Journal of Agricultural Science and Technology (JAST), 29(1)
In Press, Pre-Proof Version

Toxic Effects of Thiram on *Fusarium* phytopathogens, L929 Fibroblast Cell Lines, and Wheat Seeds

Mehmet Arslan[1], Tuğba Teker[2], Büşra Nur Çetin[1], Emre Yörük[3], and Gülruh Albayrak[4]*

Abstract

Antifungal and cytotoxic effects of thiram (THR) were investigated on *Fusarium graminearum* PH-1 and *F. culmorum* FcUK99, and mouse fibroblast cell line L929, respectively. THR's minimum inhibitory concentration (MIC) against PH-1 and FcUK99 was 165 µg/mL. The MIC_{25}, MIC_{50}, and MIC_{75} significantly decreased the linear growth rates (LGRs) of both strains (P<0.0001) and reduced cell viability by 18.30%, 32.06%, and 45.45% in PH-1 and 33.53%, 51.68%, and 76.1% in FcUK99, respectively. The dose-dependent increase in intense green fluorescence observed in both *Fusarium* strains after 2',7'-dichlorodihydrofluorescein diacetate staining, indicated oxidative stress. The apoptosis-like process was visualized through the increase in orange fluorescence emissions with acridine orange/ethidium bromide dual staining. THR reduced deoxynivalenol (DON) and 15-ADON production in PH-1 while DON and 3-ADON production in FcUK99. THR doses (0.32-165 µg/mL corresponding to 1.34-686.24 µM) decreased cell viability of the L929 cells by 4.56% (P<0.0001)-97.95% (P>0.05) at 24th hour and from 4.88% (P>0.05)-88.68% (P<0.0001) at 48th hour. The MIC did not significantly affect (P> 0.05) the germination rates of wheat seeds, root and plantlet lengths, and water loss. Although THR decreased the growth and mycotoxin production of phytopathogens and didn't adversely affect plant development, because of its inhibitory effect on the cell line, it must be evaluated as a potential risk to public health, and its utilization ought to be regulated through legislation.

Keywords: Apoptosis, Cytotoxicity on animal cell line, Fungal phytopathogens, Oxidative stress, Thiram.

Introduction

Fusarium graminearum and *F. culmorum* are the dominant species causing both Fusarium Head Blight and Crown Rot diseases. Due to the disease, grains of various cereal crops become small and shriveled. Crown Rot affects the roots and lower stems. The disease can be fatal as

[1] Programme of Molecular Biotechnology and Genetics, Institute of Graduate Studies in Sciences, Istanbul University, Istanbul, Türkiye.
[2] Department of Molecular Biology and Genetics, Faculty of Engineering and Natural Sciences, Istanbul Atlas University, Istanbul, Türkiye.
[3] Department of Molecular Biology and Genetics, Faculty of Arts and Science, Istanbul Yeni Yuzyil University, Istanbul, Türkiye
[4] Department of Molecular Biology and Genetics, Faculty of Science, Istanbul University, Istanbul, Türkiye.
*Corresponding author; e-mail: gulruh@istanbul.edu.tr

it restricts the flow of water and nutrients to developing heads. When Crown Rot is not deadly, it produces pre-harvest in pinched grains or grainless ears. The reduction of food quality due to both diseases results in economic losses. Grains contaminated with these pathogens, or their mycotoxins also threaten public health due to their mycotoxin production capabilities (Ghimire et al. 2020). Deoxynivalenol (DON), nivalenol (NIV), and zearelanone (ZEN) are commonly detected fusaria-mycotoxins in grains and processed foods (Yli-Mattila et al. 2022). The control strategies developed against *Fusarium* species will also accelerate control approaches against *Microdochium* spp., which were recently identified as another causal agent of Fusarium Head Blight, although they have different chemical control products (Bouaicha et al. 2022; Alisaac and Mahlein 2023). In contrast to *Microdochium* spp., the ability of the two *Fusarium* species to produce various mycotoxin types simultaneously is the reason why they are considered a serious threat not only to crop yields but also to public health (Xu et al. 2008).

The Food and Agriculture Organization of the United Nations (FAO) determined that 72% of the world's grains are contaminated with mycotoxins, and the rate of food crops is approximately 25% (FAO, 1977; Eskola et al. 2020). FAO also predicted that this average would remain consistent over the years (Smith et al. 1995; Streit et al. 2013). Cargill, an American global food corporation, and Dutch State Mines (DSM) have detected those rates above FAO's predictions. Cargill reported that 92% of the sampled crops were contaminated with at least one mycotoxin and 84% with more than four (Cargill 2022; DSM 2022). Animals that consume mycotoxin-contaminated crops and grains transmit these toxins to other animals and humans that consume them. Phytopathogenic fungi can survive in the air, water, and soil habitats, and they easily spread in extreme conditions in agricultural areas (Rose et al. 2003; Sobrova et al. 2010; Cinar and Onbasi 2020; Shikhaliyeva et al. 2020). For these reasons, chemical methods based on the use of fungicides with growth inhibitory or lethal effects on fungi are the most widely used methods in the fight against both *Fusarium* species.

Fungicides with a wide variety of chemical structures and properties have been classified by the Fungicide Resistance Action Committee according to cross-resistance patterns and mode of action. Thiram (THR) is a bistidiocarbamate fungicide that has been frequently used in modern agriculture to ensure food safety against fungal pathogens. THR functions through the formation of a complex with heavy metal ions, inhibiting spore germination and mycelial growth, and multi-site activity (Horsfall 1956, PPDB 2025). In addition, its high doses are used as a repellent for birds, rabbits, rodents, and deer in fields and orchards due to its aversive taste (Liu et al. 2022; PPDB 2025).

Antifungal compounds are increasingly important general pollutants of the ecosystem as they are increasingly being used for global food security (Zubrot et al. 2019). Investigating the toxicity and action mechanisms of THR is important not only for public health but also for the environment (Liu et al. 2022; PPDB 2025). The effects of THR applications, alone and/or in combination with other agents, on the growth and virulence of various *Fusarium* spp. have been studied (Fravel et al. 2005, Zhang et al. 2009; Osman et al. 2012; Tobih et al. 2015; Jamil and Ashraf 2020). However, there is still limited information about its molecular and cellular mechanisms in *Fusarium* spp.

The effects of THR on cell viability, oxidative stress, apoptosis, and DON production in *F. graminearum* and *F. culmorum* reference strains, and its inhibitory effect on the L929 cell line were investigated in this study. The physiological responses to THR treatment were also evaluated in *Triticum aestivum* L. cultivar KATE-1.

Materials and Methods

Culture conditions of reference strains and cell line L929

Fusarium PH-1 reference strain (GenBank: GCA_000240135.3), predominantly produced DON and 15-ADON, and FcUK99 reference (GenBank: GCA_900074845.1), predominantly produced 3-ADON, were grown on potato dextrose agar (PDA) (Biolife, 4019352) at 26±2 °C with 55-60% moisture for 7 days. However, both references produce trace amounts of DON, NIV, and their acetylated derivatives (Akpinar et al. 2025). L929 mouse fibroblast cells were seeded in Dulbecco's Modified Eagle Medium (DMEM; PANBIOTECH, P04-0590) supplemented with 10% (v/v) fetal bovine serum (Gibco, 10270-106), 1.25 µg/mL amphotericin B (Capricorn, AMP-B), 50 U/mL penicillin, and 50 µg/mL streptomycin (Gibco, 15140-122) and grown at 37°C in an air atmosphere with 5% CO_2. The medium was refreshed every two days, and the cells were trypsinized when they reached 80% confluency. Well-grown L929 cells were seeded into 96-well plates containing 200 µL DMEM at a density of 1×10^4 cells/well 12 hours before THR application.

