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Allelopathic potential of secondary metabolites produced by *Alternaria brassicicola* and physiological mechanisms on *Amaranthus tricolor*

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Abstract The results revealed that all isolated fungal strains significantly reduced germination and early growth of amaranth compared to control. The original metabolites (1.00X) from the fungus coded UNK03, which was identified as a strain of *Alternaria brassicicola* (98.44% similarity, accession number LC440588.1), showed the highest inhibition rate and suppressed with the maximum inhibitory level (78.75%) compared to control. In a similar pattern, the growth of the tested plant was significantly reduced by the cultural filtrates by 70.45% and 89.38% in shoot and root, respectively. Based on efficacy in the seed bioassay, the *A. brassicicola* metabolites (CF03) were selected for exposure with the tested seed to determine the mechanism of action (MOA). The results show that the *A. brassicicola* metabolites inhibited seed imbibition and α-amylase activity of the tested seed with dose-dependent effects, strongly suggesting the potential of the metabolites to inhibit seed germination and seedling growth. Therefore, this work indicates that the fungal metabolites contain potent herbicidal compounds that can potentially be used to control *A. tricolor*.

Keywords: *Alternaria brassicicola*, Herbicidal activity, Mechanism of action (MOA)

Introduction

Thailand is an outstanding producer of agricultural products such as food, fiber, and derivatives in the world market economy. However, crop plantation often involves the problem of pests, such as weeds, insects, and pathogens. Weeds are a special problem; they are a big nuisance for commercial crop growers due to their broad diversity and competitive power (Daba *et al.*, 2021). They compete with crops for space, nutrients, water and light (Wu *et al.*, 2017). Several weed control problems exist, such as the ever growing and evolving resistance in the weed population, and the high cost of developing and registering new chemical herbicides (Bastos *et al.*, 2017). Consequently,

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previously used chemical reagents on the market, particularly when used at the same concentrations, cannot be used in subsequent outbreaks. Weed management will change in the future because the environment and human health are primary concerns in modern agriculture (Araújo *et al.*, 2008). For these reasons, many countries are attempting to reduce their reliance on chemical herbicides. Taking Thailand as an example, Thailand authorities have enforced a ban on glyphosate and paraquat, which are substances often found in herbicides. Nowadays, there are no alternate approaches that offer long-term sustainable weed suppression. This situation has produced a growing body of research into new bioherbicides that have safer toxicological and environmental profiles.

The secondary metabolites produced by fungi via submerged fermentation have emerged as a body of compounds that display almost endless biological activities. From this perspective, the allelochemicals derived from secondary pathways have been investigated for their allelopathic potential, and in particular their ability to inhibit seed germination and development of various plants (Fritz *et al.*, 2007; Silva *et al.*, 2012). Various pathogenic fungi have bioactive compounds such as terpenoids, flavonoids, steroids, alkaloids, and quinones, some of which can be utilized industrially, and included are a range of enzymes and solvents (Zheng *et al.*, 2006). These enzymes which include pectinases, cutinases, hemicellulases, cellulases, ligninases, amylases, phospholipases and lipases, can degrade plant cell walls and membranes, allowing them entry or dispersion into their host (Ghorbani *et al.*, 2005). Fungal metabolites fit into this situation because they usually produce mycotoxins with the potential to inhibit or control the growth of host plants. Recently, the natural products derived from fungi have been tested for their potential use in numerous fields such as agriculture, industry, and medicine. We here are primarily interested in their application as green actives that can replace chemical herbicides (Guo *et al.*, 2020). One alternative is the production of stable formulated bioherbicides by extracting the herbicidal compounds from fermented fungal broth and mixing them with adjuvants or surfactant agents. This strategy has become one of the favored methods for weed control because direct use of a fungus as a biocontrol agent is limited by the survival of the fungus and its ability to continue to spread in an uncontrolled fashion in the environment. So, using bioherbicides is a preferred option to using biocontrols. The herbicidal activity of fungal culture filtrates against various weeds was reported by Sica *et al.* (2016). Two species within the fungal genus *Sclerotinia,* and the genus *Phoma* sp. have received attention as potential agents for biological weed management. Evidente *et al.* (2006) used *Drechslera gigantea,* a fungal pathogen isolated from *Digitaria sanguinalis*, to produce phytotoxins

