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Original Article

Media and temperature effects on the allelopathic potential and chemical diversity of *Fusarium pseudensiforme* extract

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Abstract

Fungi produce phytotoxic metabolites that can be utilized in natural herbicide development, but fungal growth and metabolite production are influenced by numerous factors. This study investigated the chemical profiles of *Fusarium pseudensiforme* extracts under different culture conditions and evaluated their herbicidal potential against *Phaseolus lathyroides*. Crude ethyl acetate extracts were obtained from *F. pseudensiforme* grown in submerged fermentation using four culture media, potato dextrose broth (PDB), malt extract broth (MEB), Czapek Dox broth (CDB), and yeast extract sucrose broth (YSB), at 25-35 °C for 14 days. The results revealed that increasing incubation temperature led to a marked reduction in fungal growth, crude yield, and herbicidal efficacy across all media. Incubation at 25 °C resulted in the highest values for all parameters, particularly in YSB medium ($p < 0.05$). Morphological analysis of treated *P. lathyroides* seedlings indicated that YSB extract significantly inhibited hypocotyl and lateral root development. GC-MS analysis revealed that PDB, MEB, CDB, and YSB extract contained 34, 27, 23, and 18 chemical components, respectively, with 17 common across all media. These variations in chemical profiles likely contribute to the observed differences in phytotoxic performance. Notably, as incubation temperature increased, YSB extracts exhibited higher accumulation of alkylated benzene derivatives, which are known to exhibit low herbicidal activity, thereby reducing overall efficacy, highlighting temperature-induced alterations in metabolite biosynthesis. Taken together, these results provide insights that could facilitate the scaling up of fungal allelochemical production and enhance the practical application of fungal-derived natural herbicides in weed management.

Keywords: Allelochemical, Fungal allelopathy, Weed control, Chemical diversity, *Fusarium pseudensiforme*

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Introduction

Replacing synthetic herbicides with natural herbicides is a major demand in global agriculture. One promising avenue of exploration is the phytotoxic secondary metabolites produced by various microorganisms (Bordin et al., 2021; Cavalcante et al., 2021). Fungi are particularly notable as subjects of investigation due to their ease of isolation, rapid growth on diverse culture media, low cultivation costs, and prolific production of secondary metabolites. *Fusarium* species are especially known for causing significant crop diseases worldwide. Most *Fusarium* species produce toxic allelochemicals and have been widely investigated for their potential in weed control (Reveglia et al., 2018). Recently, *F. fujikuroi* isolated from the Brazilian Pampa biome was shown by Daniel et al. (2018) to exhibit both pre- and post-emergent herbicidal activity against *Cucumis sativus* and *Sorghum bicolor*. The literature indicates that *Fusarium*-derived allelochemicals, such as zearalenone, can stimulate electrolyte leakage, inhibit H^+ extrusion (causing acidification), and reduce root length. Another compound, fusaric acid, increases reactive oxygen species levels, suppresses antioxidant enzymes like catalase and ascorbate peroxidase, and induces programmed cell death (Singh and Upadhyay, 2014). Foliar application of fusaric acid has also been shown to decrease chlorophyll pigment content in tomato, impairing photosynthesis, metabolism, and cell structure, eventually leading to wilt (Singh et al., 2017). However, the induction of allelochemical production by fungi remains incompletely understood. Fungal synthesis of bioactive metabolites is known to be strongly influenced by various chemical and physical parameters, including pH, temperature, incubation period, and the carbon and nitrogen sources in the culture medium (Bhavana et al., 2014). Altering these fermentation conditions can significantly modify the profile of secondary metabolites; for instance, changing nutrient sources or environmental conditions can drastically affect both growth and metabolite output (Kalyani et al., 2021). In the industrial setting, optimizing culture conditions is a basic step in maximizing metabolite production; however, such optimization is strain-specific and often challenging, as conditions favorable for one microorganism may reduce metabolite diversity in another. In our preliminary studies, we isolated a *Fusarium pseudensiforme* strain whose crude extract exhibited herbicidal activity. We hypothesized that (i) this strain produces different allelochemicals depending on the culture medium and incubation temperature, and (ii) these variations in chemical composition lead to corresponding differences in the herbicidal activity of the crude extracts. Guided by this hypothesis, the objective of this study was to determine the allelochemicals present in *F. pseudensiforme* extracts produced under varying culture conditions using gas chromatography-mass spectrometry (GC-MS) and to evaluate their herbicidal effects on the representative weed *Phaseolus lathyroides*. These results contribute to advancing the development of fungal-based natural herbicides.

Material and Methods

Fungal identification

Fusarium isolate KM-DFa was isolated from durian branches exhibiting dieback symptoms using the tissue transplanting method and purified through single-conidia isolation. The isolate was cultured on potato dextrose agar (PDA) at 25 °C in the dark for seven days and its colony and micromorphological characteristics evaluated. Genomic DNA was extracted from mycelia by the CTAB method (Zhang et al., 2010), with the final concentration adjusted to 50 ng/µl for use as a template for PCR reactions. Target regions of translation elongation factor 1-alpha (*tef-1*) and the RNA polymerase second-largest subunit (*rpb2*) were amplified by PCR using the respective primer pairs EF1/EF2 (O'Donnell et al., 1998) and RPB2-5F2/RPB2-7cR (O'Donnell et al., 2010). Each 50 µl PCR reaction contained 1× Green PCR Master Mix Direct-Load (biotechrabbit GmbH, Berlin, Germany), 0.2 µM of each primer, and 50 ng of genomic DNA. PCR amplification was carried out in a GeneMax Thermocycler (Hangzhou Bioer Technology Co., Ltd, Zhejiang, China) with the following conditions: an initial denaturation at 95 ºC for 2 min; 35 cycles of 30 s at 95 ºC, 30 s at 50 ºC (*tef-1*) or 52 ºC (*rpb2*), 1 min at 72 ºC; and a final extension at 72 ºC for 5 min. The PCR products were analyzed by 1% agarose gel electrophoresis and purified using the PCR Clean-up Gel Extraction Kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany) following the manufacturer's instructions. The purified products were then bidirectionally sequenced by Macrogen, Inc. (Seoul, South Korea). The consensus sequences of *tef-1* and *rpb2* generated in this study were deposited in GenBank and the BLASTn search tool available from the NCBI (http://blast.ncbi.nlm.nih.gov/) was

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used to identify closely related species. From those search results, 41 concatenated *tef-1* and *rpb2* sequences from strains of the *Fusarium solani* species complex (FSSC) were selected for use in phylogenetic analysis along with the combined sequence from this study (Table S1). Multiple sequence alignment was performed using MAFFT version 7 and improved manually if necessary in BioEdit v.7.2. Phylogenetic analysis of the combined *tef*-1 and *rpb2* dataset was then performed using the maximum likelihood and Bayesian inference methods, following the approach described by Nuangmek et al. (2023). The resultant phylograms were visualized using FigTree v1.4.4 and edited in Adobe Illustrator 2025 (Adobe Inc., San Jose, CA, USA).

