activity ($p < 0.05$), and increased accumulation of malondialdehyde (85.52%) and hydrogen peroxide (141.10%). Moreover, activity of the antioxidant enzymes superoxide dismutase (EC 1.15.1.1) and guaiacol peroxidase (EC 1.11.1.7) was upregulated (67.24 and 61.62%, respectively), while catalase (EC 1.11.1.6) activity was downregulated (-33.75%). The inference is that an imbalance in ROS levels combined with reduced antioxidant potential drives the gradual accumulation of oxidative damage in seed cells and consequent loss of seed viability. All told, these results confirm the *Diaporthe* extract to induce oxidative stress and inhibit antioxidant enzymes. This study clearly demonstrates the oxidative damage associated with *Diaporthe* allelochemicals.

KEYWORDS

allelochemicals, barnyardgrass, *Diaporthe* sp., oxidative damage, phytotoxicity

1 Introduction

Herbicides appear to be a cost-effective way to control weeds and contribute to crop productivity. However, the overuse of synthetic herbicides has led to the spread of herbicide-resistant biotypes (Saini and Singh, 2019). This resistance reduces the efficacy of existing herbicides, necessitating the exploration of alternative weed control strategies. Integrated weed management (IWM) approaches, which combine various weed control methods, offer a strategic way to reduce reliance on synthetic herbicides (Nazarkov and Gerasimova, 2023). In this context, natural herbicides derived from allelochemicals are of interest as potential complementary tools in IWM (Saini and Singh, 2019; Gao and Su, 2024). These natural products can be integrated with other weed control methods to achieve broad-spectrum weed control (Bailey, 2014) and, due to their multiple modes of action, can help prevent herbicide resistance (Hazrati et al., 2017). Compared to synthetic options, natural herbicides generally have lower toxicity and shorter environmental persistence (Gao and Su, 2024). By expanding research in this area, the current study aims to explore and discover new sources containing toxic compounds that could enhance weed control, ultimately contributing to the development of effective natural herbicides. *Diaporthe* is a large fungal genus belonging to the Diaporthales and Ascomycota, one for which new species are continually being reported (Huang et al., 2021; Sun et al., 2021). Notably, *Diaporthe* fungi are important sources of active natural products, with many reports of terpenes (Luo et al., 2021; Zhang et al., 2021), alkaloids, anthraquinones (Tian et al., 2018; Xu et al., 2021), and other metabolites with unique structures having been identified from this genus. In the past 20 years, more than 200 such compounds have been demonstrated to possess significant antitumor, antibacterial, herbicidal, and other biological activities. Consequently, reduced use of chemical products can be achieved by using natural herbicides containing natural fungal compounds or fungal active components. Eco-friendly herbicides have been developed using these chemicals as potential candidates.

Weed physiology during exposure to allelochemicals has lately become an area of research interest, with studies to date having highlighted impacts to significant metabolic processes such as those related to cell division, nutrient uptake, antioxidants, stress-mediated hormones, and other metabolites that control seed germination and growth (Radhakrishnan et al., 2018). It has been reported that application of herbicides induces the generation of reactive oxygen species (ROS) in plants, including superoxide radicals ($O_2^{\cdot-}$), hydroxyl radicals (OH^{\cdot}), and hydrogen peroxide (H_2O_2) (Wu et al., 2021; Dmitrieva et al., 2024). This accumulation of ROS can induce oxidative stress, which has adverse consequences for proteins, DNA, and lipids (Choudhary et al., 2020). Especially, ROS action on membrane lipids results in lipid peroxidation, which in turn causes cell membrane damage (Laosinwattana et al., 2018). Accordingly, plants have a mechanism for defending from oxidative stress via the antioxidant enzyme system. Antioxidant enzymes scavenge or eliminate a variety of free radicals, including those generated during biological processes. The main antioxidant enzymes are superoxide dismutase (SOD), catalase (CAT), and guaiacol peroxidases (POD) (Mishra et al., 2023). SOD maintains

the cellular levels of $O_2^{\cdot-}$ within physiological concentrations by converting $O_2^{\cdot-}$ to H_2O_2 . As H_2O_2 may then oxidize cell membranes and cellular components to OH^{\cdot} , its removal is a crucial concern. CAT is an H_2O_2 decomposing enzyme that metabolizes H_2O_2 to O_2 and H_2O (Fujita and Hasanuzzaman, 2022). POD then provides additional mechanisms for maintaining redox balance and removal of toxic metabolites (Zhu et al., 2023).

In a previous study, we collected 21 fungi from weed-infected tissues and screened them for herbicidal activity. Among them, *Diaporthe* sp. isolate EC010 (GenBank accession No. OR143425) showed herbicidal effects of its secondary metabolites on target plants, including pre-emergence herbicidal effects in the form of inhibition of *Amaranthus tricolor* (dicotyledonous weed) seed germination. We hypothesized the *Diaporthe* extract to induce osmotic stress and thereby restrict both amylase activity, which is required for energy supply to root and shoot growth, and mitotic activity (Manichart et al., 2023a). In this study, we investigated the oxidative damage caused by *Diaporthe* sp. extract on the monocotyledonous weed barnyardgrass (*Echinochloa crus-galli*) and studied how treated plants respond via the antioxidant enzyme system. Moreover, we identified the chemical components of the extract.