Determining the antifungal effects

The minimum inhibitory concentrations (MIC) of THR on PH-1 and FcUK99 were determined using the agar dilution technique (Albayrak et al. 2023), along with the values required to inhibit growth by 25% (MIC_{25}), 50% (MIC_{50}), and 75% (MIC_{75}). THR (T24201, Sigma-Aldrich) was added to the PDA ranging from 0.5 to 165 µg/mL concentrations. The culture grown in the medium without the antifungal agent was used as the non-treated control, while

the culture grown in the medium containing acetone, the solvent of THR, was used for normalization in the experiments. The cultures' diameter values were measured on the 4th and 7th day, and the linear growth rates (LGRs) were determined as mm/day (Teker et al. 2024). The cytotoxicity of MIC_{25}, MIC_{50}, and MIC_{75} THR doses on reference strains was assessed by WST-1 analysis (Albayrak et al. 2023). All experiments were carried out three times using independent biological replicates.

Apoptosis and oxidative stress were displayed through the staining with acridine orange/ethidium bromide (Ao/Eb) and 2′,7′-dichlorodihydrofluorescein diacetate (DCF-DA), respectively (Teker et al. 2021). Data from fluorescence images (Olympus BX-53) were processed by Olympus Cellsens 1.9 microscopy software. Experiments were conducted in triplicate using three biological replicates, each with three technical replicates.

Determining the growth inhibition on L929 cell line

L929 cells were treated with THR concentrations in µM corresponding to 0.32-165 µg/mL approximately 12 hours after seeding on DMEM. The µM concentrations were calculated using the following formula [Concentration (µM) = mass (g) / volume (L) × molecular weight (g/mol)) × 10^6]. In this formula, mass represents the amount of THR in grams, volume denotes the volume of the DMEM medium in liters, and molecular weight refers to the molecular weight of THR expressed in g/mol. The multiplication by 10^6 was performed to convert the concentration from molar to micromolar units. Cell viability was analysed from the experimental sets, the untreated (non-treated) negative control group and the acetone treated group, treated with only 0.83% acetone. After 24 and 48 hours of THR exposures, 0.25 mg/mL Mosmann's Tetrazolium Toxicity (MTT) was added to each well and then incubated for 3 hours in the dark at room temperature. The medium was removed. Formazan crystals, formed by reducing tetrazolium salt, were dissolved by adding 200 µL of DMSO to each well. Absorbance (Abs) was measured at 570 nm. Cell viability percentages were calculated using the following formula: ($Abs_{Experiment}/Abs_{Untreated\ control}$) × 100. The experiment was performed in eight independent biological replicates.

Moreover, L929 cell morphologies were investigated through an inverted microscope (Olympus CKX31) at the 24th and 48th hours in the experimental sets, negative control, and acetone treated groups.

Evaluating the effects of THR on T. aestivum cultivar KATE-1 wheat physiological parameters

The KATE-1 seeds, susceptible to *Fusarium* diseases, were treated with 2% sodium hypochlorite (NaOCl) for 5 minutes, washed three-times with double deionized water (ddH_2O), soaked in 165 µg/mL THR (control group seeds only in ddH_2O) for 12 hours, and then dried on filter paper. Subsequently, 30 seeds were planted in 15 cm Petri dishes containing water agar (1.5% w/v) and kept at 4°C for 24 h. The seeds were cultivated at 25°C, 75% humidity, under 1400 lux fluorescent light for 5 days. The number of germinated seeds was counted. Plantlet and root lengths were measured using a digital caliper. The fresh weights (FW) of the leaves were determined (time 0) and the weight (W24) was recorded after plantlets were placed on filter paper for 24 hours. The dry weight (DW) was determined by drying at 60°C for 24 hours. The water loss rate (WLR) was calculated using the following formula: WLR ($g \cdot h^{-1} \cdot g^{-1} \cdot DW$) = (FW - W24) / (DW x 24). The experiments were conducted in triplicate.

Mycotoxin extraction and thin layer chromatography (TLC)

Mycelium (0.1 g) collected from the control, acetone treated, and experimental groups was homogenized using mortar and pestle with 5 mL ethyl acetate. The homogenate was centrifuged at 5000 rpm for 5 min, and the ethyl acetate was removed by evaporation. The remaining residue containing DON and its acetylated derivatives was suspended in 100 µL of acetonitrile.

Silica gel F_{254} and ethyl acetate: toluene (1:1) mix were used as the stationary and mobile phases, respectively, in the TLC. 5 µL samples were loaded on silica gel with the following standards: DON (Sigma; D0156) and B-trichothecene mix (Sigma; 34134), and run for 15 min. After the silica gel was dried and aluminum chloride: ethanol (1:99 (w/v) was sprayed, silica gel plates were visualized under UV light at 365 nm and the retention factor (Rf) values of both standards and each sample were calculated by dividing the distance travelled by the compound spot (cm) by the distance travelled by the solvent front (cm), measured from the point of sample application. The following formula was used to calculate the Rf values [Rf = distance travelled by the compound / distance travelled by the mobile phase]. Experiments were performed in triplicate using biological replicates.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 9 software. Differences among groups were analysed using one-way analysis of variance (ANOVA) followed by Tukey's post

hoc multiple comparison test. Data are presented as mean ± standard error of the mean (SEM). The confidence interval was 0.05. Normal distribution was assessed using the Shapiro-Wilk test. The Pearson's correlation test was used to determine the strength and direction of the relationship between the tested groups.

Results

Effects of THR on fungal growth and cell viability

The MIC of THR was found to be 165 µg/mL for both reference strains. The MIC_{25}, MIC_{50}, and MIC_{75} were calculated as 41.25 µg/mL, 82.5 µg/mL, and 123.75 µg/mL, respectively. The growth inhibition values increased in both references as the THR doses increased (Online Resource Fig. S1). The dose-dependent decrease in LGRs in both PH-1 and FcUK99 cultures was determined (Fig. 1a). The differences between the LGRs of control and experimental groups for both references were statistically significant ($P<0.0001$) while those between control and acetone treated groups were non-significant. While the cell viabilities of both strains were affected in a dose-dependent manner THR doses decreased the cell viability of FcUK99 more than PH-1. The cell viability values of PH-1 were determined as 81.70%, 67.94%, and 54.55% at MIC_{25}, MIC_{50}, and MIC_{75} doses, respectively. Cell viability values for FcUK99 were 66.47%, 48.32%, and 23.90% at these doses. The one-way ANOVA showed a statistically significant ($P<0.0001$) decrease in cell viability in the experimental groups of both strains compared to their control groups (Fig. 1b). Differences among experimental groups were also determined statistical significance ($P<0.0001$) (Fig. 1b). The samples fit a normal distribution. A positive correlation between LGR and proliferation values was found ($P<0.05$).

Imaging of oxidative stress and cell death

THR-induced apoptotic-like process was determined in experimental groups in which fluorescent emissions displayed a dose-dependent range from yellow to orange after dual staining with Ao/Eb (Fig. 2). The control and acetone treated groups of both strains emitted green fluorescence.

An increase in the number of intensely green-fluorescence cells in a dose-dependent manner in all experimental sets after DCF-DA staining indicated that THR caused oxidative stress. The control and acetone treated groups of both strains emitted low-green fluorescence intensity (Fig. 3).

Effects of THR on L929 cell viability and morphology

All THR concentrations equal to 1.34, 2.68, 5.36, 10.72, 21.45, 42.89, 85.78, 171.56, 343.12, 686.24 µM were found to reduce the viability of L929 cells. There was no statistically significant difference in cell viability between the control and acetone treated groups (treated with 0.83% acetone) at both the 24th and 48th hours (P>0.05). Neither negative nor positive correlations were detected between dose increases and L929 cell viability values at the 24th and 48th hours of exposure. The cell viability ranged from 4.56% (P<0.0001) to 97.95% (P>0.05) at 24th-hour exposure, while from 4.88% (P>0.05) to 88.68% (P<0.0001) at 48th hour (Fig. 4). The THR treatment affected fibroblast morphology and caused L929 cells to acquire a rounded structure. The number of round cells increased with the dose increase (Fig. 5).