in liquid and agar mediums as potential myco-herbicides for grassy weed control. Moreover, Javaid *et al.* (2013b) suggested that culture filtrates of different *Trichoderma* species contained active herbicidal compounds that could be used for parthenium control. Natural products with allelopathic activity based on plant or fungal extracts have been tested on plant models like *Lactuca sativa* (lettuce), *Solanum lycopersicum* (tomato) and *Cucumis sativus* (cucumber) (Dos Santos *et al.*, 2019), and via Petri dish assays of seed germination and plantlet development (Tur *et al.*, 2010). In this study, *Amaranthus tricolor* (amaranth) was used as a plant model to evaluate the allelopathy of fungal metabolites. In fact, members of the *Amaranthus* genus (*A. retroflexus*, *A. hybridus* and *A. powelii*) have become severe cropland weeds in several regions worldwide. Importantly, *A. tricolor* presents sensitivity to allelochemicals at low concentrations, and shows rapid germination and development of root and linear shoot parts. The objective was to isolate and identify the fungi present in the infected lesions of weed leaves. The fungi were selected which based on the seed germination bioassay, and then their two mechanisms of action (MOA) and seed imbibition and α-amylase activity were investigated using *A. tricolor* as a plant model.

Materials and methods

Isolation of fungi

Diseased weed leaves were collected from agricultural areas in Ladkrabang district, Thailand, and the phytopathogenic fungal strains were isolated by the tissue transplanting method. After collection, the leaves were thoroughly washed in running tap water for 5 min to remove dust and debris, and the samples were allowed to air dry. The cleaned plant samples were surface sterilized with 70% ethanol, followed by 3% NaOCl solution for 4 min, immersed in 70% ethanol again, and washed twice with ddH_2O. All sterile plant samples were cut under sterile conditions into small pieces (1×1 cm). Each infected tissue was transferred to a Petri dish containing water agar (WA) medium, then incubated at 25°C for 1-7 days until the fungal mycelia were observed. The emergent hyphal tips of the fungi from the plant tissue were cut and placed on Potato Dextrose Agar (PDA) medium (Merck, Germany) to obtain pure cultures.

Fungal fermentation

Fungal metabolites were prepared for each strain by inoculating a fungal disc (5 mm) of the isolated fungal strain to autoclaved fermentation broth (FB)

in 250 mL flasks under aseptic conditions. The fermentation medium was composed of (g/L): potato extract (50.0), glucose (20.0), beef extract (10.0) and peptone (10.0). The final pH was adjusted to 5.1±0.2. The inoculated flasks were incubated at 25°C for 28 days. After incubation, each fungal mat was separated from the broth through a sterilized muslin cloth, and this was followed by further filtration using Whatman® Millipore No. 1 filter paper (150 mm dia.). Filtrates were centrifuged at 600 rpm for 5 minutes and filtered through sterilized filter papers (45 μm, MS®, UAS). The original fungal metabolites (1.00X) were diluted with autoclaved distilled water to prepare lower concentrations (0.50X and 0.25X). Each filtered sample (culture filtrate; CF) was used to evaluate its herbicidal activity in the bioassays.

Seed germination bioassays

The seeds of *Amaranthus tricolor* were purchased from Chia Tai Co. Ltd, Bangkok, Thailand. Germination tests were done using 5 mL of CF in a germination paper with 20 seeds of culture filtrate. The experiments were kept in a growth chamber (LAC-1075-N, Longyue, Shanghai) at 27 ± 2°C, 12/12 hrs light/dark, and around 80% relative humidity. The fermentation broth (FB) without cells and distilled water served as a control. The germination of tested seeds, root and shoot length (cm) were recorded after the 7 days. The inhibition percentage was then determined as follows:

$$\text{Inhibition (\%) over control} = \left(1 - \frac{\text{culture filtrate treatment}}{\text{control (ditilled water)}}\right) \times 100$$

The selection of potential fungi was made by seed germination bioassay. A total of 4 isolated fungi were codified (as UNK01, 02, 03 and 04) and used. All isolated fungi were grown in submerged fermentation (FB) in the first step. The culture mediums that presented the highest inhibition of *A. tricolor* germination were selected for further experiments.