2 Material and methods

2.1 Fungal culture and isolation of fungal allelochemicals

The fungi used in this study (*Diaporthe* sp. EC010) were initially collected by Manichart, Laosinwattana (Manichart et al., 2023a) in September 2021 from the Ladkrabang district of Bangkok, Thailand. The isolated fungi were cultured via submerged fermentation in potato dextrose broth medium. After the culture period, the biomass was collected and incubated in a hot air oven (45°C). The obtained mycelia were sequentially extracted using organic solvents with increasing polarity, according to Poonpaiboonpipat, Krumsri (Poonpaiboonpipat et al., 2021). Briefly, the dried mycelia were soaked with hexane for 24 hours, then the resulting mixture was filtered through filter paper, and the remaining residue was re-extracted. Next, the solutions were combined and evaporated to obtain the crude hexane extract fraction. The leftover residue was then extracted with ethyl acetate (EtOAc) and ethanol (EtOH) following the same process, resulting in EtOAc and EtOH extract fractions. The phytotoxicity of all extract fractions was assessed using a seed germination assay.

2.2 Seed germination assay

Firstly, each sticky fungus crude extract was dissolved in the corresponding solvent (hexane, EtOAc, or EtOH) at concentrations of 0.625, 1.25, and 2.50 mg/mL. The concentrations were selected based on preliminary experiments that showed these concentrations affected seed germination. Four replicates were maintained per treatment for each of the organic solvent in a completely

randomized design (CRD); treatments with distilled water were used as negative controls. For the germination tests, according to Teerarak, Laosinwattana (Teerarak et al., 2010), 5 mL of each extract solution was added to a Petri dish (9-cm diameter) containing a double layer of germination paper and allowed to evaporate to complete dryness at room temperature. Then, the germination paper in the Petri dish was moistened with 5 mL of distilled water. Twenty healthy seeds of barnyardgrass were placed in each dish. The dishes were kept in a growth chamber (LAC-1075-N, Longyue, Shanghai) with a temperature of $27 \pm 2^\circ\text{C}$, 12/12 h light/dark photoperiod, and relative humidity of about 80%. The germination rate of the grass seeds and the length of seedling roots and shoots (cm) were assessed after seven days of treatment. The optimal crude fraction with the highest inhibitory efficacy on germination and seedling growth was selected for further experiments.

2.3 Chemical characterization via GC-MS

Components of the *Diaporthe* EtOAc crude extract were identified using GC-MS. The extract was diluted in ethyl acetate before being analyzed on an Agilent 6890 N gas chromatograph equipped with an Agilent 5973 mass detector and an HP-5 silica capillary column (30 m x 0.25 mm ID, 0.25 m film thickness). The column temperature program started at 40°C for 3 minutes, then increased to 100°C at a rate of $10^\circ\text{C}/\text{min}$, and then further to 260°C at a rate of $5^\circ\text{C}/\text{min}$, which was held for 5 minutes. The flow rate was 1 mL/min, with helium used as the carrier gas. The MS analysis was performed with a detection range of 30-500 amu. The sample (0.2 μm) was injected using a 50:1 split ratio. The injector and detector were maintained at respective temperatures of 250°C and 270°C . Individual compounds were identified by comparison of MS profiles to a reference library (National Institute of Standards and Technology, NIST, 2014). A percentage peak area relative to the total peak area was used to express the relative amounts of the various components of the total crude extract. Peaks of less than 0.1% area were excluded, whereas all those comprising more than 0.1% were included.

2.4 Seed imbibition and α -amylase activity

The EtOAc crude extract was evaluated for its effect on seed imbibition. Seed imbibition was carried out according to Turk and Tawaha (2003) with modification. In brief, the original grass seed weight (W_1) was recorded, and the seeds were then immersed in the fungal extract for a designated incubation period of 12, 24, or 36 hours. Since barnyardgrass takes approximately 36 hours to complete the germination process (Takao et al., 2011), these time points were selected to monitor the physiological response at each stage of germination. After incubation, the seeds were washed and weighed (W_2) again and water absorption values were determined using the formula:

$$\text{Seed imbibition (\%)} = (W_1/W_2) \times 100.$$

The activity of α -amylase (EC 3.2.1.1) was determined using the 3,5-dinitrosalicylic acid (DNS) test. α -amylase activity was measured as reported by Sadasivam and Manickam (1996). For the enzyme extraction, the treated seeds were grained with 0.1 M CaCl_2 (4 mL) in an ice bath, and centrifuged at $9600 \times g$ for 20 min at a temperature of 4°C . This yielded crude enzyme. Subsequently, the reaction was carried out by adding 1.0 mL of crude enzyme to 1.0 mL of 0.5% soluble starch. The mixture was incubated, and then 1.0 mL of DNS reagent was added, after which the reaction solution was immediately placed in a boiling water bath for 5 minutes. Finally, light absorption at 560 nm was measured and used to calculate the α -amylase activity, represented in $\mu\text{mol maltose}/\text{min}/\text{g}$ (fresh weight, FW).

2.5 Measurement of MDA and H_2O_2 contents

The thiobarbituric acid reactive substance (TBARS) assay was used to determine lipid peroxidation based on estimating malondialdehyde (MDA) content. The sample (0.5 g FW) was ground with 6.0 mL of 3.0% trichloroacetic acid (TCA) and centrifuged for 20 minutes at 4°C at $9600 \times g$. The supernatant (1.0 mL) was then mixed with 2.0 mL of 0.5% thiobarbituric acid (TBA) in 20% TCA. This reaction mixture was heated in a boiling water bath for 20 minutes before cooling on ice for 5 minutes (He et al., 2014). Finally, the absorbance was measured at 532 and 600 nm, and an extinction coefficient of 155 $\text{m}/\text{M}\cdot\text{cm}$ was used to calculate the MDA content. Hydrogen peroxide (H_2O_2) content was calculated following Velikova, Yordanov (Velikova et al., 2000). The treated seeds (0.5 g FW) were homogenized with 6.0 mL of 3.0% TCA and centrifuged ($9600 \times g$) for 20 minutes. Then, 1.0 mL of supernatant was added to 3.0 mL of reaction mixture (10 mM phosphate buffer pH 7.0 and 500 mM potassium iodine). After 15 minutes, the absorbance at 390 nm was measured.