Effects of THR on wheat

The germination percentages of 5-day-old KATE-1 seeds were calculated as 95.56 ± 4.44% and 97.78 ± 3.85% for the control group and 165 µg/mL THR treatment, respectively. The mean stem and root length of the control group were 70.7 ± 2.52 and 82.61 ± 1.23, respectively, while those of the experimental group were 66.2 ± 1.51 and 83.57 ± 1.56, respectively. WLR was determined to be 0.057 ± 0.004 $gh^{-1}g^{-1}$ and 0.056 ± 0.004 $gh^{-1}g^{-1}$ for the stem in the control and experimental groups, respectively. No statistically significant difference was determined between the control and experimental groups according to germination rate, stem and root lengths, and WLR (P>0.05) (Fig. 6).

Effect of THR on the production of DON and its acetylated derivatives

Rf values of DON, 15-ADON, and 3-ADON found in the B-trichothecene mix standard solution on the TLC plate were calculated as 0.16, 0.4, and 0.5, respectively. Spots of the control and acetone treated specimens were congruent with the Rf value of DON. However, those of FcUk99 were relatively indistinct. The spots with Rf values corresponding to 15-ADON in the control and acetone treated groups of PH-1, while 3-ADON of FcUK99. Spots disappeared from PH-1 and FcUK99 treated with THR (Fig. 7). Consequently, it was monitored that THR reduced the production of DON and its acetylated derivatives among B-trichothecenes in the PH-1 and FcUK99 experimental groups to a certain extent that could be qualitatively observed in TLC.

Discussion

The MIC value of THR was determined to be 165 µg/mL on both PH-1 and FcUK99. Its MIC_{25}, MIC_{50}, and MIC_{75} doses inhibited the LGRs of PH-1 by 67.01%, 86.94%, and 93.15%, respectively, while those of FcUK99 by 31.40%, 60.91% and 84.81%, respectively, at the 7th day (Fig. 1). Previously, Djekoun et al. (2016) demonstrated that THR was the most effective compound among their testing fungicide-active compounds and found that 0.42 µg/mL THR inhibited the growth of *Fusarium* spp. by 81%. Jamdagni et al. (2018) reported that the MICs of THR were in the range of 2-4 µg/mL against five different plant pathogens, including *F. oxysporum*. Similarly, Jamil and Ashraf (2020) also informed that THR at a concentration of 50 µg/mL reduced the growth of *F. oxysporum* by 98.13%. It is seen that the inhibitory concentrations obtained from this study were found to be significantly higher than those previously reported. All these outcomes are important to indicate that MIC values of THR may vary depending on species or genotype; in addition, phytopathogens may lose their susceptibility to this compound over time. For these reasons, screening of the growth inhibitory concentrations of antifungals, determined on reference strains, on field isolates, will be important for providing the use of these compounds at effective doses for agricultural sustainability.

Cell viability can be assessed using tetrazolium salt-based (MTT, WST-1, XTT, etc.) assays. They are accepted as powerful tools for investigating antifungal activity (Meletiadis et al. 2001; Wiederhold and Lewis 2009; Ghasemian et al. 2012). Tetrazolium salts are reduced to formazan crystals by activation of dehydrogenases and oxidoreductases in mitochondria. Their activities enable the indirect detection of the number of viable cells, particularly during the exponential growth phase (Berridge et al. 2005).

Meletiadis et al. (2001), in their study on *Aspergillus* species, demonstrated a correlation between the cell viability and the amounts of formazan crystals by using XTT tetrazolium salt. Lewis et al. (2005) observed that reducing the formazan crystals depends on the increase in dose-dependent activity of various fungicides on *Fusarium* spp. Although WST-1 can be reduced extracellularly by the reaction with mitochondrial oxidoreductases- due to its net negative charge (Berridge et al. 2005)- WST-1 was successfully used to determine the effect of THR on the cell viability of *Fusarium* strains in this study. The growth inhibition values increased in both references as the THR doses increased. Cell viability data were compatible with the LGRs, and their relationship was statistically significant according to Pearson's correlation test ($P<0.05$). When all THR-treated experimental groups of both strains were

compared, it was revealed that FcUK99 had higher LGR values than PH-1, and the cell viability of FcUK99 was significantly lower than that of PH-1.

Reactive oxygen species (ROS) generated by a healthy cell dramatically increase in oxidative stress conditions. Excess ROS levels also increase oxidative stress by causing damage to various cellular processes. Consequently, it negatively affects cell viability (Su et al. 2020). Oxidative stress can be monitored via DCF-DA staining (Albayrak et al. 2023). Since non-fluorescent DCF-DA emits green fluorescence when oxidized by ROS, cells emit intense green fluorescence in the presence of increased ROS levels. This study revealed that THR increased oxidative stress levels in a dose-dependent manner in both *Fusarium* strains.

Healthy fungal cells emitted a green fluorescence after dual Ao/Eb staining. Since the Eb can penetrate only apoptotic cells with damaged membranes, apoptotic cells emit red fluorescence (Renvoize et al. 1998; Ribble et al. 2005). In this study, control groups of PH-1 and FcUK99 emitted a green fluorescence while THR treated group underwent an apoptosis-like process displaying yellow-orange fluorescence because they took both Ao and Eb dyes. The number of apoptotic cells increased with increasing THR concentrations. Findings implied that oxidative stress triggered by THR could contribute to inducing apoptosis-like cell death. Since inappropriate and/or lethal antifungal doses could cause the development of resistance in pathogens to them over time, Wang et al. (2019) proposed that the utilisation of antifungals that suppress the growth, such as THR, could be advantageous.

Furthermore, it is known that some antifungals used can raise mycotoxin production while inhibiting the growth of pathogens. So, it should be tested whether antifungals increase mycotoxin production or not. In this study, TLC was employed to observe DON and its acetylated forms (3-ADON and 15-ADON) due to its low cost, simplicity, and suitability for simultaneous analysis of multiple samples. This method can detect compounds at very low levels (micrograms) depending on their physicochemical properties, and the quality of the plate used, etc. However, quantitative data is not provided by using it. Within this scope, PH-1 was verified as a DON/15-ADON producer and FcUK99 as a DON/3-ADON producer. TLC results were consistent with the literature data related to the chemotypes of PH-1 (Wang et al. 2021) and FcUK99 (Oufensou et al. 2021). THR exposure reduced the production of DON/15-ADON in PH-1 and DON/3-ADON in FcUK99. Similar to our results, Zhang et al. (2009) reported, in their field trial, that THR affected DON biosynthesis of *F. graminearum* by decreasing the production of DON, 3-ADON, and 15-ADON. On the contrary, Becher et al. (2010) and Duan et al. (2020) reported that different fungicidal compounds dramatically increased mycotoxin (DON, NIV and ZEN) production in wild-type *F. graminearum* isolates, although their

inhibitory concentrations were at very low levels compared to THR. These studies draw attention to the investigation of the antifungal effects not only on growth inhibition but also on mycotoxin production.

It has been reported that seeds treated with fungicides such as THR are protected from pathogen infection, and the active compound promotes plant growth (Freiberg et al. 2021). Furthermore, some researchers demonstrated that THR supported germination and seedling development (Shivankar et al. 2000; Ergin et al. 2021). Unlike those reports, our wet lab experiments indicated that the THR concentration that inhibits *Fusarium* spp. did not affect the germination rate of wheat seeds, stem and root lengths of seedlings, or their WLR ($P > 0.05$ for all parameters).

The extensive use of THR in agriculture and its improper handling or storage lead to contamination of terrestrial ecosystems, water, and groundwater resources. The THR is degraded approximately 15 days in soil, but its persistence is influenced by environmental factors such as pH and moisture (Sharma et al. 2003). So, it has become one of the significant environmental risk factors for humans and animals (Sankowska et al. 2017). It can be exposed to fungicidal active compounds through direct skin contact, consumption of fungicide-contaminated foods, and use of fungicide-contaminated water for irrigation and drinking (Montoya et al. 2024; Liu et al. 2022; PubChem 2025).