Cultural conditions for biomass and phytotoxic metabolite production

To optimize cultural conditions and promote the growth of and maximum herbicidal metabolite production by *F. pseudensiforme*, two parameters were varied: the incubation temperature, and type of culture media. Liquid media were selected to represent a broad range of nutrient sources, and included potato dextrose broth (PDB; Himedia), malt extract broth (MEB; Himedia), Czapek Dox broth (CDB; Himedia), and yeast extract sucrose broth (YSB; 20 g yeast extract, 150 g sucrose, 0.5 g $MgSO_4 \bullet 7H_2O$, and 1 L H_2O). Each culture medium (100 mL) was allocated into 240 mL culture bottles, autoclaved at 121 °C for 30 min. Bottles were then inoculated with spore suspension (1×10^6 spores/mL) and incubated without stirring in the dark at different temperatures (25, 30, or 35 °C). The experiment consisted of 12 treatments formed by the combination of four culture media and three incubation temperatures. All treatments were arranged in a completely randomized design (CRD) with three replicates per treatment. Fungal growth and production of extracellular phytotoxic metabolites were evaluated after 14 days of incubation. The following parameters were assessed: biomass (mycelium dry weight; mg/mL), extraction yield (mg), and seed germination inhibition activity (%) on *P. lathyroides*.

Extraction of fungal cultures

Mycelia were removed from culture broth through initial filtering using a sterilized muslin cloth and subsequent re-filtering with filter paper (Whatman No.1). The filtrate was then incubated at 45 °C in a hot air oven (Binder World FP400UL-208 V, Binder, Germany) for 48 hours to air-dry, and the mycelium dry weight was determined. Extraction of fungal cultures was carried out according to Gill et al. (2023) with modification. Specifically, the cultured filtrate was extracted three times with an equal volume of ethyl acetate in a separatory funnel. The top organic layer was collected after each extraction. Subsequently, the combined organic layers were added with 2 g of anhydrous magnesium sulfate ($MgSO_4$) as a desiccant, filtered, and evaporated under reduced pressure using a rotary evaporator (Buchi Rotavapor R255, Buchi, Lausanne, Switzerland) to obtain a crude extract. This crude extract was weighed to determine the extraction yield and stored at 4 °C in a dark container to minimize light- and temperature-induced degradation before its use in the bioassay.

Seed germination bioassay

The phytotoxicity of 12 crude extracts of cultured *F. pseudensiforme*, representing four different media and three temperature conditions, was evaluated on the seed germination of *Phaseolus lathyroides* (wild pea) using a CRD with four replications. Wild pea seeds were collected from an agricultural field in Phitsanulok province (16.7479° N, 100.1919° E), Thailand. To break dormancy, the seeds were exposed to full light conditions for 72 hours, followed by incubation at 50 °C for 24 hours (Wichittrakarn et al., 2025). Empty and undeveloped seeds were removed by floating them in tap water. For the formulation of crude extracts, an anionic surfactant mixture was first prepared comprising Tween® 80 and N,N-dimethylformamide (DMF) in a 1:2 (v/v) ratio. Each crude extract was dissolved in this surfactant mixture at a 1:2 (w/v) ratio with homogenization using a mechanical stirrer, and the resulting liquid solution was used as a fungal-based herbicidal product. Each product was diluted with distilled water to a concentration of 10 mg/mL and directly used in the bioassays. For the seed germination tests, 3.0 mL of the tested solution was added to germination paper placed in glass Petri dishes. Uniform and fully developed wild pea seeds (10 seeds/dish) were then added to the dishes. The surfactant mixture and distilled water alone served as check and control treatments, respectively. After seven days of treatment, germination count, root length, and shoot length (in cm) of the tested seeds were recorded and the percentage inhibition of seed germination and root and shoot growth was calculated.

Analysis of fungal metabolite profiles by GC-MS

Constituents of the selected cultured extracts were identified using GC-MS with a Scion 436 gas chromatograph coupled to a triple quad (Bruker, USA) mass spectrometer. Operating parameters were as follows: helium flow rate of 1 ml/min; detection range of 30-500 amu; starting oven temperature of 50 °C (2 min); ramping to 250 °C (20 °C/min); and then holding for 18 min. The HP-5MS capillary column (30 m, film 0.25 µm, ID 0.25 mm) was filled with 1 mL sample in splitless mode. The temperature of the transfer line was 250 °C and that of the ion source 230 °C. Individual ingredients were identified by comparison of obtained mass spectra (molecular mass and fragmentation pattern) with the internal reference library (National Institute of Standards and Technology, NIST, 2014). Components were quantified in terms of the percentage peak area relative to total peak area. Peaks with a relative area less than 0.5% were excluded.

Statistical analysis

The data were expressed as mean value ± standard deviation (SD). Significant differences were determined through one-way analysis of variance (ANOVA) followed by comparisons made using Tukey's test ($p < 0.05$). These analyses were conducted using the SAS version 9.4 software (SAS Institute Inc, Cary, NC).

Results and Discussion

Fungal identification and morphological characterization

The initial colony, grown on PDA at 25 °C in the dark for seven days, was pale straw in color with white aerial mycelium, pale straw at the center, and white at the margin on the reverse. Its diameter ranged from 84 to 86 mm (Figure 1). The isolate produced both microconidia and macroconidia. Microconidia were hyaline, thin-walled, aseptate, oval or obovoid with a truncate base, formed on aerial conidiophores, and ranged in size from 7.85-15.60 × 3.31-5.98 µm (average ± SD: 12.27 ± 2.21 × 4.52 ± 0.70 µm; n = 50). Macroconidia were hyaline, elongate, and slender, with two to seven septa, and ranged in size from 36.62-82.68 × 5.83-8.05 µm (average ± SD: 63.41 ± 10.92 × 7.02 ± 0.65 µm; n = 50). Chlamydospores were hyaline, globose, rough-walled, and solitary, with a size range of 6.37-8.69 × 6.39-8.13 µm (average ± SD: 7.39 ± 0.70 × 7.16 ± 0.52 µm; n = 30). These morphological features are consistent with the description of *Fusarium* (Ekwomadu and Mwanza, 2023). To confirm the species, a phylogenetic analysis was performed on combined *tef-1* and *rpb2* sequences from 41 taxa from the FSSC and the isolate from this study. The aligned concatenated sequence comprised 1521 bp, including gaps (*tef-1*: positions 1-702; *rpb2*: positions 703-1521). The outgroups were *F. setosum* and *F. decemcellulare* from the *F. decemcellulare* species complex. The isolate KM-DFa was found to cluster within the clade of *F. pseudensiforme* (CBS 125729 and CMW 59665), with a bootstrap support (BS) of 100% and a Bayesian posterior probability (PP) of 1.0 (Figure 2). This confirms that the isolate used in this study is *F. pseudensiforme*.