2.6 Extraction and determination of antioxidant enzyme activity

The crude enzyme extract was prepared according to the procedure described by Mir, John (Mir et al., 2018). Briefly, 6.0 mL of 50 mM phosphate buffer containing polyvinylpyrrolidone (PVP) and 0.5 mM ethylenediaminetetraacetic acid (EDTA) was used to extract the treated barnyardgrass seeds (1.0 g FW). The homogenates were centrifuged, and the resulting crude enzyme extracts were used to test enzyme activity. The absorbance values of soluble proteins were determined colorimetrically at 595 nm using the Bradford method (Bradford, 1976). All the biochemical indexes obtained were determined in four replicates. Superoxide dismutase (SOD), catalase (CAT), and guaiacol peroxidases (POD) were determined via colorimetric assay following the method described by Chen and Zhang (2016). SOD (EC 1.15.1.1) activity was determined at 560 nm using nitro-blue tetrazolium (NBT) photoreduction. The reaction

mixture was composed of 100 mM potassium phosphate (pH 7.8) containing 130 mM methionine, 750 μ M NBT, 100 mM EDTA and 20 μ M riboflavin. Enzyme extract (1.0 mL) was added to the reaction mixture, except in the case of the control. The mixture was then incubated in light for 30 minutes and the specific activity determined, expressed in SOD unit/mg protein. One unit of SOD is defined as the amount that inhibits the photoreduction of NBT by 50%. Similarly, to determine CAT (EC 1.11.1.6) activity, the crude enzyme extract (1.0 mL) was added to a CAT reaction mixture, which included phosphate buffer (100 mM, pH 7.0) and H₂O₂ (10 mM). The change in absorbance because of H₂O₂ consumption was immediately monitored at 240 nm at every 15 seconds for 1 minute, looking for steady average alteration. The enzyme activity was then calculated and represented as unit/mg protein. One unit of CAT is defined as the amount of enzyme that decreases the absorbance at 240 nm by 0.1 per minute. Finally, guaiacol was used to measure POD (EC 1.11.1.7) activity. In this assay, 1.0 mL of enzyme extract was added to a mixture of phosphate buffer (100 mM, pH 7.0), EDTA (0.1 mM), guaiacol (0.5%), and H₂O₂ (1.0 mM). The increase in absorbance caused by guaiacol oxidation was immediately measured at 470 nm every 15 seconds for 1 minute. The determined enzyme activity was expressed as unit/mg protein. One unit of POD is defined as the amount of enzyme that increases the absorbance at 470 nm by 0.01 per minute.

2.7 Statistical analysis

Experiment was arranged in a completely randomized design with four replicates and were repeated two times. Each sample was collected in four replicates for biochemical analyses. The results are shown as means. All data were analyzed using the SAS software and subjected to analysis of variance (ANOVA) and a comparison of means by Tukey's multiple range tests ($p \leq 0.05$). Means followed by the same letter(s) are not different.

3 Results and discussion

3.1 Effect on *E. crus-galli* germination and early growth

Diaporthe sp. EC010 powdered mycelia were weighed and subjected to sequential extraction. The highest extraction yield was obtained from the EtOH fraction, followed by the EtOAc and hexane fractions respectively (data not shown). Table 1 shows the effect of these extracts on *E. crus-galli* germination and seedling growth. For all extracts, germination percentage decreased as the extract concentration increased. The EtOAc fraction had significantly greater impact on barnyardgrass germination compared to the other solvents; at the highest tested concentration of 2.50 mg/mL, the respective inhibition percentages were 81.01%, 24.05%, and 12.66% for the EtOAc, EtOH, and hexane fractions (Figure 1). Apart from its impact on seed germination, the EtOAc fraction also had an effect on the growth of *E. crus-galli* seedlings. Physical examination of the shoot and root lengths showed that the EtOAc fraction treatment decreased both of them across the concentration

TABLE 1 Respective effects of sequential *Diaporthe* solvent extracts on the germination and growth of *Echinochloa crus-galli* seeds.

Solvent (Concentration; mg/mL)	Inhibition level (% over control)		
	Germination	Shoot	Root
Hexane (C ₆ H ₁₄) fraction 0.625 mg/mL 1.25 mg/mL 2.50 mg/mL	0.00 e	-3.71 d	-5.92 d
	1.27 de	2.14 cd	-0.68 d
	12.66 cd	3.42 c	19.36 c
Ethyl acetate (C ₄ H ₈ O ₂) fraction 0.625 mg/mL 1.25 mg/mL 2.50 mg/mL	8.86 d	23.11 b	35.42 b
	54.43 b	73.04 a	82.35 a
	81.01 a	84.74 a	89.18 a
Ethanol (C ₂ H ₆ O) fraction 0.625 mg/mL 1.25 mg/mL 2.50 mg/mL	1.27 de	-10.13 d	3.64 d
	7.59 d	8.70 c	19.13 c
	24.05 c	20.40 b	40.89 b

Different letter(s) in the same column indicate significant differences according to Tukey's multiple range test at the $p < 0.05$ level.

