
Inhibitory effects of 8 toxic mushroom strains on growth and germination of *Alternaria alternata*

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The inhibitory effects of culture, filtered culture and ultra-sonified mycelial extracts of 8 toxic mushroom strains [*Amanita virosa*] *Lepiota clypeolaria*] *Lactarius vellereus*, *Amanita pachycolea*, *Amanita* sp., *Ramaria ephemero-derma*, *Clitocybe dealbata*, *Lepiota cristata*] on *Alternaria alternata* were studied. The culture extracts and filtered culture extracts inhibited the *in vitro* growth of *Alternaria alternata*. With the exception of *Ramaria ephemero-derma* and *Lepiota cristata*, ultra-sonified mycelial extracts also inhibited the growth of *Alternaria alternata*. With the exception of *Amanita* sp. and *Ramaria ephemero-derma*, the culture extracts also inhibited *Alternaria alternata* conidial germination. *Lactarius vellereus* had the strongest inhibitory effects on growth and germination of *Alternaria alternata*; the growth inhibitory rate and germination inhibitory rate of the culture extracts were 61.44 to 90%.

Key words: *Alternaria*, *biocontrol*, *poisonous mushrooms*, *popular leaf blight*, *toxins*

Introduction

Mass chemical control of forest tree diseases has caused serious environmental pollution, destruction of forest ecosystem balance and problems to human health and livelihood. Disease caused by popular leaf blight has caused serious problems in nurseries of northeastern China. Popular leaf blight is caused by *Alternaria alternata* (Fr.) Keissler (anamorphic *Pleosporales*). The pathogen infects leaves and stems of poplar seedlings, and causes leaf blight, shoot blight and stem canker. It has caused significant economical and ecological losses (Xu and Sun, 1986). The disease is mainly present in nurseries. Disease control using fungicides is important because the disease in nurseries rapidly spreads and is prevalent. It is therefore important to find new effective biological pesticides with low toxicity.

Various mushrooms are toxic and may cause poisoning if eaten (Meng *et al.* 1997). Most of toxic mushrooms belong to the Basidiomycotina, with many

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in the genus *Amanita* (Hymenomycetes, *Amanitaceae*), *Inocybe* (*Cortinariaceae*), *Panaeolus* (*Coprinaceae*) and *Russulaceae* (Zhang *et al.*, 2004). Presently, more than 190 species of toxic mushrooms belonging to 58 genera and 26 families are known from China. Of these, 179 species are Basidiomycotina belonging to 50 genera and 20 families. More than 40 species are highly toxic, and about 30 species may result in death if consumed. More than 20 other species have slight toxicity and symptoms can be treated if eaten; they are called conditional edible mushrooms (Mao, 1980, 1987, 1991, 2000).

Research on the harmful affect of toxic mushrooms on humans is limited. There are many active substances in toxic mushrooms, some of which have anti-biotic, microbiostatic and anti-viral properties; the active substances of these toxic mushrooms have great potential for application in biocontrol.

There have been many reports that various mushrooms produce substances that may be used in biocontrol. Examples include *Amanita hemolysin* extracts that have a specific effect against actin; antibiotics produced by *Lentinus squarrosulus* that can inhibit the growth of *Rigidoporus lignosus*, *Saccharomyces cerevisiae* and *Bacillus subtilis*; biformine produced by *Coriolus biformis* that has inhibitory activity against G^+ and G^- bacteria and fungi; phenolic compounds produced by *Armillariella mellea* that have inhibitory effects against G^- bacteria, fungi and viruses (Min, 1996). Extracts from *Agaricus xanthodermus* can also inhibit *Staphylococcus aureus*; nebularine from *Clitocybe nebularis* can inhibit the growth of *Mycobacterium tuberculosis* and bacteriophages; cultural extracts from *Amanita pantherina* and *A. kwangsiensis* produce high mortality in flies; extracts of *Russula laurosi* and *R. emetica* have inhibitory effects on various moulds; a type of clitocybin isolated from *Leucopaxillus giganteus* and *L. candidus* has inhibitory effects against G^+ and G^- bacteria; clitocybin isolated from fermented broth of *Clitocybe illudens* has inhibitory effects against moulds and also anti-tumour effects. Lampterol isolated from the fruiting body of *Lampteromyces japonicus* is inhibitory against various moulds. *Russula emetica* and *Panallus stypticus* have inhibitory effects against the mouse poliomyelitis virus; phallotoxin and amanita toxins from *Russula emetica* and *Amanita verna* can efficiently kill red spiders; ibotenic acid from *Amanita verrucosivolva* is a lethal chemical toxic preparation against flies, but is not harmful to humans (Meng *et al.*, 1997, Zhang *et al.*, 2004, He *et al.*, 2002). Sesquiterpene enol from *Lactarius vellereus* and *L. rufus* have an anti-feeding effect against some insects (Kopczacki *et al.*, 2001). These examples show that it is practicable to control plant harmful organisms by using toxins from poisonous mushrooms.