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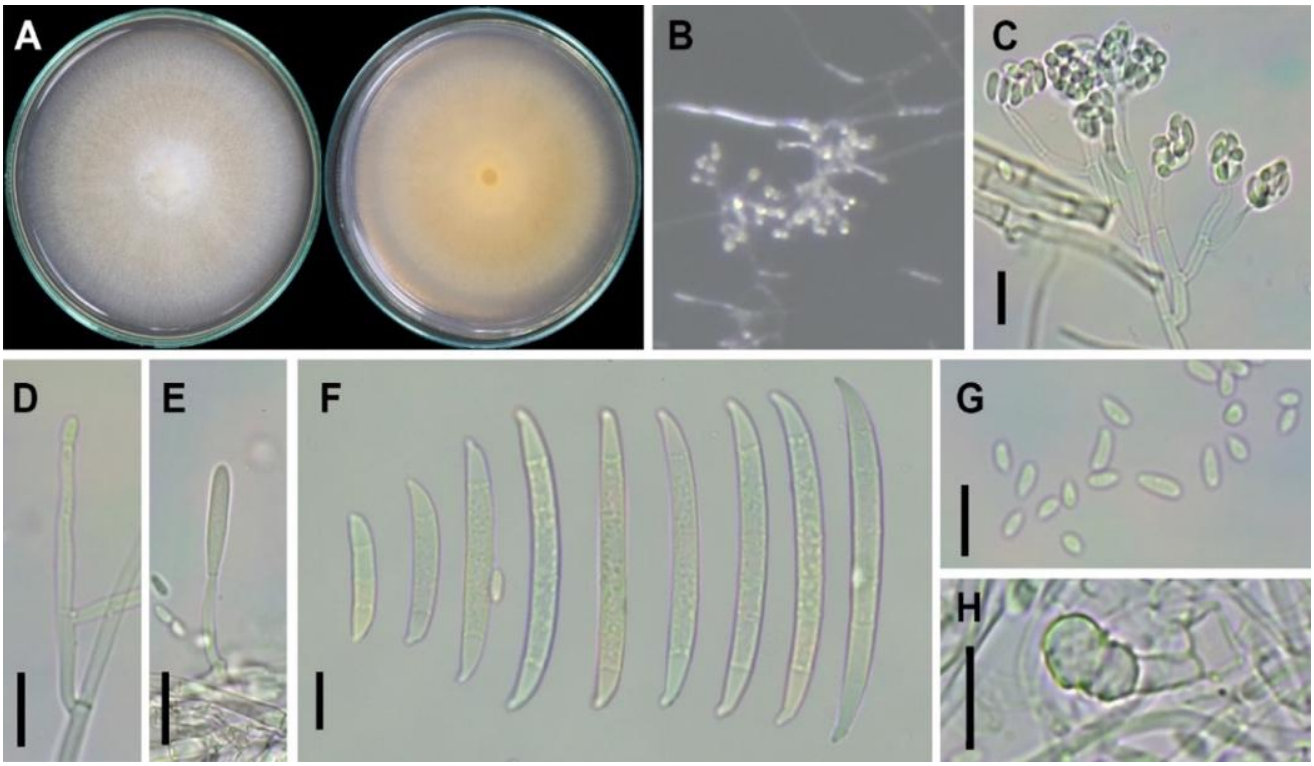

Figure-1. Morphological characteristics of *F. pseudensiforme* isolate KM-DFa. (A) Colony on PDA after seven days of incubation at 25 °C in the dark (left, obverse view; right, reverse view). (B-C) Conidiophore of microconidia. (D-E) Conidiophore of macroconidia. (F) Macroconidia. (G) Microconidia. (H) Chlamydospore. Scale bars = 10 μm.

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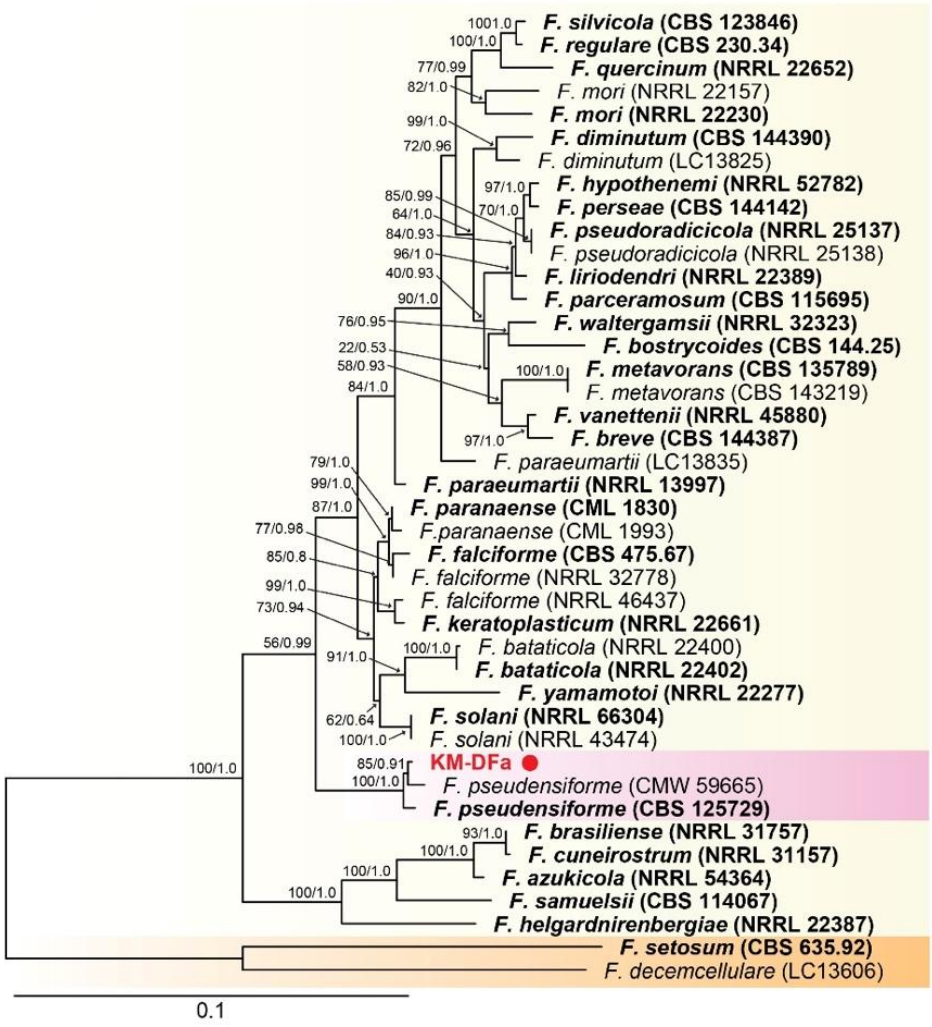