range. Notably, grass seedling roots were extremely restricted compared to the shoots (Laosinwattana et al., 2018; Somala et al., 2022). Because the root of the *E. crus-galli* seedlings soaked in Petri dishes containing the extract, the inhibitory effect of the extract on the root of the seedlings was found to be greater than that on the shoot. Teerarak, Charoenying (Teerarak et al., 2012) similarly studied the effect of *Aglaia odorata* EtOAc extract in wettable powder formulation on *E. crus-galli* germination and initial growth, in which treated seeds showed lower imbibition and α -amylase activity. Regarding the differing results of the three extracts tested here, differences in inhibitory efficacy may be caused by several factors such as composition, solvent solubility, and solvent polarity. Thus, inhibitory activity is well-established as varying across extracts made using different solvents (Li et al., 2006; Luthria et al., 2007; Garcia et al., 2010). These reports support that it is imperative to use suitable solvent solutions when performing selective extraction on natural sources in order to get fractions with high allelopathic potential and high crude extract yield. As the assay results indicated the EtOAc fraction to have the most powerful inhibitory efficacy, that fraction was used in the subsequent experiments.

3.2 Chemical constituents of the *Diaporthe* EtOAc crude extract

Table 2 lists the chemical components identified by GC-MS in the *Diaporthe* mycelia EtOAc crude extract. This analysis revealed the extract to contain a variety of phytochemicals (Figure 2), with 40 identified components collectively accounting for 86.44% of the total crude extract. The main constituents (Figure 3) were linoleic acid, butyl ester (9.69%), hexadecenoic acid (7.99%), and 14-pentadecenoic acid (7.86%). These findings are consistent with the prior reports of da Rosa, Sauzem (da Rosa et al., 2021) and dos Reis, da Rosa (dos Reis et al., 2019), who found fatty acid esters and diaza-compounds to be the major chemical constituents of *Diaporthe schini* extracts. Notably, the specific activities of metabolic enzymes (Winter et al., 2011; Tanapichatsakul et al.,

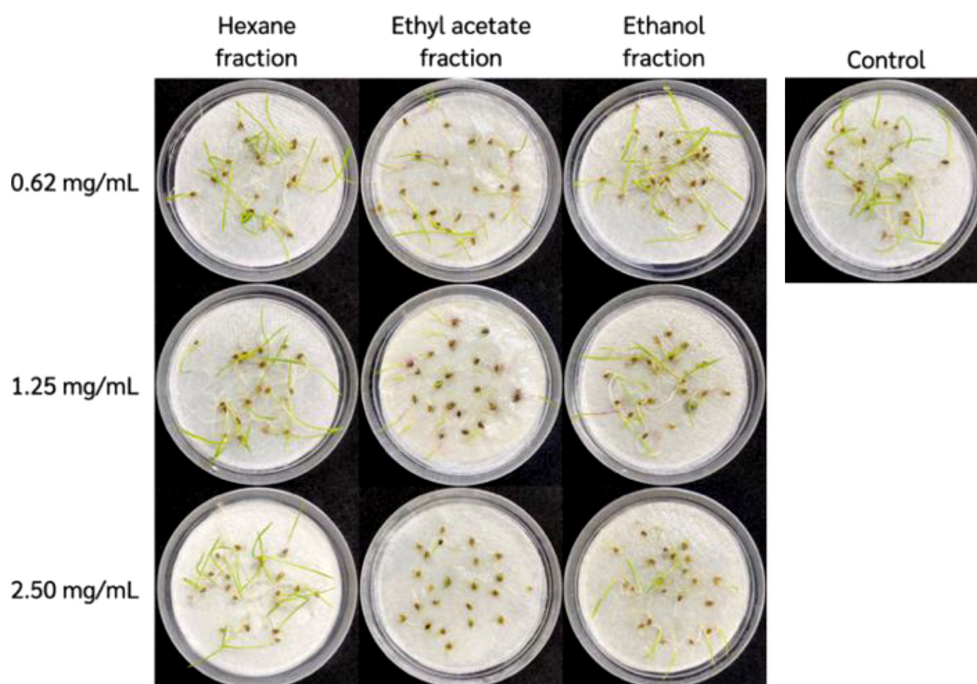


FIGURE 1
Inhibitory effects observed during bioassays with sequential solvent extracts of *Diaporthe sp. mycelia*.

TABLE 2 Constituents of the *Diaporthe sp.* ethyl acetate (EtOAc) crude extract.

No.	Constituent	RT ^a	% of total ^b
1	Butanoic acid	3.49	1.24
2	Cyclopentacycloheptene	10.73	0.77
3	1,1-Dimethyl-2-propylcyclohexane	12.46	1.07
4	4-Isopropyl-1,3-cyclohexanedione	12.58	1.28
5	2-Ethyl-1,1,3-trimethylcyclohexane	12.70	1.22
6	4,6-Dimethylundecane	14.52	0.75
7	Phytane	14.87	0.54
8	2,6,10,15-Tetramethylheptadecane	14.95	0.58
9	2-Propyl-1-heptanol	15.49	1.30
10	2-Octyldodecanol	15.75	0.70
11	Hexadecane	16.17	0.72
12	Asarone	16.46	0.67
13	Pentadecane, 2,6,10-trimethyl-	17.06	0.52
14	Heneicosane, methyl-	17.16	0.59
15	Heptadecane	17.30	0.92

(Continued)

2019) and species diversity may lead to chemical constituent differentiation. The phytotoxicity observed in this study could be associated with these identified chemicals and possible synergies among them.