The aim of this research was to screen extracts from toxic mushroom strains for the control of the pathogen causing popular leaf blight, in order to explore the basis for exploiting possible biological pesticides.

Materials and methods

Eight isolates of toxic mushroom were tested comprising *Amanita virosa* Lamb. ex. Secr. *Lepiota clypeolaria* (Bull. Ex Fr.) Quil. *Lactarius vellereus* (Fr.) Fr., *Amanita pachycolea* Stuntz, *Amanita* sp., *Ramaria ephemerotheca* Sacc. & Syd., *Clitocybe dealbata* (Sow. ex Fr.) Gill. and *Lepiota cristata* (Bolt: Fr.) Quel. The isolates were obtained from the Northeast Forestry University culture collection. The pathogen isolate of *Alternaria alternata* was also obtained from the Northeast Forestry University culture collection.

The culture medium used in the experiment were:

1. Modified PDA: potato 200 g, glucose 20g, MgSO₄ 1.5g, NaH₂PO₄ 3g, agar 20 g, water 1000 ml.
2. PD: as above but no agar.
3. Double strength PDA: except for water, the other components of medium are doubled.

Preparation of culture extracts

Toxic mushroom isolates were inoculated in liquid medium and cultured with a shaker (120rpm/min, 25°C) for 20 days using modified methods of Cheng *et al.* (1995), Ke (2002) and Wang *et al.* (2004).

In order to provide some idea to the origin of the inhibitory substance total culture extracts, filtered culture extracts and ultra-sonified mycelial extracts were prepared. Total culture extracts utilised the raw broth and culture extracts were obtained by filtering through 8 layers of sterilized tissue paper to extract mycelium (Cheng *et al.*, 1995; Lin, 2003). Ultra-sonified mycelial extracts were prepared by washing the mycelium filtered from the broth in sterilized water several times, followed by ultra-sonification for 2 hours and then filtered (Lin *et al.*, 2003, Ling *et al.*, 2000; Xu *et al.*, 2003).

Assay for growth inhibition

The effect of extracts on the growth rates of *Alternaria alternata* was used in determine the growth inhibition effect (Xiang, 1991; Fang, 1998). Petri dishes with media were prepared using the same quantity of filtered culture extracts or ultra-sonified mycelial extracts. A 0.5 cm diameter plug from the

growing edge of a 7-day-old colony of the pathogen cultured on PDA was placed in the centre of the assay plate. The plate was incubated at 25°C for 7 days, and the colony radius was measured. Plates prepared without the extract and water acted as the control. Each treatment was replicated 5 times.

Growth inhibition rate =

$$\frac{\text{mean radius of control} - \text{mean radius of plate with extracts}}{\text{mean radius of control}} \times 100$$

Inhibition of germination

A conidial suspension (1 ml) in water (3×10^6 /ml) was placed on a slide and 1 ml of filtered culture extracts or ultra-sonified mycelial abstract was added and mixed thoroughly (Xiang 1991 and Fang 1998). Slides were placed in a moist chamber at 25°C and checked every 4 hours for up to 24 hours and the number of germinating spores recorded. In the control, sterile water with 2% glucose and PD media was used. In order to establish the source of the inhibition of germination, culture filtered extracts and ultra-sonified mycelial extracts from the most effective toxic mushroom, *Lactarius vellereus*, were added to the suspension. PD medium was used as the control. The inhibition of germination was also assayed over 5 days using diluted extracts (50%, 25%, 10%) to establish the effect of different concentrations of toxin. Extracts were also sterilised at 121°C for 30 minutes to establish whether the toxins in the extracts could be deactivated with heat.