Figure-2. Maximum likelihood phylogenetic tree constructed based on combined *tef-1* and *rpb2* sequences of the present isolate and 41 taxa within the *F. solani* species complex. The isolate from this study is indicated in red, and ex-type strains are indicated in bold. The outgroups were *F. setosum* (CBS 635.92) and *F. decemcellulare* (LC13606). Bootstrap support values and Bayesian posterior probabilities (BS/PP) are shown at each node. The scale bar represents the number of expected nucleotide changes per site.

Effect of temperature and culture media on fungal growth and extraction yield

This study investigated the influence of temperature and culture media on the growth of *F. pseudensiforme*, focusing on biomass production (mycelial dry weight). Visual differences in fungal growth were immediately noticeable across culture conditions, with significant variations in mycelial morphology. At 25 and 30 °C, the mycelium grew densely and remained primarily above the liquid medium. At 35 °C, the mycelium formed agglomerated precipitate-like pellets and was immersed in the medium, as illustrated in Figure 3A. From mycelial dry weight measurements, the highest biomass was observed at 25 °C (6.34 mg/mL), followed by 30 °C (4.98 mg/mL), and then the lowest biomass at 35 °C (0.54 mg/mL), suggesting that higher temperatures negatively impact fungal growth (Figure 4A). Various studies have shown that temperature is a major factor affecting fungal growth and enzymatic reactions in biosynthetic pathways. The present results align with a previous report that an incubation temperature of 20-25 °C is optimal for the mycelial growth of *Rhizoctonia solani* (Ritchie et al., 2009). Regarding media selection, in general, fungal growth media consist of carbon and nitrogen sources along with trace amounts of fungal-specific elements. This

study evaluated four common culture media: PDB, MEB, CDB, and YSB. Among these, YSB was identified as the most effective for maximizing fungal biomass, with a mean dry weight of 9.40 mg/mL; at the other extreme, MEB resulted in the lowest biomass production. These differing results are likely due to the nutrient composition of each medium, reflecting that sugar source and abundance affects fungal growth. Specifically, fungi use saccharides for energy and as a carbon source, contingent on availability of sugar, cultural conditions, and adaptation of the strain on the substrate. Accordingly, higher carbon supplementation, such as the 150 g/L sucrose in YSB and 30 g/L sucrose in CDB, contributed to increased biomass yield. These results suggest that sucrose may be the most effective carbon source for biomass production. Several studies have similarly confirmed the importance of sucrose over glucose and other carbon sources in maximizing fungal growth yield (Wattanachaisaereekul et al., 2014), including medium optimization studies on *Xylaria* sp. BCC 1067 (Jayasekara et al., 2022) and *Xylaria* sp. 2508 (Xiaobo et al., 2006).

Extraction yield in this study followed a similar trend as for biomass production, with temperature and culture media significantly influencing metabolite yield. The highest extraction yield was obtained from YSB at 25 °C, with yield declining as the temperature increased (Figure 4B). The observed trends indicate that incubation temperature has a greater influence than culture media on both fungal biomass production and crude extract yield in *F. pseudensiforme*. Both parameters showed a consistent pattern of decline as temperature increased, regardless of the type of culture medium used. These findings align with previous reports highlighting temperature as a key environmental factor modulating fungal growth and secondary metabolism (Al-Zaban, 2023; Medina et al., 2017). Overall, these findings suggest that a temperature of 25 °C and the YSB medium provide the most favorable conditions for maximizing *F. pseudensiforme* growth and crude extract yield.

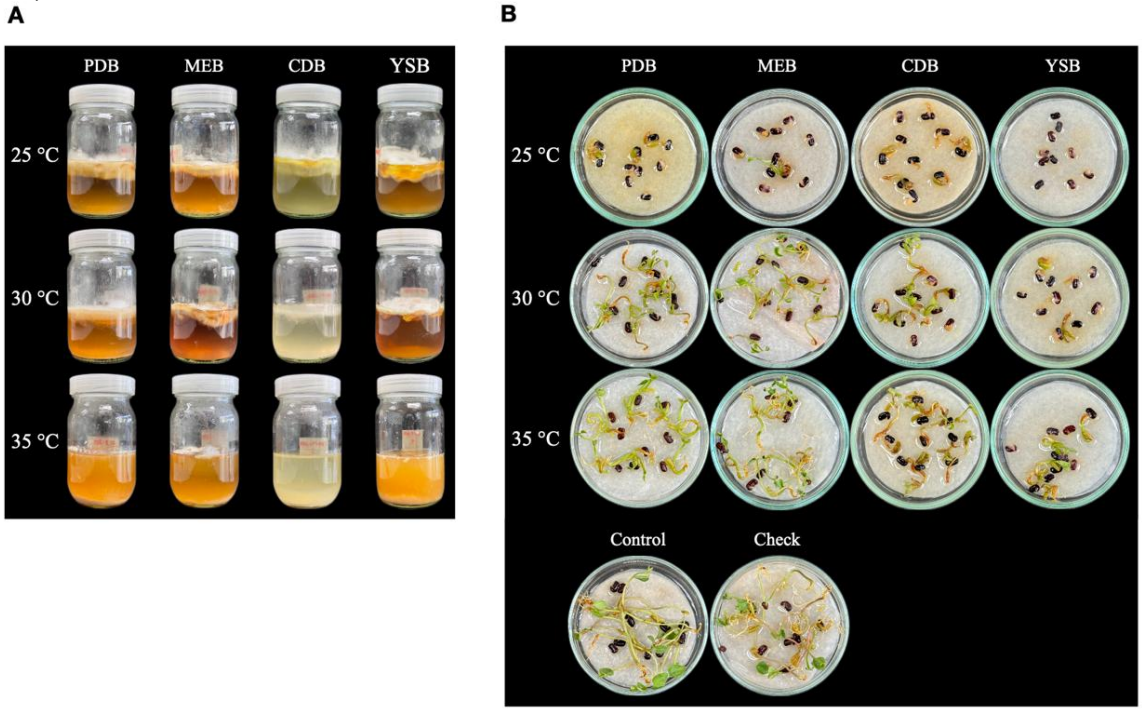

Figure-3. Effect of temperature and culture medium on (A) mycelial growth of *F. pseudensiforme* cultured under submerged conditions for 14 days and (B) the inhibitory effect of 10 mg/mL fungal extract on *P. lathyroides* seed germination at 7 days.