3.3 Effect on seed imbibition and α -amylase activity

We first investigated whether seed imbibition, the initial phase in the seed germination process, is disrupted by the *Diaporthe* EtOAc crude extract. In the first 12 hours following immersion of grass seeds in the extract or distilled water, the absorbing rate was high and not significantly different ($p > 0.05$) from the control. From 12 to 24 hours, the seeds absorbed more water than in the first stage. Then, from 24 to 36 hours, *Diaporthe* extract clearly disrupted seed imbibition, depending on the treatment concentration and duration (Figure 3A). This can be attributed to the extract changing the water potential, which caused osmotic stress (Manichart et al., 2023a). Decreased imbibition suggests that water and mineral nutrient uptake is limited, which restricts seedling growth (Kamran et al., 2020), as supported by previously published studies (Teerarak et al., 2012; Laosinwattana et al., 2018; Manichart et al., 2023b).

We further investigated the effect of the *Diaporthe* extract on α -amylase activity, shown in Figure 3B. During seed germination, the main energy source is the endosperm starch and its breakdown. This process depends upon several enzymes, including α -glucosidase, debranching enzyme, and the α - and β -amylases

TABLE 2 Continued

No.	Constituent	RT ^a	% of total ^b
16	Hexadecane, 2,6,11,15-tetramethyl-	17.37	0.49
17	3-Pyrrolidin-2-yl-propionic acid	17.47	2.86
18	2-Methylcyclododecanone	17.77	1.22
19	Cyclopropanemethanol, 2-methyl-	17.86	1.89
20	1,4-Diaza-2,5-dioxobicyclo [4.3.0]nonane	17.96	6.25
21	(-)-4-Terpineol	18.07	2.33
22	Cyclopentane, propyl-	18.17	1.39
23	Nonahexacontanoic acid	18.31	0.84
24	Celidoniol, deoxy-	18.40	0.65
25	Cyclohexane, 1-ethyl-2-propyl-	18.45	0.77
26	1,4-Diaza-2,5-dioxo-3-isobutyl bicyclo[4.3.0]nonane	18.67	2.08
27	Octadecane	19.68	0.61
28	2-Benzylidenemalononitrile	19.97	0.90
29	Cyclo(Pro-Leu)	20.23	1.41
30	Hexadecanoic acid	20.66	7.99
31	Ethyl palmitate	21.25	1.97
32	Linoleic acid	24.23	3.32
33	Ethyl linoleate	24.78	4.79
34	9-Octadecenoic acid (Z)-, ethyl ester	24.88	2.55
35	Hexadecanamide	25.07	1.77
36	t-Butyl palmitate	25.19	6.10
37	Linoleic acid, butyl ester	27.55	9.69
38	14-Pentadecenoic acid	27.61	7.86
39	Octadecanoic acid, butyl ester	27.92	3.15
40	Octyl phthalate	29.99	0.69
	Total		86.44%

^aRetention time.

^bRelative area percentage (peak area relative to the total peak area, %).

(Amato et al., 2023), of which α -amylase functions throughout the germination process to break down starch into smaller molecules, thereby providing the nutrients and energy required for germination (Li et al., 2019). After treating grass seeds with *Diaporthe* extract, α -amylase was extracted from the seeds and its activity determined by *in vitro* generation of maltose. As a result, degradation of α -amylase activity was inhibited by all concentration of the extract correlate to the germination (Table 1). Overall, α -amylase activity was decreased in extract-treated grass seeds compared to controls. Moreover, the extract inhibited α -amylase activity in a dose-dependent manner, with the highest tested concentration (2.50 mg/mL) demonstrating a remarkably

extensive decrease of α -amylase activity at 36 hours after treatment ($p < 0.05$). This finding is compatible with the seed imbibition assay results, and aligns with the literature on the herbicidal effects of fungal extracts from *Alternaria brassicicola* and *Diaporthe* sp. isolate EC010 on amaranth, a dicotyledonous weed plant (Manichart et al., 2023a; Manichart et al., 2023b). According to the current study's findings, one of herbicidal properties of this extract is the inhibition of α -amylase activity. Because starch fails to break down into tiny molecules to support growth and development, this impact may in turn result in inhibition of seed germination and seedling growth.

3.4 Effect on MDA, ROS generation, and activities of antioxidant enzymes

The degree of ROS activity in plant cells is contingent on the ability of ROS to move across cell membranes. Among ROS, H_2O_2 is the most stable and easily moves through membranes over relatively long distances, even in dry seeds. Ratajczak, Malecka (Ratajczak et al., 2015) and Kurek, Plitta-Michalak (Kurek et al., 2019) demonstrated a significant correlation between H_2O_2 levels and reduced germination ($r = -0.9471$, $p = 0.004139$). Accordingly, in this study, we focus on the accumulation of H_2O_2 in *E. crus-galli* seeds as a window into the role of oxidative stress in the mechanism of action of the *Diaporthe* sp. EtOAc fraction (Figures 4A, B). Plant cell membranes are composed of fatty acids and other lipids, and thus free lipids could be released from degraded cell membranes as the result of oxidative action (Scrivanti et al., 2003). Lipid peroxidation of fatty acids ultimately leads to accumulation of MDA (Marnett, 1999). In the present study, seed MDA and H_2O_2 content were observed to be significantly elevated after 24 and 36 hours of exposure to 2.50 mg/mL of the fungal extract, with 84.47% and 85.52% increases in MDA and 137.17% and 141.10% increases in H_2O_2 concentration, respectively. Thus, treatment with the extract significantly increased H_2O_2 production and caused lipid peroxidation, resulting in induced oxidative damage to the *E. crus-galli* seeds (Oracz et al., 2009; Ma et al., 2016). H_2O_2 has been considered both a beneficial (Oracz et al., 2009; Bahin et al., 2011) and detrimental factor with regard to cellular events in seed germination. As it regulates gene expression and phytohormone signaling (abscisic acid, gibberellins, auxins, and ethylene) (Bailly, 2019), a moderate level of H_2O_2 is beneficial and promotes germination, whereas excessive H_2O_2 content induces oxidative damage, which prevents or delays germination (Kurek et al., 2019). This dual role has been observed in cotton seeds, for which higher levels of H_2O_2 and lipid peroxidation correlated with reduced germination (Goel and Sheoran, 2003), and in aging sunflower seeds by Kibinza, Vinel (Kibinza et al., 2006), who established a linear association between H_2O_2 content and germination. Likewise, declining viability of wheat seeds was found to be dependent on both H_2O_2 production and the level of lipid peroxidation (Kong et al., 2015), which modifies membrane permeability and thereby decreases seed viability.