Results

Mycelial growth inhibition

The total culture extracts of all 8 mushroom strains inhibited the radial growth of *Alternaria alternata* (Fig. 1). *Lactarius vellereus* caused the highest growth inhibition (61.44% as compared to the control), followed by *Amanita* sp. (35.67%), while the others ranged from 15.97-1.44%. The filtered extracts from all 8 mushroom strains also inhibited the radial growth of *Alternaria alternata in vitro* (Fig. 2). *Lactarius vellereus* caused the highest growth inhibition (49.56% as compared to the control), followed by *Amanita* sp. (48.69%), while the others ranged from 15.26-2.18%.

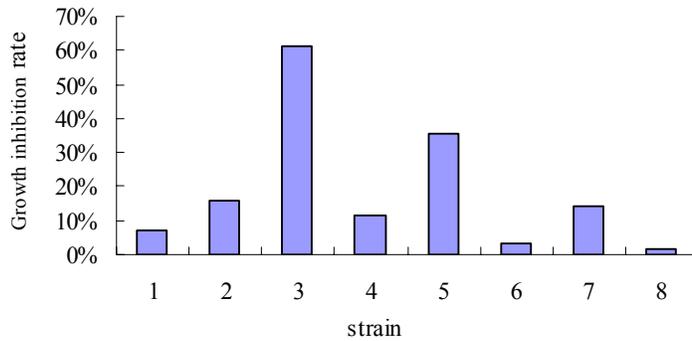


Fig. 1. Inhibition of growth of *Alternaria alternata* by extracts from toxic fungi. 1. *Amanita virosa*. 2. *Lepiota clypeolaria*. 3. *Lactarius vellereus*. 4. *Amanita paehycolea*. 5. *Amanita* sp. 6. *Ramaria ephemeroherma*. 7. *Clitocybe clealbate*. 8. *Lepiota cristata*.

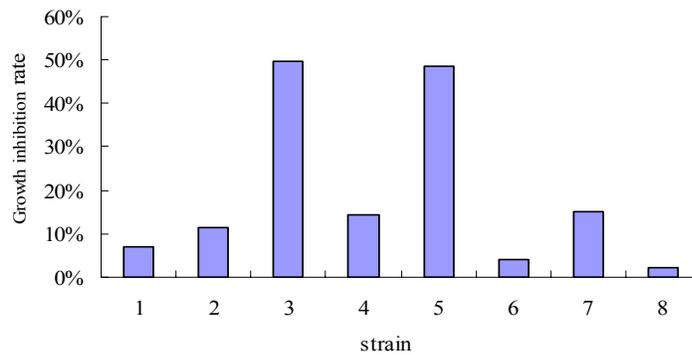


Fig. 2. Growth inhibition of radial growth of *Alternaria alternata* *in vitro* by filtered culture extracts of 8 toxic mushroom strains. 1. *Amanita virosa*. 2. *Lepiota clypeolaria*. 3. *Lactarius vellereus*. 4. *Amanita paehycolea*. 5. *Amanita* sp. 6. *Ramaria ephemeroherma*. 7. *Clitocybe clealbate*. 8. *Lepiota cristata*.

With the exception of *Ramaria ephemeroherma* and *Lepiota cristata* the ultra-sonified mycelial extracts from the toxic mushroom strains inhibited the radial growth of *Alternaria alternata* *in vitro* (Fig. 3). *Lactarius vellereus* caused the highest growth inhibition (39.97% as compared to the control), while the others ranged from 22.53-6.54%.