Although the cytotoxicity of THR has been investigated in various mammalian cell models, there is still limited knowledge about its toxicity on humans and animals. In the present study, cytotoxicity of THR doses, which inhibited the growth of PH-1 and FcUK99, was investigated on the mouse fibroblast cell line (L929). Mouse fibroblasts, which are found generally in the dermal layer of the skin, are members of the connective-tissue cell family originating from mesenchymal stem cells (MSCs), essential components of connective tissue, and are responsible for the healing of skin injuries (Ghafourian et al. 2015; Singh et al. 2017). Also, they retain their plasticity capabilities. Therefore, they can differentiate into other connective-tissue cell family members, proliferate, and have immunomodulatory capacities like MSCs. Hence, L929 has been frequently preferred for the cytotoxicity screening studies. The MIC_{25} and its higher doses (>171.56 µM) strongly inhibited (>90 %) the viability of L929 cells at both the 24th and 48th hours. Concerning that, Radko et al. (2017) examined its cytotoxicity on human (HepG2), chicken (LMH), rat (FaO) hepatoma cell lines, and rat myoblast cell lines (L6). They concluded that THR decreased cell viability in all cell cultures. Similarly, Grosicka et al. (2005) reported that 200 µM is the 50 % lethal dose for Chinese hamster fibroblast cells. Fibroblasts spreading throughout the human body are intrinsically differentiated as they

mature, and each of them is responsible for a specialized function. Therefore, the transformation capacity of mature fibroblasts is more restricted than that of immature fibroblasts. Cereser et al. (2001) may have determined that the human skin fibroblast cells were more sensitive to low THR doses (5 µg/mL corresponding to 20.8 µM) than rodent fibroblasts. They observed a dose- and time-dependent reduction in cell viability, and informed that cells with normally spindle-shaped morphology became shrunken and dispersed. Similarly, THR caused L929 cells to lose their spindle-like shapes and to change into round structures of in the cultures, in the current study. Cereser et al. (2001) reported that the apoptotic mechanism occurred by decreased glutathione levels, as well as increased protein oxidation and lipid peroxidation.

THR is still used as a filler compound for fungicides and in the coating of seeds in most countries, despite its prohibition in agriculture in the European Union. Unlike previous studies, this study showed that the THR dose that inhibits the growth of phytopathogenic *Fusarium* species by inducing oxidative stress and apoptosis-like cell death was not toxic to wheat seeds and seedlings but toxic to animal cell lines. These results indicate that THR could affect animal and human health if it accumulates in soil and on agricultural products. In conclusion, this study provides an integrative understanding of the effects of THR across different kinds of organisms. Also, it indicates the necessity of investigating the safe application limits and potential toxicity to other organisms that may be exposed to this compound in the same ecosystem.

Acknowledgments

The authors would like to thank Dr. Tapani Yli-Mattila (University of Turku) and Dr. Pierre Hellin (Walloon Agricultural Research Centre), for fungal strains *F. graminearum* PH-1, *F. culmorum* FcUK99. This study was funded by TÜBİTAK [Project number: 119Z366] and the Scientific Research Projects Coordination Unit of Istanbul University [Project number: 29782].

References

1.Akpinar, R., Arslan, M., Teker, T., Shikhaliyeva, I., Albayrak, G., Ozkorucuklu, S.P. 2025. Evaluation of B-type Trichothecene Production by *Fusarium graminearum* and *F. culmorum* strains. *J Food Compos Anal.*, 148.

2.Albayrak, G., Yörük, E., Teker, T. and Sefer, Ö. 2023. Investigation of antifungal activities of myrcene on *Fusarium* reference strains. *Arch. Microbiol.,* 205 (3):82.

3.Alisaac, E. and Mahlein, A.K. 2023. Fusarium Head Blight on Wheat: Biology, Modern Detection and Diagnosis and Integrated Disease Management. *Toxins,* 15 (3):192.

4.Becher, R., Hettwer, U., Karlovsky, P., Deising, H.B. and Wirsel, S.G. 2010. Adaptation of *Fusarium graminearum* to tebuconazole yielded descendants diverging for levels of fitness, fungicide resistance, virulence, and mycotoxin production. *Phytopathol.,* 100 (5):444-53.

5.Berridge, M.V., Herst, P.M. and Tan, A.S. 2005. Tetrazolium dyes as tools in cell biology: new insights into their cellular reduction. *Biotechnol. Annu. Rev.,* 11:127-152.

6.Bouaicha, O., Laraba, I. and Boureghda, H. 2022. Identification, *in vitro* growth and pathogenicity of *Microdochium* spp. associated with wheat crown rot in Algeria. *Plant Pathol. J.,* 104 (4):1431-1442.

7.Cargill: Cargill World Mycotoxin Report Feeding Intelligence. 2022. https://www.cargill.com/2022-cargill-world-mycotoxin-report. Accessed 18 May 2025

8.Cereser, C., Boget, S., Parvaz, P. and Revol, A. 2001. An evaluation of thiram toxicity on cultured human skin fibroblasts. *Toxicol.,* 162 (2):89-101.

9.Cinar, A. and Onbaşı, E. 2020. Mycotoxins: The Hidden Danger in Foods. *IntechOpen*, 1-21.

10. Djekoun, M., Berrebbah, H., Saib, A. and Djebar, M.R. 2016. Determination of median effective inhibitory concentration of three fungicides widely used for treatment of wheat on the target pest *Fusarium* sp. *Res. J. Environ. Toxicol.,* 10:109-114.

11. DSM: World Mycotoxin Survey 2022 Report. 2022. https://www.dsm.com/anh/news/downloads/whitepapers-and-reports/dsm-world-mycotoxin-survey-2022-report.html. Accessed 18 May 2025

12. Duan, Y., Lu, F., Zhou, Z., Zhao, H., Zhang, J., Mao, Y., Li, M., Wang, J. and Zhou, M. 2020. Quinone outside inhibitors affect DON biosynthesis, mitochondrial structure and toxisome formation in *Fusarium graminearum*. *J. Hazard Mater.,* 398:122908.

13. Ergin, N., Kulan, E. G., & Kaya, M. D. (2021). The effects of fungicidal seed treatments on seed germination, mean germination time and seedling growth in safflower (Carthamus tinctorius L.). *Selcuk Journal of Agriculture and Food Sciences*, 35(2), 139-143.

14. Eskola, M., Kos, G., Elliott, C. T., Hajšlová, J., Mayar, S., and Krska, R. 2020. Worldwide contamination of food-crops with mycotoxins: Validity of the widely cited 'FAO estimate'of 25%. *Crit. Rev. Food Sci*. 60(16), 2773-2789.

15. FAO. 1977. Perspective on mycotoxins Food and Agriculture Organization of the United Nations, Rome. Accessed 05 February 2026

16. Fravel, D.R., Deahl, K.L. and Stommel, J.R. 2005. Compatibility of the biocontrol fungus *Fusarium oxysporum* strain CS-20 with selected fungicides. *Biol. Control,* 34 (2):165-169.
17. Freiberg, J.A., Ludwig, M.P. and Girotto, E. 2021. Does seed treatment affect wheat yield components?. *Cad. Ciên. Agrá.,* 13:1-5.
18. Ghafourian, M., Tamri, P. and Hemmati, A. 2015. Enhancement of human skin fibroblasts proliferation as a result of treating with quince seed mucilage. *Jundishapur J. Nat. Pharm. Prod.,* 10 (1):e18820.
19. Ghasemian, E., Naghoni, A., Tabaraie, B. and Tabaraie, T. 2012. *In vitro* susceptibility of filamentous fungi to copper nanoparticles assessed by rapid XTT colorimetry and agar dilution method. *J. Mycol. Med.,* 22 (4):322-328.
20. Ghimire, B., Sapkota, S., Bahri, B.A., Martinez-Espinoza, A.D., Buck, J.W. and Mergoum, M. 2020. Fusarium Head Blight and Rust Diseases in Soft Red Winter Wheat in the Southeast United States: State of the Art, Challenges and Future Perspective for Breeding. *Front. Plant Sci.,* 11:1080.
21. Grosicka, E., Sadurska, B., Szumiło, M., Grzela, T., Łazarczyk, P., Niderla-Bielińska, J. and Rahden-Staroń, I. 2005. Effect of glutathione depletion on apoptosis induced by thiram in Chinese hamster fibroblasts. *Int. Immunopharmacol.,* 5 (13-14):1945-1956.
22. Horsfall, J.G. 1956. The Principles of fungicidal actions. USA: Massachusetts and Hafner Publishing Company, p. 279.
23. Jamdagni, P., Rana, J.S. and Khatri, P. 2018. Comparative study of antifungal effect of green and chemically synthesised silver nanoparticles in combination with carbendazim, mancozeb, and thiram. *IET Nanobiotechnol.,* 12 (8):1102-1107.
24. Jamil, A. and Ashraf, S. 2020. Utilization of chemical fungicides in managing the wilt disease of chickpea caused by *Fusarium oxysporum* f. sp. cicero. *Arch. Phytopathol. Plant Prot.,* 53 (17-18):876-898.
25. Lewis, R.E., Wiederhold, N.P. and Klepser, M.E. 2005. *In vitro* pharmacodynamics of amphotericin B, itraconazole, and voriconazole against *Aspergillus*, *Fusarium*, and *Scedosporium* spp. *Antimicrob. Agents Ch.,* 49 (3):945-951.
26. Liu, K., Li, Y., Iqbal, M., Tang, Z. and Zhang, H. 2022. Thiram exposure in environment: A critical review on cytotoxicity. *Chemosphere,* 295:133928.
27. Meletiadis, J., Mouton, J.W., Meis, J.F., Bouman, B.A., Donnelly, J.P., Verweij, P.E. and EUROFUNG Network 2001. Colorimetric assay for antifungal susceptibility testing of *Aspergillus* species. *J. Clin. Microbiol.,* 39 (9):3402-3408.