Antioxidant enzymes play an important role in redox regulation by balancing ROS levels. We found SOD activity to be significantly

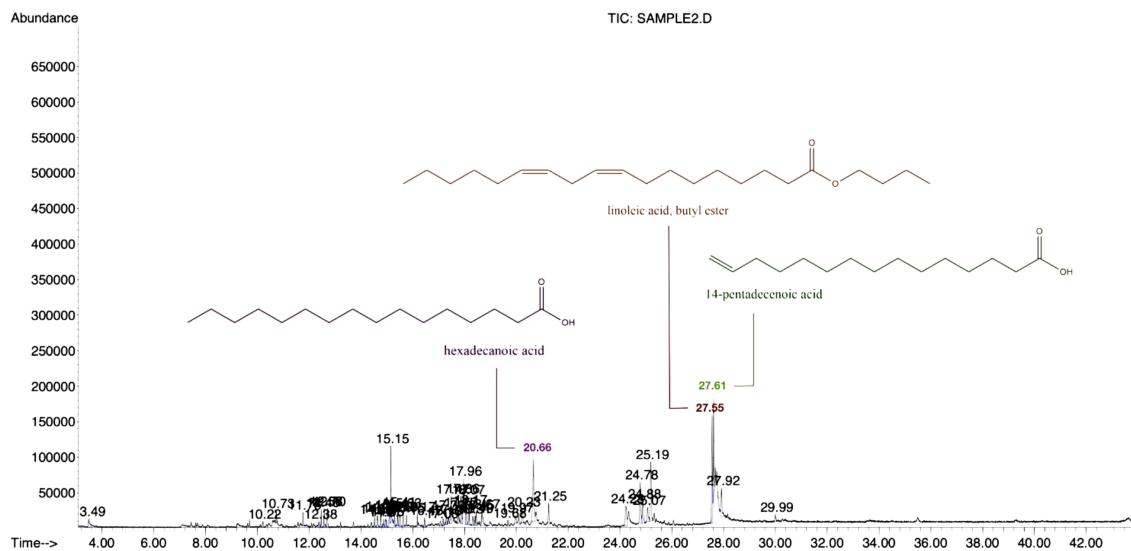
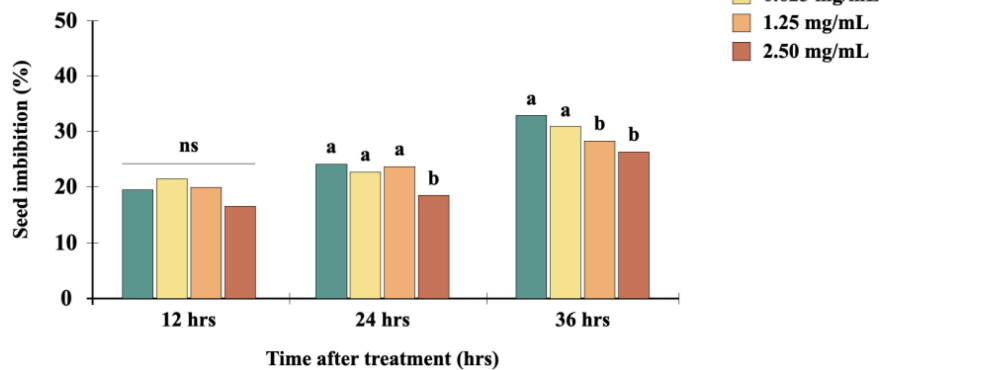


FIGURE 2 GC-MS chromatogram and chemical structure of the main compounds of the *Diaporthe* sp. EtOAc crude extract showing the retention times (min) of the peaks.

(A) Seed imbibition



(B) α-amylase activity

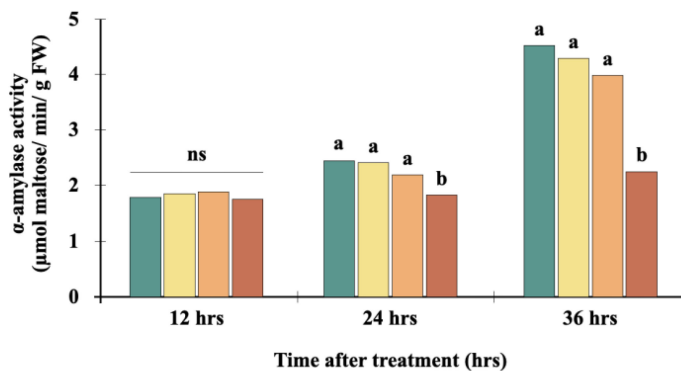


FIGURE 3 Effect of the *Diaporthe* sp. EtOAc crude extract on imbibition (A) and α-amylase activity (B) of *E. crus-galli* seeds. For a given exposure time, bars with different letters (a, b) among treatments are significantly different at $p < 0.05$ according to Tukey's multiple range test. "ns" indicates no significant difference. Data are mean values (n=4).