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Allelopathic effect of fungal extract on *P. lathyroides* seed germination

Figure 3B illustrates the inhibitory effect of ethyl acetate extracts from culture filtrates of *F. pseudensiforme* on *P. lathyroides* seed germination. Overall, the phytotoxic effect followed a similar trend as for fungal growth. All crude ethyl acetate extracts obtained from culture filtrates incubated at 25 °C exhibited highly significant inhibitory effects on all growth parameters, with the strongest effect being exerted by the YSB extract, at 93.33% inhibition relative to distilled water. Extracts from MEB, PDB, and CDB ($p > 0.05$) exerted intermediate effects, reducing seed germination by more than 50% compared to the control (Figure 4C). Inhibitory effects were reduced when *P. lathyroides* was treated with extracts obtained from culture filtrates incubated at higher temperatures (30 and 35 °C), showing the same trend across all culture media. Similarly, prior reports have found extracts from *F. solani*, *Aspergillus terreus*, and *A. fumigatus* to exhibit maximum antibacterial activity at 25 °C, with an incubation temperature range of 25-30 °C enhancing secondary metabolite production, whereas lower (15 °C) or higher (35 °C) temperatures resulted in reduced metabolite production (Jain and Pundir, 2011; Kalyani et al., 2023; Merlin et al., 2013). Also consistent with biomass findings, YSB extracts consistently exhibited the highest phytotoxic effect across all temperatures. In general, the degree of growth parameter inhibition followed the order: root length > shoot length > seed germination (Figure 4D, E). These results align with previous studies reporting the herbicidal activity of other *Fusarium* species extracts, such as *F. fujikuroi* (Daniel et al., 2018) and *F. equiseti* (Laosinwattana et al., 2024), as well as cultivated extracts of *Mycoleptodiscus indicus* (Ortaça et al., 2024), which similarly inhibited seed germination and root growth in plant species.

The varying degrees of inhibition observed in this study may be attributed to differences in the bioactive compounds present in the extracts. Beyond carbon source, allelochemical production in fungi is also significantly influenced by the nitrogen source (Kalyani et al., 2023), as nitrogen is an essential component of amino acids that are required for biosynthesis (Sharma et al., 2018). MEB contains both peptone and malt extract as nitrogen sources, while in YSP, yeast extract serves as the nitrogen source. In contrast, CDB and PDB respectively contain only sodium nitrate and potato extract as the nitrogen source. Yeast extract provides a broad range of nutrients and growth factors, including carbon, sulfur, trace elements, and members of the vitamin B complex. In a prior report, yeast extract did not visibly affect fungal growth, but did significantly enhance production of secondary metabolites in *F. pseudograminearum*, *F. graminearum*, *F. avenaceum*, and *F. fujikuroi* (Sørensen and Sondergaard, 2014). Our finding also aligns with the report of Merlin et al. (2013) that yeast extract enhances production of antibacterial compounds in *F. solani*. Thus, the complex and nutrient-rich composition of the YSB medium may promote the growth of *F. pseudensiforme* and facilitate its production of valuable allelochemicals, including herbicidal compounds.

Allelopathic effect of YSB extract on *P. lathyroides* morphology

YSB extracts had a significant, dose-dependent effect on the growth of *P. lathyroides* seedlings ($p < 0.05$, Figure 5), with the degree of inhibition increasing with higher extract concentrations. After seven days of treatment, seed germination was inhibited by 6.67%, 16.67%, and 90.00% relative to the control at respective extract concentrations of 2.5, 5, and 10 mg/mL. A similar pattern was observed for seedling growth, with partial inhibition at lower concentrations and complete inhibition at the highest concentration of 10 mg/mL (Figure 5C). The greater sensitivity of root growth is because radicles have more permeable tissue than other organs; thus, roots are less protected than shoots, leading to higher accumulation of phytotoxic compounds in root tissues. Morphological analysis of seven-day-old seedlings treated with YSB extracts revealed that both hypocotyl and root lengths were significantly shorter than in the control (Figure 5A). Overall, YSB extracts severely hindered hypocotyl and root development. The treated seedlings also had fewer lateral roots compared to the control (Figure 5B), suggesting that YSB extracts may affect both primary and lateral root tip structures. Severe root damage in treated seedlings can significantly impact plant development, as roots are the main organs responsible for nutrient and water uptake. These results are consistent with previous investigations on the effects of natural phytotoxins found in various plant and fungal species (Jiang et al., 2021; Landi et al., 2020; Wei et al., 2024).