28. Montoya, A.M., Rodríguez-Grimaldo, J.E., López-Jácome, L.E., Bonifaz, A., Enríquez-Domínguez, E., Castañón-Olivares, L.R., Charles-Niño, C.L., Rodríguez-Rodríguez, A., Treviño-Rangela, R.J., Rojas, O.C. and González, G.M. 2024. Species distribution and antifungal susceptibility profiles of clinical and environmental *Fusarium* isolates from Mexico: A multicenter study. *Mycol.,* 116(2):258–266.

29. Osman, A.G., Sherif, A.M., Elhussein, A.A. and Mohamed, A.T. 2012. Sensitivity of some nitrogen fixers and the target pest *Fusarium oxysporum* to fungicide thiram. *Interdiscip. Toxicol.,* 5 (1):25-29.

30. Oufensou, S., Dessì, A., Dallocchio, R., Balmas, V., Azara, E., Carta, P., Migheli, Q. and Delogu, G. 2021. Molecular Docking and Comparative Inhibitory Efficacy of Naturally Occurring Compounds on Vegetative Growth and Deoxynivalenol Biosynthesis in *Fusarium culmorum*. *Toxins,* 13 (11):759.

31. PPDB. 2025. https://sitem.herts.ac.uk/aeru/ppdb/. Accessed 18 May 2025

32. PubChem. 2025. https://pubchem.ncbi.nlm.nih.gov/. Accessed 18 May 2025

33. Radko, L., Cybulski, W. and Rzeski, W. 2017. The protective effects of silybin on the cytotoxicity of thiram in human, rat and chicken cell cultures. *Pestic. Biochem. Physiol.,* 143:154-160.

34. Renvoize, C., Biola, A., Pallardy, M. and Breard, J. 1998. Apoptosis: identification of dying cells. *Cell Biol. Toxicol.,* 14:111-120.

35. Ribble, D., Goldstein, N.B., Norris, D.A. and Shellman, Y.G. 2005. A simple technique for quantifying apoptosis in 96-well plates. *BMC Biotechnol.,* 5:1-7.

36. Rose, S., Parker, M. and Punja, Z.K. 2003. Efficacy of Biological and Chemical Treatments for Control of Fusarium Root and Stem Rot on Greenhouse Cucumber. *Plant Dis.,* 87 (12):1462-1470.

37. Sankowska, M., Gajek, A., Celiński, M. and Sałasińska, K. 2017. Determination of gaseous products of thermal degradation of thiram. *J. Therm. Anal. Calorim.,* 128:1639-1647.

38. Sharma, V.K., Aulakh, J.S. and Malik, A.K. 2003. Thiram: degradation, applications and analytical methods. *JEM,* 5 (5):717-723.

39. Shikhaliyeva, I., Teker, T. and Albayrak, G. 2020. Masked Mycotoxins of Deoxynivalenol and Zearalenone- Unpredicted Toxicity. *Biomed. J. Sci. Tech. Res.,* 29: 22288-22293.

40. Shivankar, S.K., Shivankar, R.S. and Nagone, A.H. 2000. Effect of fungicidal seed treatment on the germination, shoot and root length of wheat seed infected by black point disease. *ASD,* 20 (3):205-206.

41. Singh, M., McKenzie, K. and Ma, X. 2017. Effect of dimethyl sulfoxide on *in vitro* proliferation of skin fibroblast cells. *J. Biotech. Res.,* 8:78.
42. Smith, J.E., Solomons, G., Lewis, C. and Anderson, J.G. 1995. Role of mycotoxins in human and animal nutrition and health. *Nat. Toxins,* 3 (4):187-192.
43. Sobrova, P., Adam, V., Vasatkova, A., Beklova, M., Zeman, L. and Kizek, R. 2010. Deoxynivalenol and its toxicity. *Interdiscip. Toxicol.,* 3 (3):94-99.
44. Streit, E., Naehrer, K., Rodrigues, I. and Schatzmayr, G. 2013. Mycotoxin occurrence in feed and feed raw materials worldwide: long-term analysis with special focus on Europe and Asia. *J. Sci. Food Agric.,* 93 (12):2892-2899.
45. Su, H., Hu, C., Cao, B., Qu, X., Guan, P., Mu, Y., Han, L. and Huang, X. 2020. A semisynthetic borrelidin analogue BN-3b exerts potent antifungal activity against *Candida albicans* through ROS-mediated oxidative damage. *Sci. Rep.,* 10 (1):5081.
46. Teker, T., Khalid, S.A., Yörük, E. and Albayrak, G. 2024. Physiological, genetic and transcriptional characterization of *Fusarium graminearum* isolates. *Sigma J. Eng. Nat. Sci.,* 42 (2):450-458.
47. Teker, T., Sefer, Ö., Gazdağlı, A., Yörük, E., Varol, G.İ. and Albayrak, G. 2021. α-Thujone exhibits an antifungal activity against *F. graminearum* by inducing oxidative stress, apoptosis, epigenetics alterations and reduced toxin synthesis. *Eur. J. Plant Pathol.,* 160 (3):611-622.
48. Tobih, F.O., Bosah, B.O. and Nweke, F.U. 2015. Evaluation of the efficacy of Thiram and benomyl on radial growth, spore germination and sporulation density of *Curvularia lunata* and *Fusarium semitectum*. *Int. J. Agric. Innov. Res.,* 4: 47-50.
49. Wang, L., Ren, X., Guo, W., Wang, D., Han, L. and Feng, J. 2019. Oxidative Stress and Apoptosis of *Gaeumannomyces graminis* (*Get)* Induced by Carabrone. *J. Agric. Food Chem.,* 67 (37):10448-10457.
50. Wang, L., Yan, Z., Zhou, H., Fan, Y., Wang, C., Zhang, J., Liao, Y. and Wu, A. 2021. Validation of LC-MS/MS Coupled with a Chiral Column for the Determination of 3- or 15-Acetyl Deoxynivalenol Mycotoxins from *Fusarium graminearum* in Wheat. *Toxins,* 13 (9):659.
51. Wiederhold, N.P. and Lewis, R.E. 2009. Antifungal activity against *Scedosporium* species and novel assays to assess antifungal pharmacodynamics against filamentous fungi. *Med. Mycol.,* 47(4):422-432.
52. Xu, X.M., Nicholson, P., Thomsett, M.A., Simpson, D., Cooke, B.M., Doohan, F.M., Brennan, J., Monaghan, S., Moretti, A., Mule, G., Hornok, L., Beki, E., Tatnell, J., Ritieni,

A. and Edwards S.G. 2008. Relationship between the fungal complex causing Fusarium head blight of wheat and environmental conditions. *Phytopathol.,* 98 (1):69-78.