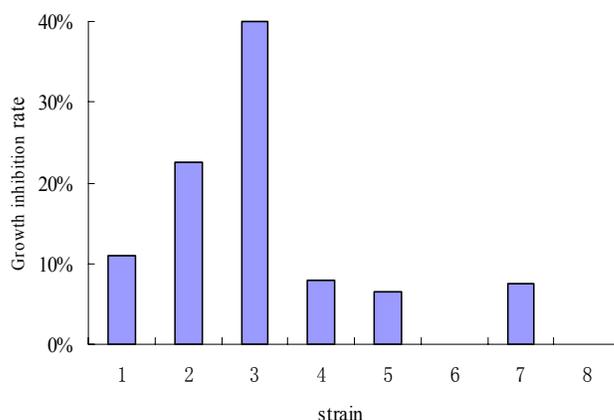


Fig. 3. Growth inhibition of radial growth of *Alternaria alternata* *in vitro* by mycelial ultrasonic extracts of 8 toxic mushroom strains. 1. *Amanita virosa*. 2. *Lepiota clypeolaria*. 3. *Lactarius vellereus*. 4. *Amanita paehycolea*. 5. *Amanita* sp. 6. *Ramaria ephemeroherma*. 7. *Clitocybe clealbate*. 8. *Lepiota cristata*.

Inhibition of growth of *Alternaria alternata* *in vitro* by heat sterilized filtered culture extracts was 48.01% which is slightly lower than that of non-sterilized extracts (49.56%); that of heat sterilized ultra-sonified mycelial extracts is 23.45% which is much lower than that of non-sterilized extracts (39.97%). The result shows that the sterilization technique had little effect on filtered culture extracts but a greater effect on ultra-sonified mycelial extracts.

Inhibition of spore germination

Germination rates of pathogen conidia differed depending on the control medium; after 24 hours this was highest in PD with 78.12% of conidia germinating, followed by 2% glucose (71.40%) and sterilized water (65.8%). Germination was therefore better in suspensions containing nutrients. The addition of extracts from the toxic mushrooms resulted in inhibition of germination (Fig. 4). Inhibition of germination by the extracts were *Lactarius vellereus* (91.45% of conidia not germinating) > *Lepiota cristata* (36.32%) > *Lepiota clypeolaria* (21.27%) > *Amanita virosa* (12.57%) > *Amanita paehycolea* (7.71%) > *Clitocybe clealbate* (1.05%) > *Amanita* sp. and *Ramaria ephemeroherma* (0%), when compared to germination rates in PD medium. When 2% glucose fluid was used as the control germination inhibition was *Lactarius vellereus* (90.64%) > *Lepiota cristata* (30.32%) > *Lepiota clypeolaria* (13.87%) > *Amanita virosa* (4.34%) > *Amanita paehycolea*, *Clitocybe clealbate*, *Amanita* sp. and *Ramaria ephemeroherma* (0%). When water was used as the

control germination inhibition was *Lactarius vellereus* (89.85%) > *Lepiota cristata* (24.39%) > *Lepiota clypeolaria* (6.53%) > *Amanita virosa*, *Amanita paehycolea*, *Clitocybe clealbata*, *Amanita* sp. and *Ramaria ephemeroderma* (0%). *Lactarius vellereus* had the greatest inhibitory effect on germination of the pathogen conidia.

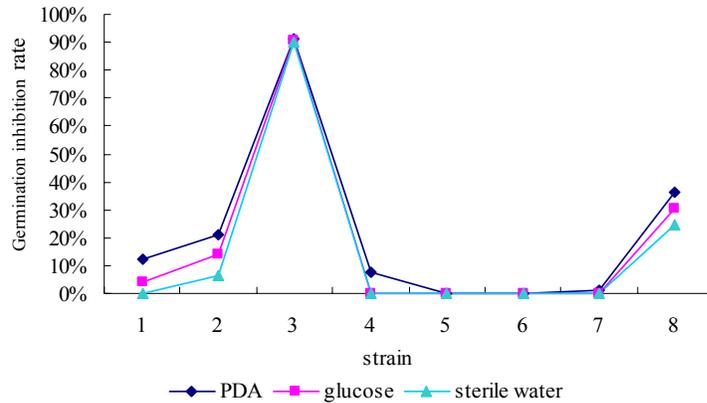


Fig. 4. Inhibition of *Alternaria alternata* conidial germination by culture extracts of toxic mushrooms. 1. *Amanita virosa*. 2. *Lepiota clypeolaria*. 3. *Lactarius vellereus*. 4. *Amanita paehycolea*. 5. *Amanita* sp. 6. *Ramaria ephemeroderma*. 7. *Clitocybe clealbata*. 8. *Lepiota cristata*. (24 hours).