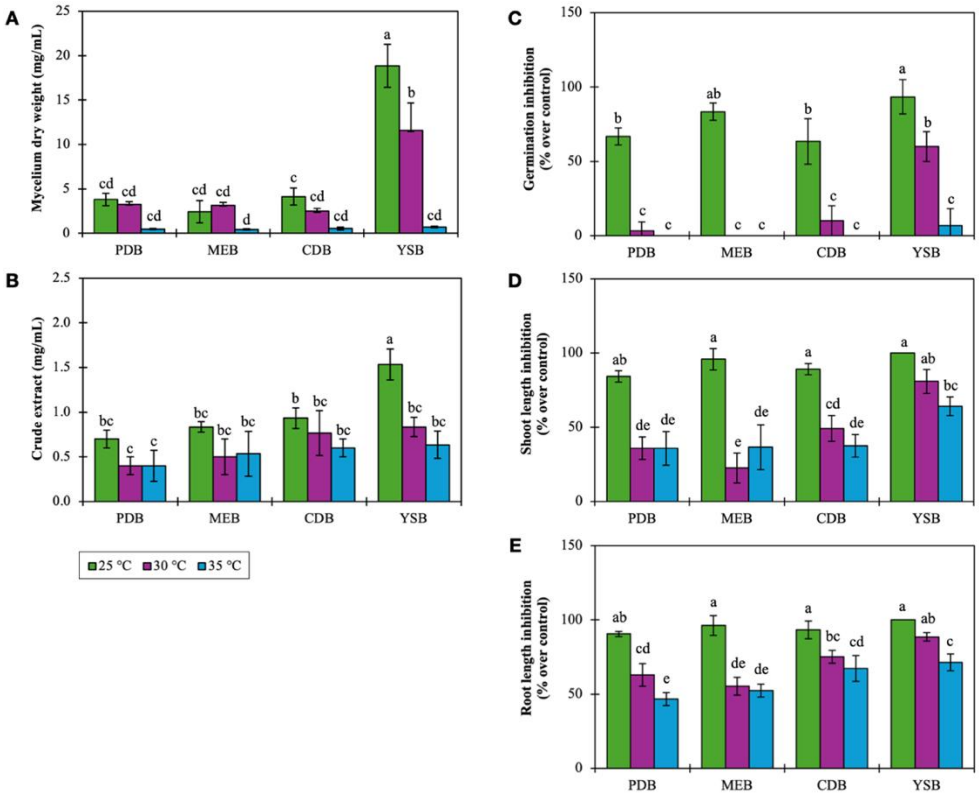

Figure-4. Effect of temperature and culture medium on (A) mycelial growth, (B) extraction yield, and (C-D) phytotoxicity of *F. pseudensiforme* extract. Fungal culture extract (10 mg/mL) was further evaluated by Petri-dish test for inhibitory effects on the (C) seed germination, (D) shoot length, and (E) root length of *P. lathyroides* at 7 days. Data are the means of four replicates, and different letters above bars indicate significant difference at $p < 0.05$ (Tukey's test).

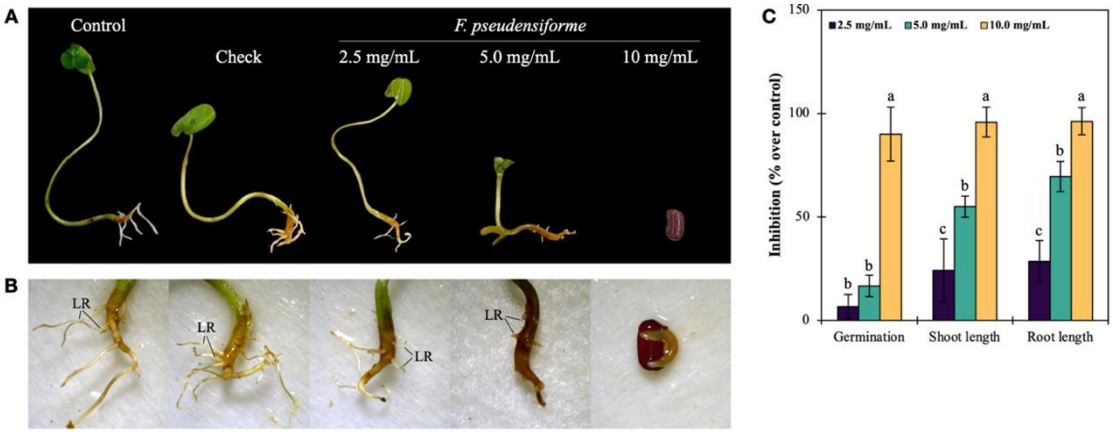

Figure-5. Growth inhibitory effects on *P. lathyroides* of the extract from *F. pseudensiforme* cultured on YSB at 25 °C, applied at 2.5, 5.0, and 10 mg/mL. (A) Morphology of *P. lathyroides* seedlings, (B) lateral roots (LR) and root tips, and (C) seed germination, shoot length, and root length after 7 days of treatment. Values are the mean of four replicates; different letters within a growth parameter are significantly different as indicated by Tukey's test ($p < 0.05$).

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Chemical profile of the phytotoxic extract

The chemical compositions of the six selected extracts were comprehensively analyzed using GC-MS. The results demonstrated that *F. pseudensiforme* produces a diverse range of metabolites when cultured under different conditions (Figure 6), with both qualitative and quantitative variations according to the culture media (Figure 7A). Specifically, for culture at 25 °C, a total of 34, 27, 23, and 18 chemical constituents were identified from fungi cultured on PDB, MEB, CDB, and YSB, respectively. These compounds accounted for relative area percentages of 97.10%, 95.12%, 98.60%, and 98.15% in the GC-MS analysis. Among these, 17 components were shared across all media (Figure 7B), suggesting a core metabolite profile. Ethyl propanoate emerged as the predominant compound in all extracts; however, its relative abundance varied markedly among the different media (Table S2). These findings suggest that carbon and nitrogen sources in culture media have critical roles in modulating allelochemical biosynthesis in *F. pseudensiforme*. The individual effects of constituent compounds on the crude extract's herbicidal activity remain unclear, and few studies have attempted to correlate specific metabolites with phytotoxic effects. The paucity of such research may be due to challenges such as difficulties in isolating specific herbicidal compounds, their low concentrations in extracts, or the possibility that herbicidal activity results from synergistic effects between multiple metabolites (Laosinwattana et al., 2025). However, prior reports of herbicidal activity have been made for some metabolites identified in this study. Wang et al. (2022) described that camphor induces phytotoxicity in tomato roots, potentially affecting nutrient absorption and leading to reduced biomass accumulation. Similarly, 2-phenylethanol, a phenolic compound, significantly inhibited the germination and development of *Allium cepa* and *Lactuca sativa* seedlings, affecting root and cotyledon size (Tena et al., 2021). Eicosane, a major component of *Bacillus inaquosorum* and *B. safensis* NL2 culture filtrates (11.01% and 14.12%, respectively), was shown to inhibit germination in *Cenchrus echinatus* (Hagaggi and Abdul, 2023). Another major group of compounds detected in this study was fatty acids and their derivatives, which are key metabolites of many fungi (Anisha and Radhakrishnan, 2017). The bioactivity of fatty acids is thought to be due to their ability to disrupt cell membranes, which may increase the uptake of other allelochemicals into plant cytoplasm. Other known phytotoxic compounds, including levomenthol (Synowiec et al., 2020), *m*-cymene (Khedhri et al., 2022), dodecane, and hexadecane (Sharma and Kumar, 2021), were also identified in all extracts.