Seed imbibition

The selected cultured medium was used for the evaluation of seed imbibition. The CF concentrations were 1.00X (original CF), 0.50X, 0.25X and 0.00X (FB). Water was the control. Seed imbibition was measured at 6, 12 and 18 hours after exposure for 100 *A. tricolor* seeds per treatment. Briefly, the initial seed weight (W1) was recorded and soaked in CF solution for each

concentration and exposure time. After incubation, the seeds were washed and weighed (W2). Seed imbibition percentage was expressed as follows:

$$\text{Seed imbibition (\%)} = \frac{W2 - W1}{W1} \times 100$$

α-amylase activity assay

The enzymatic activity of α-amylase (EC 3.2.1.1) was done by dinitrosalicylic acid (DNS) assay. After measuring the seed imbibition, the seeds were grained with 4 mL ice-cold 0.1 M $CaCl_2$ solution and centrifuged at 12,000 rpm for 20 min at 4°C. The supernatant was collected and used as the enzyme source. The reaction was done by mixing 1 mL of enzyme and 1 mL of 0.5% soluble starch as substrate. The mixture was incubated, and 1 mL of DNS reagent was added to the mixture. The reaction solution mixture was immediately heated in a boiling water bath for 5 min. Finally, the light absorption at 560 nm was measured using a UV/Vis spectrophotometer (Thermo Fisher Scientific, USA). The α-amylase activity was calculated and expressed as μmol maltose/min/g (FW).

Molecular identification of the fungus

The most promising fungi to produce bioherbicide was identified using a molecular technique according to the method of White *et al.* (1990). Fungal mycelia were ground to a fine powder using a sterile mortar and pestle. The fungal genomic DNA was isolated using the CTAB method. The internal transcribed spacer region (ITS) was amplified with the specific universal primers ITS1 and ITS4. Polymerase chain reaction (PCR) amplifications were performed using a thermal Cycler (BioRad®, USA). Afterward, amplicons were analyzed in 1.5% agarose gel in 1X TBE buffer, colored with Blue Green Loading Dye I® (LGC Biotecnologia, Cotia, Brazil), and visualized in ultraviolet light. The PCR samples were sent to be purified and sequenced at Marcogen™ (Seoul, Korea). For the identification of fungi, the sequences were compared with available sequences in the NCBI database using BLASTn tool. The ITS sequences were concatenated and aligned by the ClustalW algorithm with sequences from the GenBank database for the phylogenetic analysis. A Maximum Likelihood tree method was constructed based on the analysis of the ITS regions, employing MEGA11 software.

Statistical analysis

The whole experiment was performed in a complete randomized design (CRD) with three replications. The experiment was repeated two times, and the averaged data were used for analysis. All treatments were subjected to analysis of variance (ANOVA), and the treatment means were compared using Tukey's test at $p<0.05$.

Results

Seed germination bioassay

In the first step, 4 samples of isolated fungi (UNK01, 02, 03 and 04) were cultured under submerged fermentation to produce fungal metabolites. After filtering, each cultured filtrate (CF), which was codified as CF01, 02, 03 and 04, was tested as a potential bioherbicide precursor in the pre-emergence of *A. tricolor*. The results obtained are shown in Figure 1.