53. Yli-Mattila, T., Yörük, E., Abbas, A. and Teker, T. 2022. Overview on Major Mycotoxins Accumulated on Food and Feed. In: Deshmukh SK, Sridhareds KR (eds) Fungal Biotechnology: Prospects and Avenues, CRC Press, p. 310–343.

54. Zhang, Y.J., Fan, P.S., Zhang, X., Chen, C.J. and Zhou, M.G. 2009 Quantification of *Fusarium graminearum* in harvested grain by real-time polymerase chain reaction to assess efficacies of fungicides on fusarium head blight, deoxynivalenol contamination, and yield of winter wheat. *Phytopathol.,* 99 (1):95-100.

55. Zubrod, J.P., Bundschuh, M., Arts, G., Brühl, C.A., Imfeld, G., Knäbel, A., Payraudeau, S., Rasmussen, J.J., Rohr, J., Scharmüller, A., Smalling, K., Stehle, S., Schulz, R. and Schäfer, R.B. 2019. Fungicides: an overlooked pesticide class?. *Environ. Sci. Technol.,* 53 (7):3347-3365.

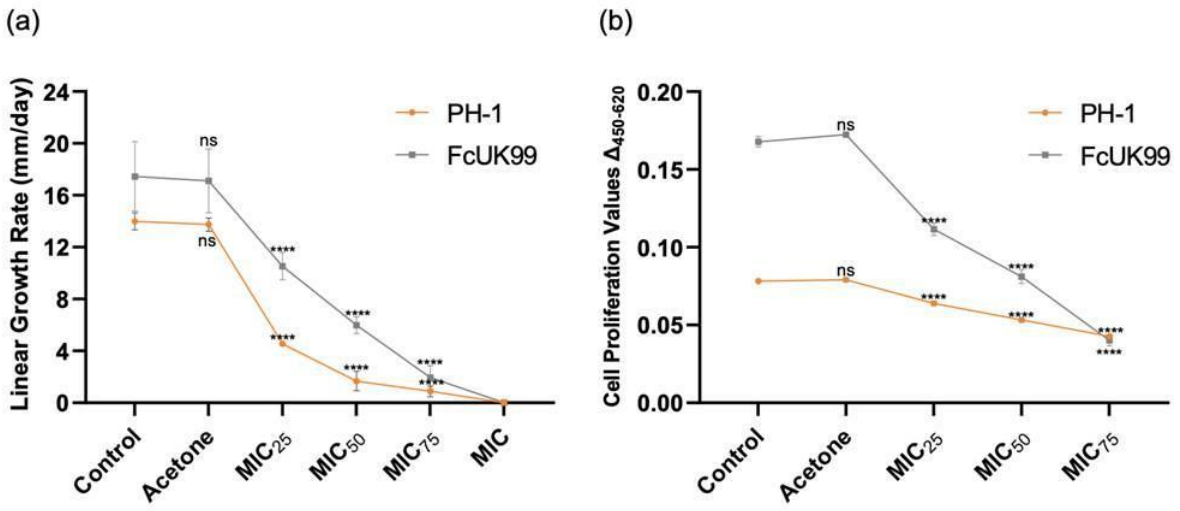

Fig. 1 Comparison of the LGR of non-treated control, the acetone treated and experimental groups treated with MIC_{25}, MIC_{50}, and MIC_{75} doses of THR (mean ± SEM) (a); Cell proliferation values of the PH-1 and FcUK99 strains (b). Data represent three independent biological replicates (n = 3). No significant difference was observed between the control and acetone treated groups (ns: non-significant, P>0.05), whereas statistically significant differences were detected at MIC_{25}, MIC_{50}, and MIC_{75} compared to the control (****P< 0.0001).

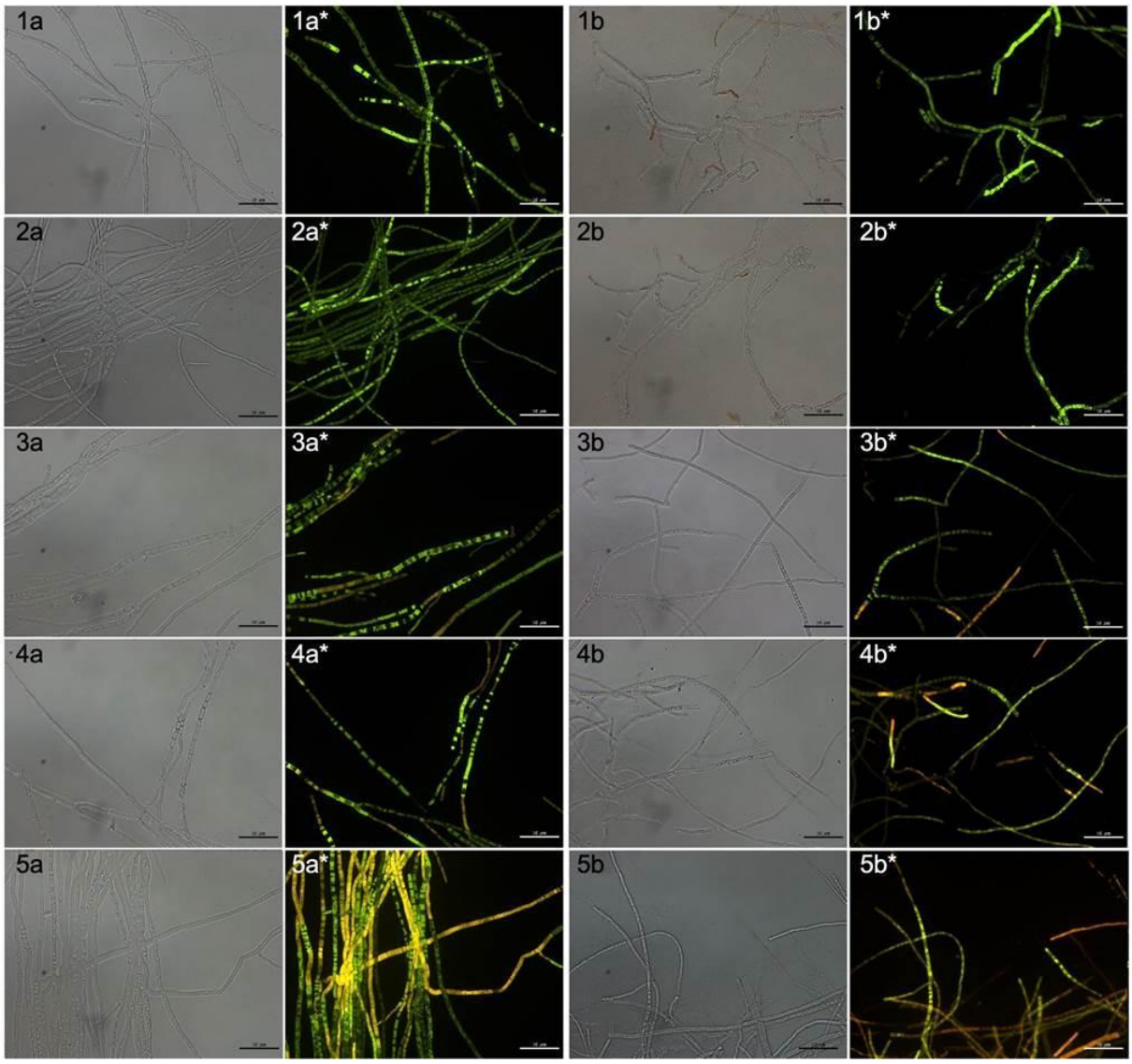

Fig. 2 Ao/Eb dual-staining images of PH-1 (a) and FcUK99 (b) strains under light and fluorescence (*) microscopy of the non-treated control (1), the acetone treated (2), MIC_{25} (3), MIC_{50} (4) and MIC_{75} (5) dose groups at 40× magnification.