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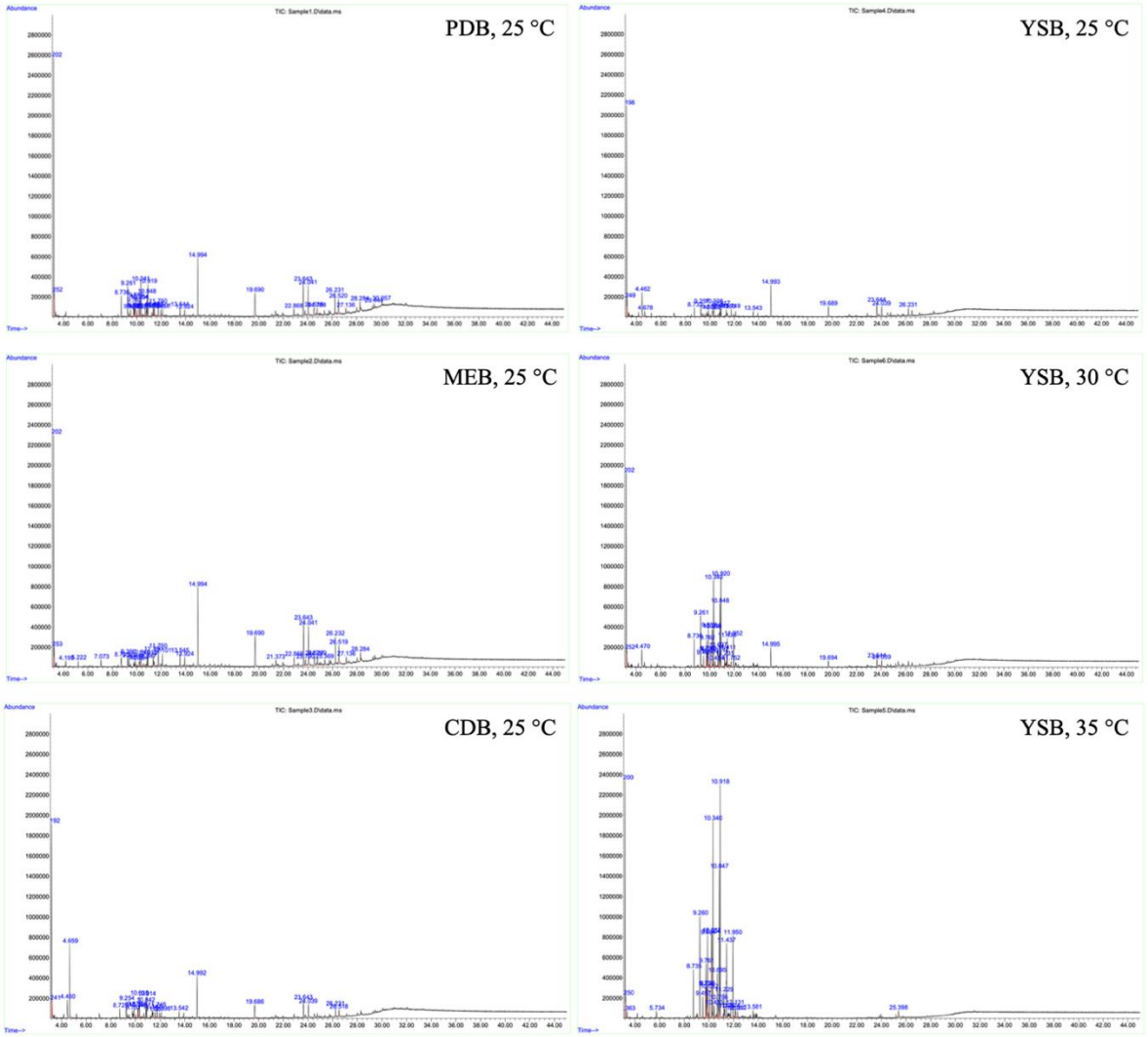

Figure-6. Chromatograms of the selected phytotoxic extracts derived from *F. pseudensiforme* cultured under different temperature and medium conditions.

The results of our study support the hypothesis that temperature influences the allelochemicals present in extracts, which in turn affects extract toxicity against plants. Specifically, extracts from fungi cultured on YSB media at 25 °C induced significantly higher phytotoxicity in *P. lathyroides* seedlings compared to extracts from cultures incubated at 30 and 35 °C. A clustering heatmap of the chemical constituents of six selected extracts (Figure 7A) revealed distinct clustering based on culture medium and incubation temperature. The main separation divided the 25 °C cluster from cultures grown at higher temperatures, which is consistent with the observed phytotoxic effects. GC-MS analysis further confirmed a temperature-dependent shift in the chemical profiles of *F. pseudensiforme* extracts (Figure 6), with increased accumulation of aromatic compounds at higher incubation temperatures. Specifically, ten benzene-related compounds were exclusively detected in the extracts from fungi cultured at 30 °C and 35 °C (Figure 7C), including benzene, 1,3-diethyl-, benzene, 1-methyl-3-propyl-, *p*-menthatriene, benzene, 1-methyl-2-propyl-, *o*-cymene, benzene, 2-ethyl-1,3-dimethyl-, benzene, 1-ethyl-2,3-dimethyl-, 2-phenylethanol, prehnitene, and naphthalene. An additional unique compound, benzene, (1-methyl-1-propenyl)-, (Z)- and benzene, 1-methyl-4-(1-methylpropyl)-. Thus, higher incubation temperatures may lead to enhanced biosynthesis of specific aromatic metabolites. The phytotoxicity of

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allelochemicals is significantly influenced by their structural characteristics. Studies emphasized that hydroxyl substitutions on the benzene ring (commonly referred to as phenols), such as carvacrol and thymol, which are abundant in essential oils, can interact with plant proteins and enzymes through hydrogen bonding and can induce oxidative damage to cellular structures (Ismail et al., 2013). These molecular features enable such compounds to interfere with plant physiology, resulting in strong phytotoxic effects. In contrast, the benzene derivatives detected in our study were mainly alkylated benzene compounds, whose substitution patterns lack reactive functional groups and therefore exert only nonspecific hydrophobic narcosis (Mohammedi, 2017). This structural simplicity, combined with their low reactivity and limited ability to specifically interact with plant cellular targets, may explain the low herbicidal activity observed under high-temperature conditions. Although specific studies on the accumulation of benzene derivatives by fungi at elevated temperatures are limited, some research indicates that certain fungi can produce or transform aromatic compounds under certain conditions. In general, benzene derivative compounds are biosynthesized via the shikimate pathway, which leads to the formation of aromatic amino acids. Previous studies have reported that using glucose as a carbon source enhances the production of aromatic compounds by stimulating biosynthesis of several aromatic intermediates (Noda and Kondo, 2017). Achimón et al. (2022) reported that *F. verticillioides* produces benzene derivatives in proportions ranging from 7.48% to 35.58% when cultured with different carbon sources. Notably, benzene, 1,3-dimethyl-, benzene, 1,2,4,5-tetramethyl-, and naphthalene were produced in higher percentages in cultures supplemented with sucrose. Additionally, *Hormoconis resinae* has demonstrated the ability to transform benzene derivatives via both oxidative and reductive pathways under acidic conditions (Mogil et al., 2023). While these studies do not directly link elevated temperatures to increased accumulation of benzene derivatives in fungi, they suggest that chemical and physical factors, including temperature, may influence metabolic pathways involved in the production or transformation of aromatic compounds in various fungal species. Overall, the observed temperature-dependent shifts in chemical composition support our hypothesis that changes in allelochemical profiles directly influence herbicidal activity. In particular, the benzene-derivative substitution patterns that predominated at higher temperatures provide a mechanistic explanation for the reduced phytotoxicity of the extracts obtained under these conditions. Further research is needed to elucidate the precise regulatory mechanisms governing temperature-dependent secondary metabolite production. Such insights could inform strategies for optimizing fungal cultivation conditions to enhance bioactive compound production at both laboratory and industrial scales.