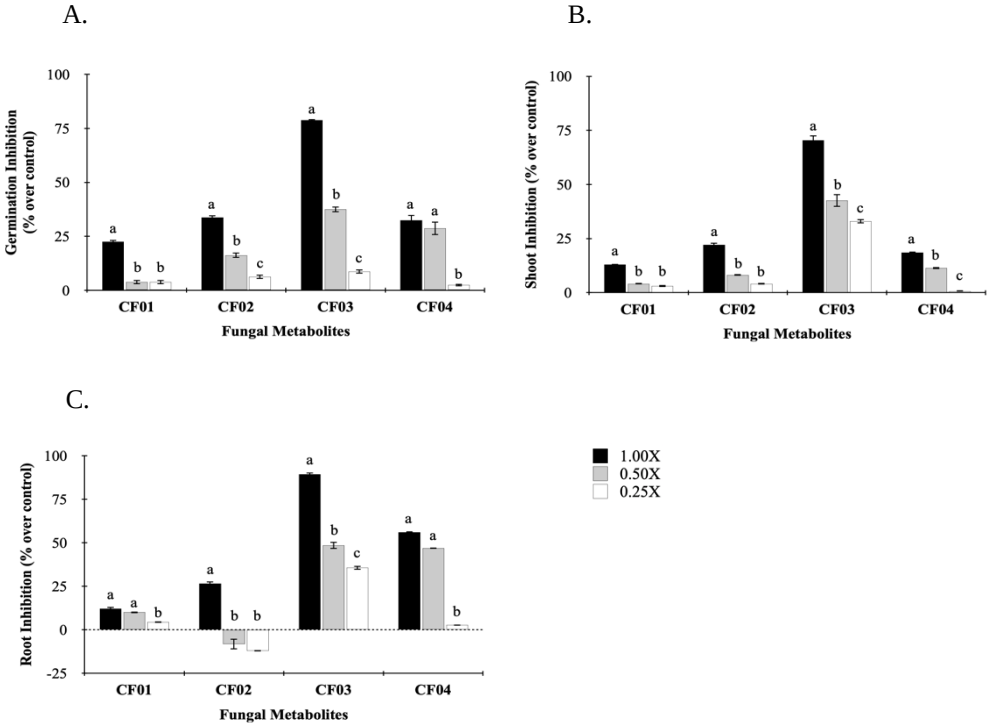

Figure 1. Herbicidal activity of original and diluted cultured filtrates (CF01-04) on *A. tricolor* emergence (A) and seedling growth (B and C)

All treatments moderately affected *A. tricolor* seed germination, ranging from 2.50 to 78.75% (Figure 1A). For the CF01, CF02 and CF04 treatments, the inhibition percentage was less than 35.00%, indicating low herbicidal activity of these cultured broths. Regarding the CF03, the germination of seeds was reduced to 78.75% in the case of the original broth (1.00X) and inhibition decreased to 37.50 and 8.75% in the diluted concentrations 0.50X and 0.25X, respectively. Application of CF03 at a high concentration level significantly (p<0.05) reduced germination compared to the lower concentration levels, showing that the CF03 exerted a dose-dependent inhibitory effect. Although the germination of fermented broth was not significantly different from the control, promotion of germination, root, and main axis growth were observed in some cases (data not shown). A similar pattern was observed for the initial seedling growth stage; all treatments were found to have an inhibitory effect on root and shoot length in tested plants, except for CF02 at diluted concentrations. The fungal metabolite treatments presented inhibitory percentages ranging from 8.14 to 70.45% and 10.03 to 89.38% in the shoot (Figure 1B) and root (Figure 1C), respectively. The initial weed growth was more affected in the case of CF03, with a reduction of 70.45% (shoot) and 89.38% (root) at the original broth concentration (1.00X), demonstrating the toxicity of metabolites for both shoot and root.