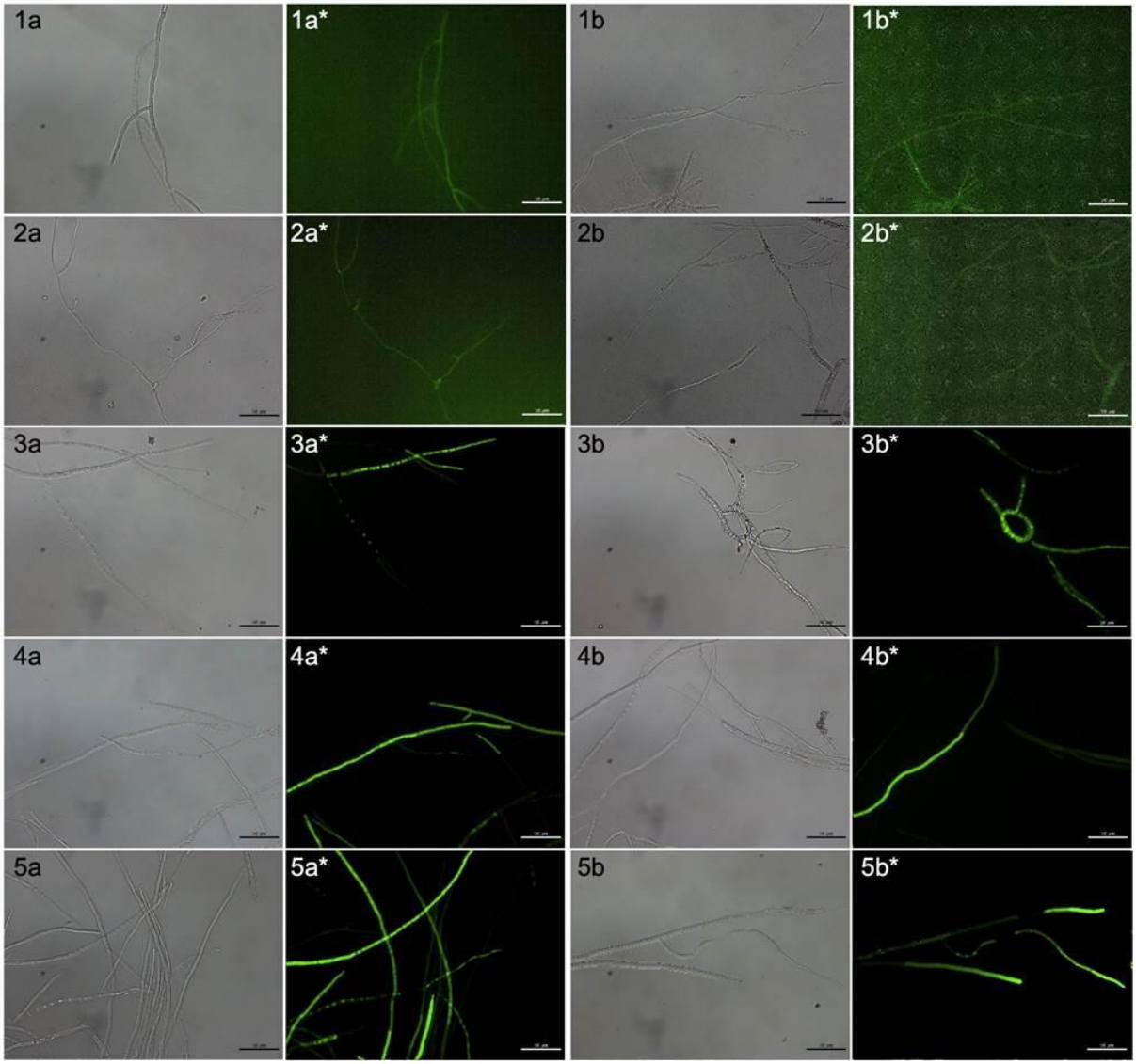

Fig. 3 DCF-DA staining images of PH-1 (a) and FcUK99 (b) strains under light and fluorescence (*) microscopy of the non-treated control (1), the acetone treated (2), MIC_{25} (3), MIC_{50} (4), and MIC_{75} (5) dose groups at 40× magnification.

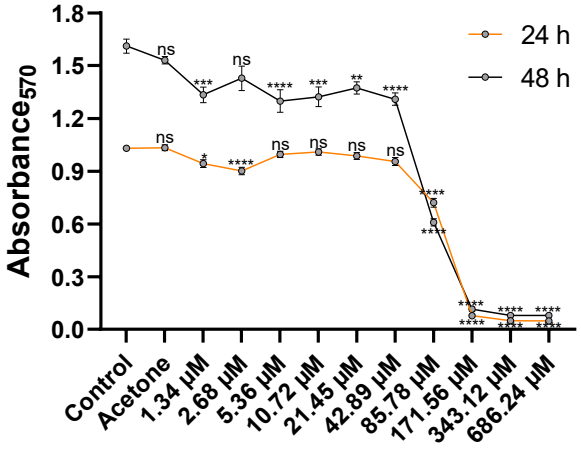

Fig. 4 Absorbance$_{570}$ values of L929 cells treated with different concentrations of THR after 24 and 48 hours. Data represent the mean ± SEM of eight independent biological replicates (n = 8). No statistically significant difference was observed between the control and acetone treated groups at either point (ns: non-significant, P>0.05). At 24 h, no significant difference was observed at 5.36, 10.72, 21.45, and 42.89 µM THR concentrations compared with the control, whereas alterations at the other concentrations were statistically significant (*P< 0.05, ****P< 0.0001). At 48 h, cell viability differed significantly (**P< 0.01, ***P < 0.001, ****P < 0.0001) from the control at all THR concentrations except 2.68 µM (ns: P> 0.05).