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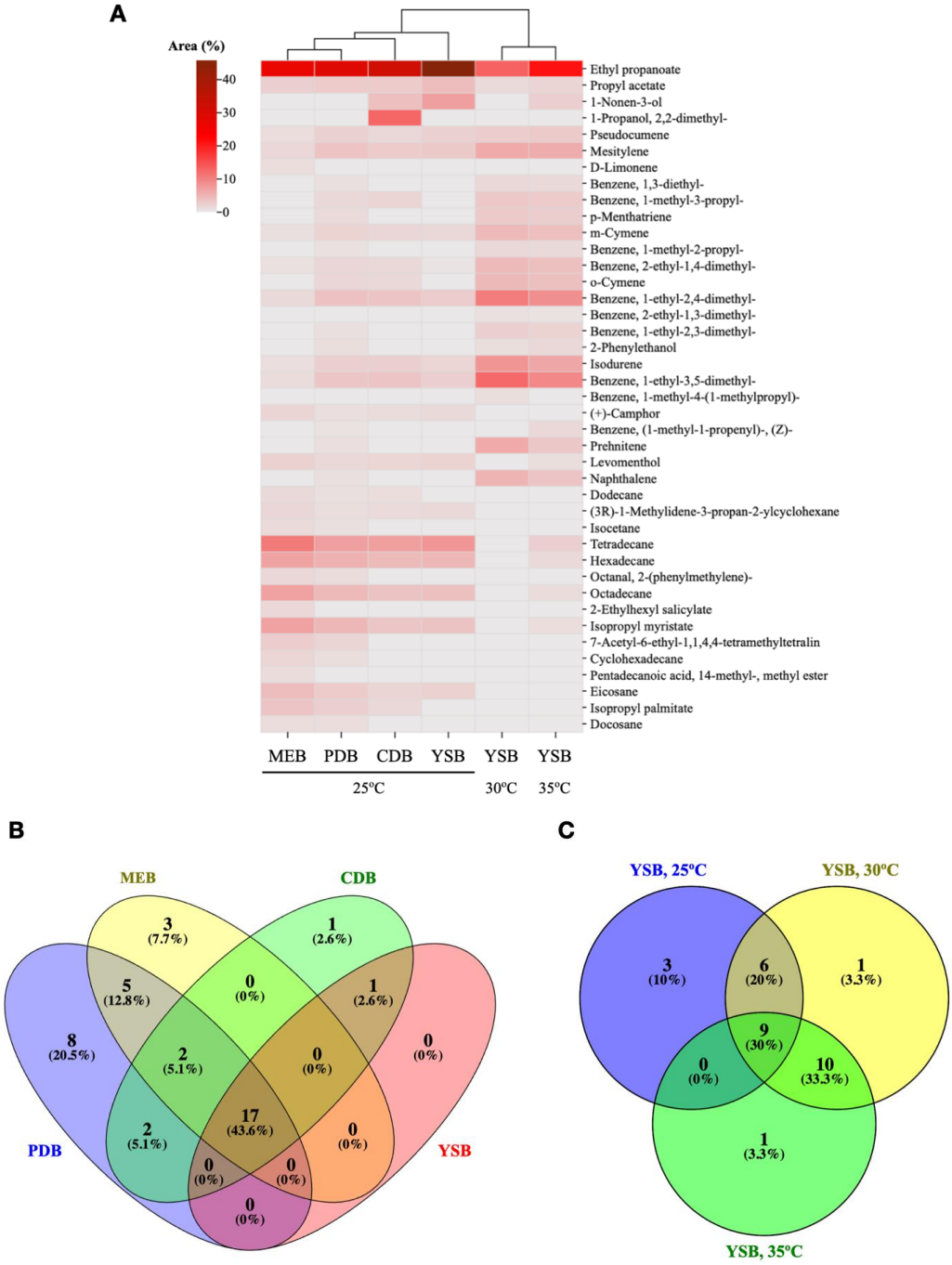

Figure-7. Chemical constituent analysis of phytotoxic extracts. (A) Clustering heatmap of the six extracts based on GC-MS peak area (%). Venn diagrams showing shared and unique chemical components in extracts from (B) each medium cultured at 25 °C and (C) YSB cultured at 25, 30, and 35 °C.

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Conclusions

Temperature directly impacts fungal growth and enzyme activity within the allelochemical biosynthesis pathway and can be precisely controlled in large-scale production systems. This study demonstrated that temperature significantly influences both the growth and metabolite production of *F. pseudensiforme*. Among the tested culture media, YSB consistently supported higher fungal biomass and extraction yield, possibly due to its rich nutrient composition promoting fungal metabolism. The resultant extract exhibited strong herbicidal activity against *P. lathyroides*. However, as incubation temperature increased, a shift in metabolic profile was observed, with increased accumulation of alkylated benzene compounds, known for their low herbicidal activity, which may contribute to the reduced efficacy. Future studies should focus on elucidating the specific mechanisms underlying temperature-dependent secondary metabolite biosynthesis and evaluating the field efficacy of these fungal extracts. Such insights could facilitate the efficient large-scale production of fungal bioactive compounds and promote the development of fungal-derived natural herbicide alternatives to chemical herbicides in weed management.

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Contribution of Authors

Poti T & Tunchai M: Investigation, methodology, formal analysis, writing-review and editing.

Manichart N: Conceptualization, investigation, project administration, writing-original draft.

Wichittrakarn P: Investigation, methodology and formal analysis.

Yoneyama K: Formal analysis, validation, writing-review and editing.

Laosinwattana C: Conceptualization, funding acquisition, resources and supervision.

All authors read and approved the final draft of the manuscript.

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