Seed imbibition and α-amylase activity

The potential herbicidal activity of CF03 was confirmed in the seed imbibition and α-amylase activity assays (Figure 2). Seed imbibition is a water absorption process in the seed germination stage. In the control and FB treatments, seed imbibition gradually increased with the imbibition period. The results in Figure 2A show that after the first 12 hours of treatment, all concentrations of tested broths showed non-significantly different imbibition percentages. On the other hand, at 18 hours, seed imbibition percentage of control, FB, 0.25X, 0.50X, and 1.00X were 45.08, 43.53, 35.91, 28.57 and 22.60%, respectively. Treatment with the highest concentration resulted in the lowest seed imbibition percentage. The α-amylase activity of seeds was found during the germination process to be degradation of carbohydrates to produce energy required for germination. Following the same trend as seed imbibition, after 12 hours, the CF03 treatment was not significantly different from the control; however, at 18 hours, α-amylase activity had significantly (p<0.05) decreased with the dose-dependent response. The α -amylase activity in amaranth seeds treated with CF03 at concentrations of 0 (control), 0.25X,

0.50X and 1.00X were 3.04, 2.30, 1.92 and 1.50 μmol maltose/min/g (FW), respectively (Figure 2B).

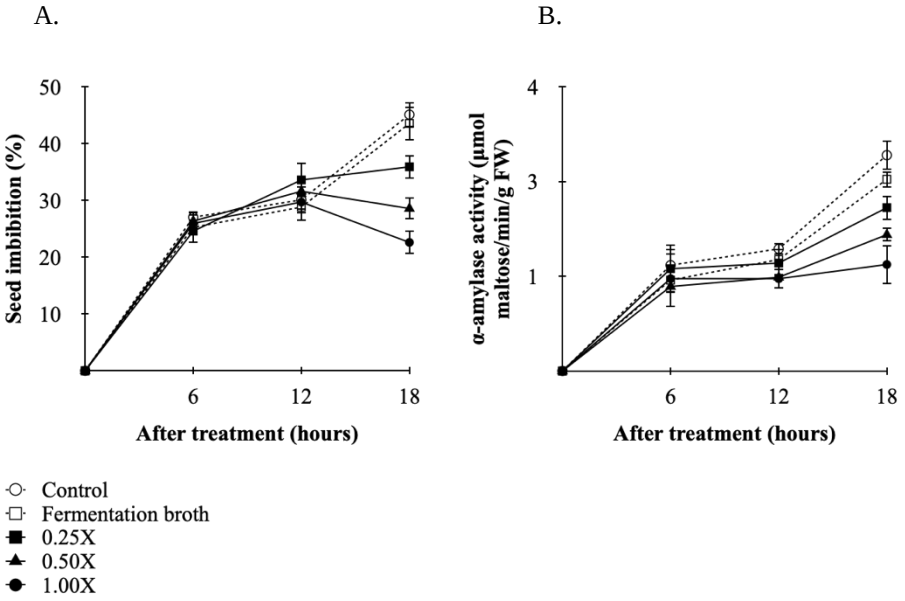

Figure 2. Effect of the UNK03 cultured filtrate (CF03) on seed imbibition (A) and α-amylase activity (B) of *A. tricolor* seeds at 6, 12, and 18 hours

Molecular identification of UNK03

Considering that the treatment with UNK03 provided the highest the inhibition seed germination percentage of the target plant, this isolate was selected for identification. Molecular analysis was carried out by sequencing the PCR products of the partial regions of ITS1, and ITS4 sequences, and it revealed that UNK03 was 98.44% identical with *Alternaria brassicicola* under GenBank Accession No. LC440588.1. A phylogenetic tree (Figure 3) was constructed by MEGA11 software by using multiple sequence alignment, and a phylogenetic relationship of UNK03 was drawn with other *Alternaria* species. It revealed that the UNK03 obtained in this work became clustered with *A. brassicicola* strains.