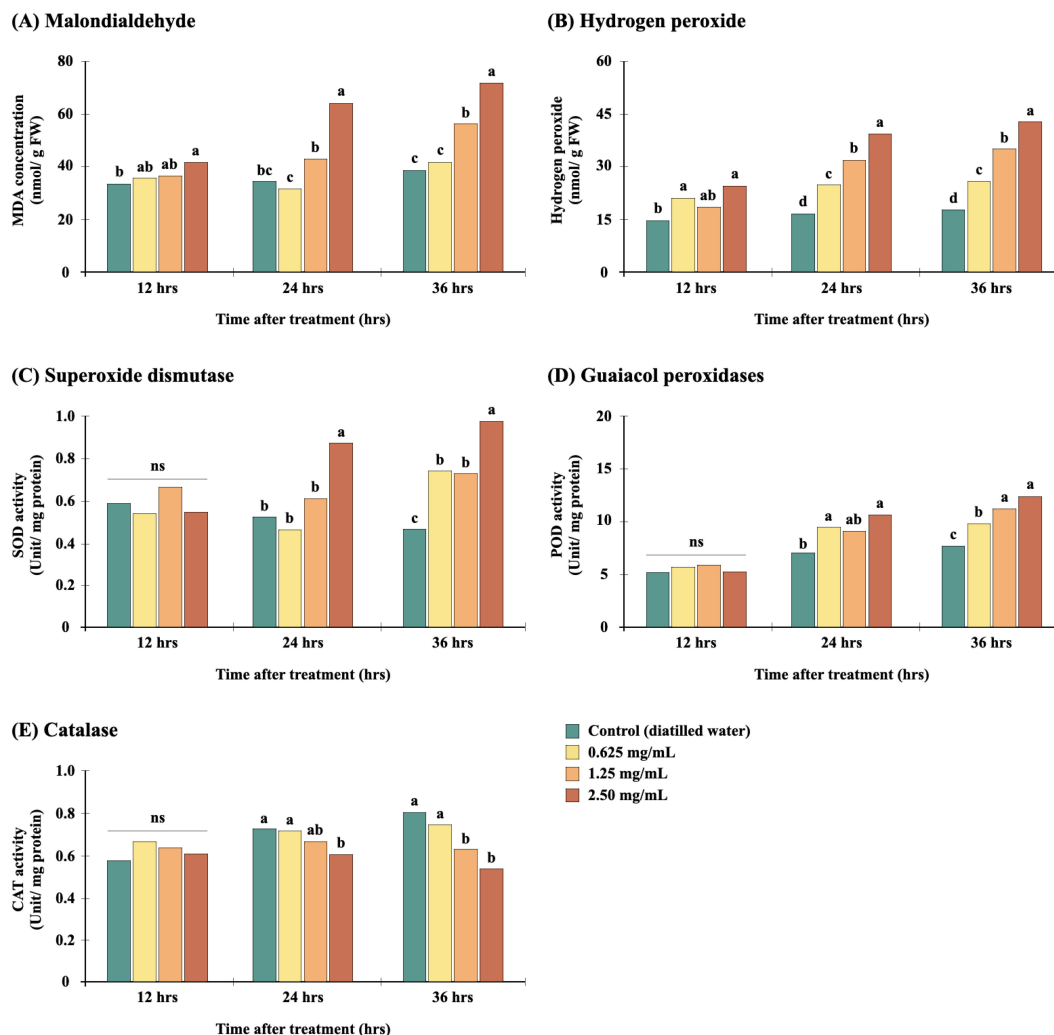
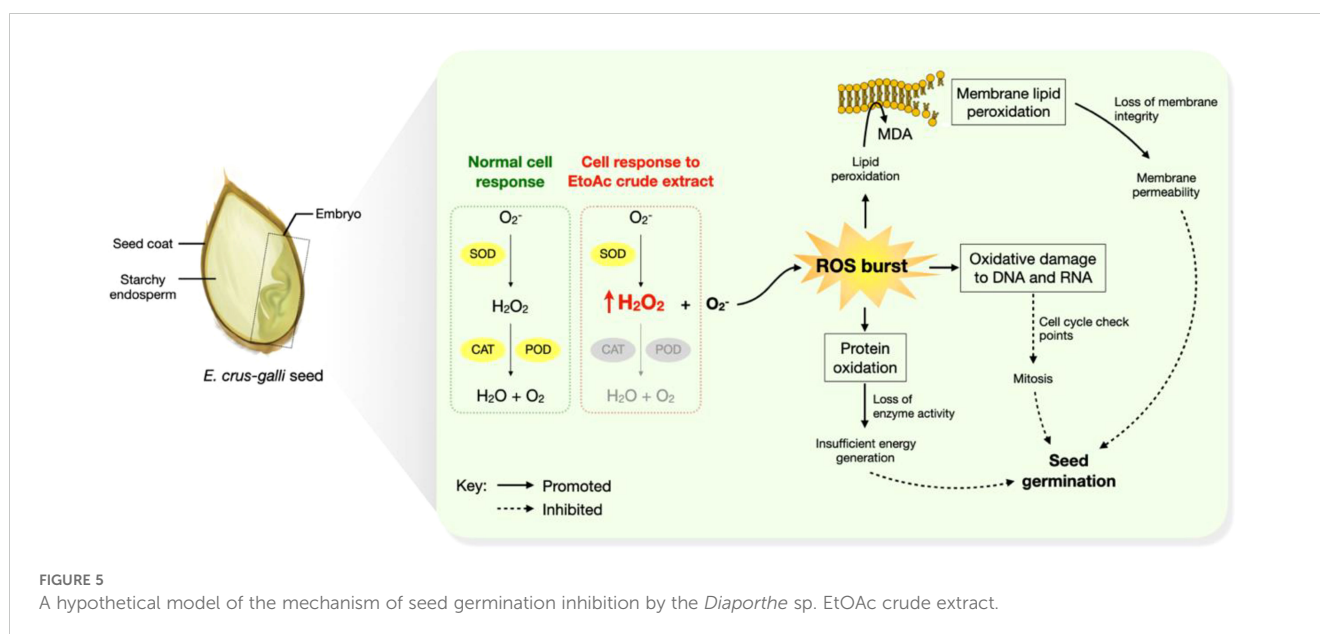