The inhibition of *Alternaria alternata* conidial germination by culture filtered extracts from *Lactarius vellereus* was 3.25%, while ultra-sonified mycelial extracts did not inhibit germination. Inhibition of germination decreased with dilution of *Lactarius vellereus* culture extracts (Fig. 5). At 24 hours, the germination inhibition rate using culture extracts was 91.59% as compared to 50% dilution (83.63%), 25% dilution (40.21%) and 10% dilution (23.34%). All spores had germinated after 37 hours when culture extracts were diluted by 10%; 84 hours when culture extracts were diluted by 25% and 122 hours when culture extracts were diluted by 50%. In 100% culture extract less spores had germinated after 5 days.

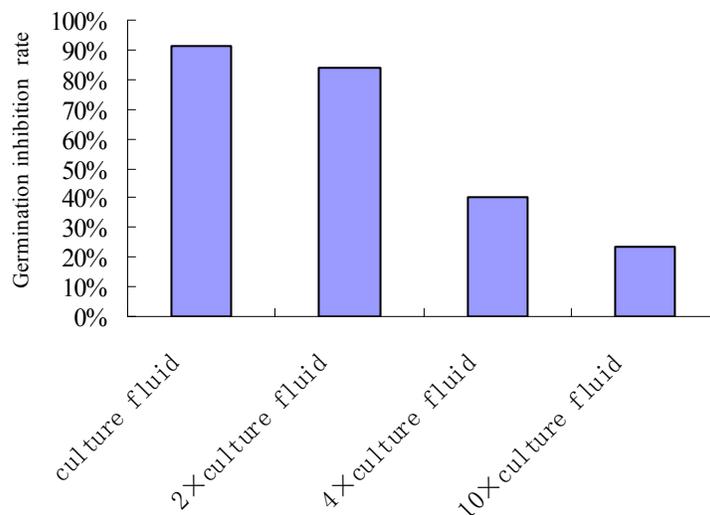


Fig. 5. Inhibition of *Alternaria alternata* conidial germination at different concentration of culture extracts from *Lactarius vellereus* (24 hours)

Discussion

The eight toxic mushroom strains all inhibited the radial growth of *Alternaria alternata* *in vitro*. Extracts from *Lactarius vellereus* produce the greatest inhibitory effects either on growth or germination. *Lactarius vellereus* may therefore have the capacity to control poplar leaf blight. The growth inhibition rate of culture extracts was 61.44%, that of filtered culture extracts 49.56% and mycelial ultrasonic extracts 39.97%. This shows that the growth-inhibiting substances from *Lactarius vellereus* is not only in the mycelium, but are also secreted into the culture extracts. There are no previous studies on the use of mushroom toxins in biocontrol. In the only other study we are aware of, the toxins of poisonous mushrooms, *Lactarius vellereus* promoted the growth of *Cytospora chrysosperma* (Pers) Fr., while *Amanita virosa* Lamb. ex Secr., *A. muscaria* (L. ex Fr.) Pers. ex Hook and *Lepiota clypeolaria* (Bull. ex Fr.) Quil. inhibited the growth (Song and Ji, 2005).

Inhibition of germination of conidia of *Alternaria alternata* by *Lactarius vellereus* culture extracts was 91.45%, while that of filtered culture extracts was 3.25%, and mycelial ultrasonic extracts had no effect. This shows that inhibition of conidial germination is caused by extracellular substances secreted by the mycelium into the culture media. There are numerous publications on the toxins of *Lactarius vellereus* (Mlinaric *et al.*, 2004),

however, this is the first report on inhibition of plant pathogen growth and germination by such toxins.

Sterilization of the culture extracts had little effect on the mushroom toxins and thus are not proteins.

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