Figure 3. Maximum likelihood phylogenetic tree relationship between the UNK03 and some reference isolates of *Alternaria* species. Bootstrap values supporting the branch were shown at the nodes

Discussion

The present work was designed to use alternative natural resources like fungal metabolites to control weeds. To this end, phytopathogenic fungi were isolated and screened for herbicidal activity. The genetic identification revealed that the selected isolate was a strain of *A. brassicicola*. For pre-emergence bioassay, both original (1.00X) and dilute (0.25X and 0.50X) metabolites of this strain significantly reduced the germination and seedling growth of *A. tricolor* weed. However, the inhibitory effects of the original metabolites were better than those of the dilutions. The results show that the herbicidal activity of the metabolites was associated with their concentration. These findings agree with those of Javaid *et al.* (2017), who noted that metabolites from *Phoma* sp. showed an inhibitory effect on *Bidens pilosa* seed germination. The bioherbicidal effect of secondary metabolites from *Aspergillus niger* (Javaid *et al.*, 2013a; Javaid *et al.*, 2014), *and Trichoderma* sp. (Mohammed, 2020), reduced parthenium seed germination by more than 90.00% at the original metabolite concentration. The genus *Alternaria* is comprised of 299 species, many of which are phytopathogenic, saprophytic, and common contaminants of crops (Pavicich *et al.*, 2022). Javaid *et al.* (2017) reported that a cultured filtrate of *A. japonica* prepared in malt extract broth (MEB) reduced parthenium seed germination by 51.00% compared with the control. On the other hand, the

metabolites prepared in potato dextrose broth (PDB) decreased germination by 80.00% over control. In this work, the adjusted broth consisted of potato extract, glucose, beef extract and peptone. The germination percentage was 78.75%. This may have been different to the result obtained in previous work because the different herbicidal effects of fungal metabolites might not only been the result of the different metabolite concentrations used but might also have been due to the quantities of herbicidal compounds in the growth (Javaid *et al.*, 2017). According to Lou *et al.* (2013), the ascomycetous fungus *Alternaria* sp. can produce over 268 metabolites from amongst its different species, and most of them show phytotoxic and antimicrobial activities. The investigation of the herbicidal effects of different *Alternaria* were reported. Tenuazonic acid in *A. alternata* was identified, and its herbicidal activity against parthenium weed was confirmed (Bhagwan Singh *et al.*, 2010). Likewise, Barash *et al.* (1981) reported that zinniol isolated from culture filtrates (CF) of the mycelia and cell walls of *A. dauci* had phytotoxic effects, causing dark brown necrotic spots on carrot leaves within 1 hour. Moreover, the ascomycetous fungus *A. macrospora* caused significant damage (leaf blight) to the leaves of the *Parthenium hysterophorus* weed. (Kaur *et al.*, 2014). In this current study, the secondary metabolites of the tested fungi exhibited variable herbicidal activity against the early growth of *A. tricolor*. The results showed that root length (89.38% inhibition) was more sensitive to metabolites than was the shoot (70.45% inhibition), as shown in Figure 1B and 1C. During the germination step, the root, known as radicle, is the first structure that grows out of the seed coat. This situation means that the radicle is exposed to the cultured filtrate for a longer time, and consequently, radicles are destroyed more than the shoots. This agrees with the findings of Akbar and Javaid (2010), who observed that the roots of tested plants were more susceptible to *Drechslera* sp. cultured filtrate than were the shoots. *Alternaria* is the genus most cited in the study of mycotoxins and phytotoxic effects on plants, and to do with the production of bioherbicides. However, there were no reports of *A. brassicicola* pre-emergence herbicidal activity and its physiological mechanism. Our study is the first one specific one in the literature. The results indicated that *A. brassicicola* metabolite inhibited the *A. tricolor* seed germination through the reduction of seed imbibition and α-amylase activity. The results agreed with the report by Teerarak *et al.* (2012), who stated that inhibitory effects on seed imbibition increased with increased metabolite concentration. Furthermore, inhibition of α-amylase activity caused stored carbohydrates not to be degraded into the soluble sugars needed to fuel the early growth and development of seedlings (Somala *et al.*, 2022). The present study concludes that submerged fermentation using *A. brassicicola* produced phytochemical compounds with herbicidal

activity. Future research is required to extract and identify the main compounds that show phytotoxic effects in these culture filtrates. Such research can further support the production of novel bioproducts.

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