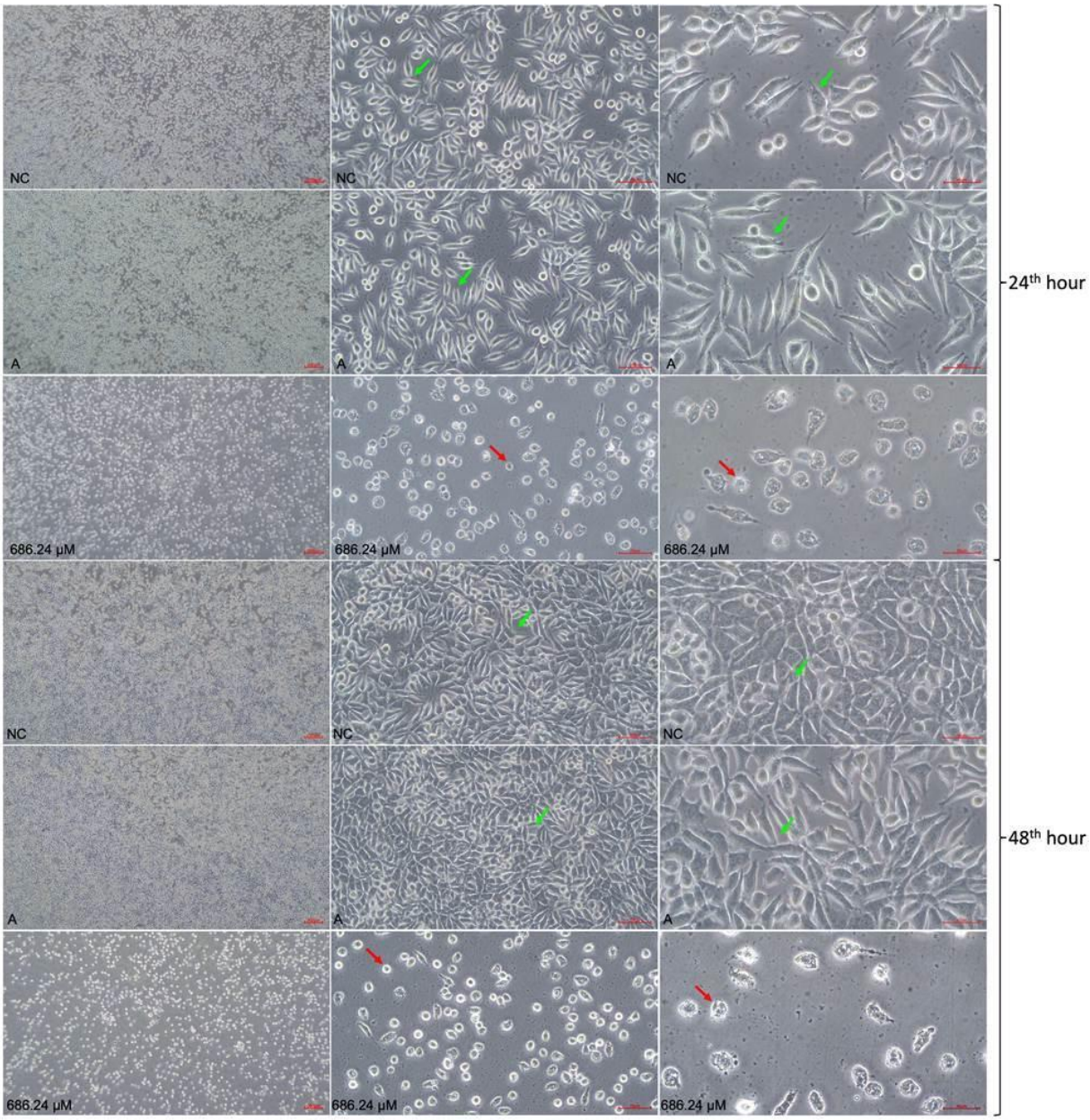

Fig. 5 Cell morphology assessment of the non-treated control (NC), the acetone treated (A), and THR-treated groups of LP29 cells after 24 and 48 hours. Images were taken under an inverted optical microscope. The first, second, and third columns represent the 4×, 20×, and 40× magnification, respectively. Green arrows indicate healthy cells with a regular shape and morphology, and red arrows indicate a round morphology.

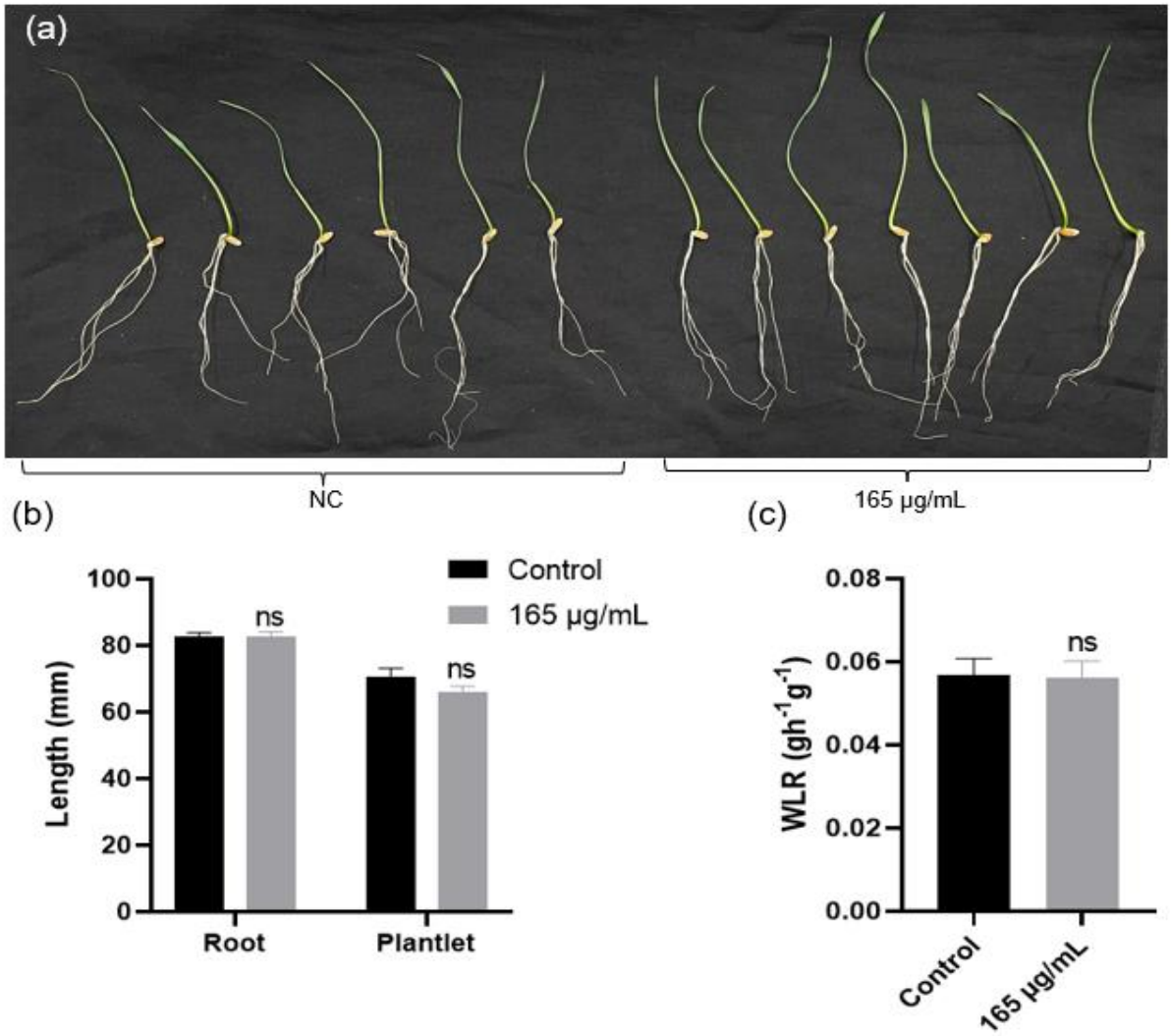

Fig. 6 Wheat seedlings in the non-treated control (NC) and experimental groups (a). Graphs represent the root and plantlet lengths of seedlings (b) and WLR (c) after seed treatment by THR. Data represent experiments conducted with 30 seeds per replicate (n = 90), with statistical analysis based on all seeds. No significant difference was observed in root, plantlet length, and WLR between the control group and the 165 µg/mL THR treated experimental group (ns: non-significant, P> 0.05).

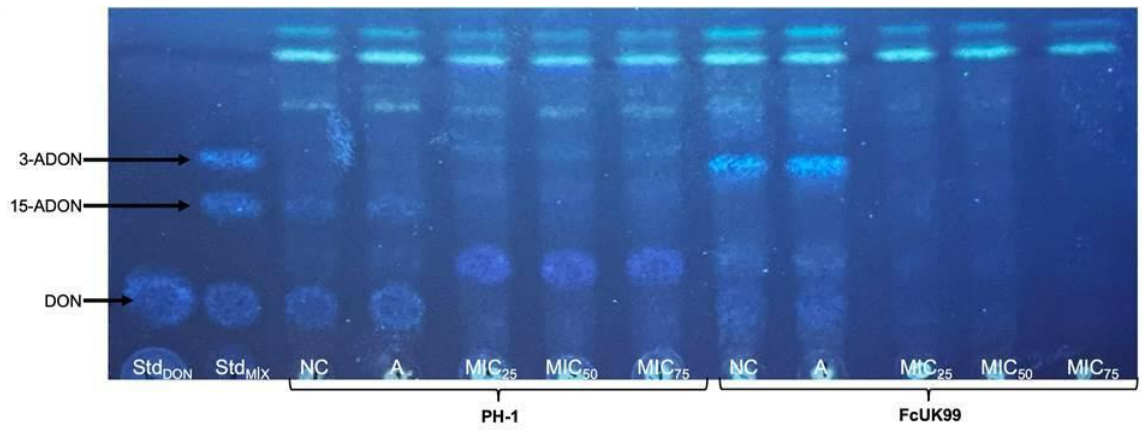

Fig. 7 TLC chromatogram of standards and mycotoxins of *F. graminearum* PH-1 and *F. culmorum* FcUK99 on silica gel F_{254}. Mycotoxins obtained from the non-treated control (NC), the acetone treated (A), and experimental groups under 356 nm UV light (arrows indicate DON, 15-ADON, and 3-ADON spots with Rf values of 0.16, 0.4, and 0.5, respectively).