FIGURE 4

Effect of the *Diaporthe* sp. EtOAc crude extract on MDA (A) and H_2O_2 (B) content and SOD (C), POD (D) and CAT (E) activity in *E. crus-galli*. For a given exposure time, different letters (a, b) above bars indicate significant differences at $p < 0.05$. "ns" indicates no significant difference. Data are mean values ($n=4$).

increased in *E. crus-galli* seed exposed to the *Diaporthe* sp. extract (Figure 4C), reaching 67.24% over control ($p < 0.05$) at 36 hours after germination. POD activity also consistently increased as the germination period progressed (Figure 4D), with the maximum dose producing increases of 49.87% and 61.62% at 24 and 36 hours, respectively. Meanwhile, unlike SOD and POD, CAT activity showed a decreasing tendency in a dose-dependent manner (Figure 4E), indicating the extract to downregulate CAT enzymatic activity. As SOD and POD are involved in ROS scavenging (Shuai et al., 2011; Bhardwaj et al., 2021), their higher activities in treated *E. crus-galli* seeds may indicate induction of these antioxidant enzymes to help cells cope with the extract-induced oxidative stress and minimize free radical development (Narayan et al., 2024). A previous study suggested higher proline activity in *Amaranthus tricolor* treated with *Diaporthe* extract (Manichart et al., 2023a). Proline also serves as a free radical scavenger, protecting against protein denaturation and membrane degradation (Kumar et al., 2018;

Kamran et al., 2020). Nonetheless, as the *Diaporthe* extract in this study demonstrated negative effects on seed germination regardless of the concentration applied, the induced SOD and POD was not sufficient to inhibit the proliferation of ROS and MDA. The significant decrease in CAT activity likely exacerbated oxidative bursts. In a similar study of sunflower seeds, CAT enzymatic activity was observed to decrease under accelerated oxidative stress (Kibinza et al., 2011). Moreover, Ratajczak, Małeczka (Ratajczak et al., 2015) reported decreased activity of CAT in beech seeds and a strong negative correlation ($r = -0.9177$) of CAT activity with the level of H_2O_2 . In our hypothetical model, shown in Figure 5, the decrease in CAT activity might have resulted in ROS accumulation, leading to lipid peroxidation and enzyme inactivation, which could in turn promote structural and functional damage to proteins and carbohydrates and modification or disruption of DNA structure (Sharma et al., 2012). Such events would explain the reduced germination percentage observed in extract-treated seeds.



Our study has demonstrated the potential of fungal allelochemicals in inhibiting weed growth through oxidative damage, contributing valuable insights to the limited literature on this topic. Future work will focus on optimizing the formulation of the extract, investigating its selectivity, and conducting comprehensive field trials. We will also assess potential environmental impacts to ensure the safety and sustainability of this approach. This pioneering study serves as a foundation for the development of fungus-derived herbicides as an alternative weed control management strategy.

4 Conclusion

This study explores the inhibitory effects of *Diaporthe* sp. strain EC010 crude extracts on barnyardgrass germination and growth. The ethyl acetate fraction most strongly reduced seed germination and grass development. GC-MS of that fraction identified its major constituents to include linoleic acid, butyl ester (9.69%), hexadecanoic acid (7.99%), and 14-pentadecenoic acid (7.86%). Treated *E. crus-galli* seeds exhibited lower imbibition, reduced α -amylase activity, increased MDA, and H_2O_2 accumulation. They also demonstrated higher SOD and POD activities but lower CAT activity, which may correlate with delayed germination and seedling growth. All told, this study constitutes the investigation of oxidative damage caused by *Diaporthe* extract. This research highlights the potential of *Diaporthe* extract as a candidate for pre-emergence herbicidal products. Although the findings are promising, they are based on laboratory assays and focus on a single weed species. Further studies are essential to determine the efficacy of *Diaporthe* extracts under field conditions, evaluate their effects on crop species, soil health, and environmental factors, and understand their roles in integrated weed management strategies.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

Author contributions

NS: Conceptualization, Investigation, Project administration, Writing – original draft. NM: Formal Analysis, Methodology, Writing – original draft. CL: Conceptualization, Funding acquisition, Resources, Supervision, Writing – review & editing. PW: Formal Analysis, Validation, Writing – review & editing. KY: Formal Analysis, Validation, Writing – review & editing. MT: Formal Analysis, Investigation, Methodology, Writing – original draft. NC: Formal Analysis, Validation, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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