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# SCREENING AND IDENTIFICATION OF ANTAGONISTIC FUNGI AGAINST PEPPER ANTHRACNOSE AND INVESTIGATION INTO ITS POT-PLANT CONTROL EFFECTS

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**Abstract**

In order to enrich the high-quality biocontrol fungi resources for pepper anthracnose, this experiment obtained 282 fungal strains isolated and purified from soil by plate coating method and screened 7 antagonistic fungi with obvious inhibitory strips by using the plate standoff method and re-screened the inhibitory effect of the fungal fermentation broth by using the method of fungal plate containing poisonous medium. The results showed that the fungal strain A192 had a better inhibitory effect, and the inhibition rate of the rescreened fermentation broth was 61.29%. Morphological identification and molecular biological identification of the fungal strain identified as *Penicillium restrictum*. The fermentation broth of antagonistic fungal strain A192 has a certain degree of inhibitory effect on 13 pathogenic fungi. It has inhibition rates of 80.18%, 79.11%, 77.35%, and 71.67% against *Sclerotinia sclerotiorum*, *Fusarium oxysporum*, *Alternaria lternata*, *Phoma arachidicola*, respectively. A pot plant control effect test was conducted using the fermentation solution of fungal strain A192, and the control effect was 85.13%.

**Key words:** Pepper anthracnose; Biocontrol fungi; *Penicillium restrictum*; Response surface methodology.

## Introduction

Chili pepper, one of the earliest vegetable crops cultivated by human beings (Zou *et al*., 2020; Pickersgill *et al*., 1969), is now grown in more than 140 countries worldwide (Zou *et al*., 2022). Its popularity stems from being a rich source of vitamin C (Meng *et al*., 2018), capsaicin (Wang *et al*., 2023), carotene (Liu *et al*., 2021), etc. along with its distinctive flavor, making it valuable both as a medicinal aid and as a food item (Singh *et al*., 2016).

Pepper anthracnose is one of the three most severe diseases of pepper caused by the fungus *Colletotrichum* (Chen *et al*., 2023). It primarily affects nearly ripe fruits and leaves, affecting the quality of fruits and seeds, and aggravating the damage by serving as a source of primary infection for the subsequent year's disease. Under conditions favorable for disease development, up to 50% of the fruit may suffer damage. As both a pre-harvest and post-harvest disease, residual pathogens on post-harvest fruit not only can infect immature fruit, but also residual pathogens on post-harvest fruit can continue to infest warehoused peppers, resulting in severe cases of chili crop extinction (Yang *et al*., 2023). The pathogens that cause anthracnose in chili peppers are more diverse, and in general, they are compound infestations, making control difficult (Wang *et al*., 2022).

Currently, chemical control remains the predominant method for preventing and controling pepper anthracnose in production (Wang *et al*., 2023), in which mycophenyl esters are more effective with 22.5% picoxystrobin suspension, 250 g/L pyraclostrobin emulsion, 30% benzyl-pyraclostrobin suspension and so on, and they are commonly applied for in the prevention and control of pepper anthracnose (Long *et al*., 2021). Agricultural control and selection of disease-resistant varieties are also widely employed to combat this disease. However, since the implementation of field measures is often delayed, and different anthracnose fungi have varying infectivity to different hosts, there are discrepancies in disease resistance among pepper varieties, complicating the selection and breeding of resistant varieties (Ridzuan *et al*., 2018). Additionally, with the improvement of people's living standards and the advancement of science and technology, human thinking about the relationship between human beings and nature is also more and more in-depth, people are increasingly aware of the need to protect the environment prompting exploration into pesticide environmental safety standards for a series of exploration (Gao *et al*., 2023). Consequently, there is increasing interest in utilizing biological substances and methods to control plant diseases such as pepper anthracnose due to their environmental friendliness, health and safety benefits, marking an inevitable trend in plant control with promising prospects for development.

Currently, there is a growing body of research focusing on microorganisms used for biological control of pepper anthracnose, including bacteria, fungi and actinomycetes. The commonly used bacterial biocontrol fungi are mainly *Bacillus* spp. and *Pseudomonas* spp., while Trichoderma spp. are predominant among fungal biocontrol fungi, and Streptomycetaceae spp. are frequently utilized among actinomycetes biocontrol fungi (Yang *et al*., 2023). Sandani *et al*. (2019) screened five strains of *Burkholderia* spp. and *Pseudomonas* spp. exhibiting potent inhibitory effects on *Colletotrichum truncatum*, which were able to deform the mycelial development of the pathogen and thus inhibit the spread of the disease (Sandani *et al*., 2019); Zhao *et al*., (2023) discovered a strain of *Bacillus beleriensis* ZF438, which was 68.26% effective in the control of pepper anthracnose (Zhao *et al*., 2023). Additionally, in 2023, Yarida *et al*. found a strain of marine wood mold *Trichoderma asperellum* KUFA 0155, which can protect chili peppers against anthracnose (Yarida *et al*., 2023); In 2023, Yulmira *et al*. isolated and screened eight strains of actinomycetes with 100% inhibition of pepper anthracnose, and five of them promoted chili peppers' growth (Yanti *et al*., 2023).

In this experiment, the target pathogenic fungus is *Colletotrichum scovillei*, and a fungal strain with antagonistic effect on chili pepper anthracnose was obtained by isolation and screening from the soil of vegetable planting land. In addition, it was thoroughly characterized and its bacteriostatic effect and broad-spectrum were determined, providing new strain resources for the biological control of pepper anthracnose.

## Material and Methods

**Fungal strains and media:** *A. longipes, A. lternata, Botrytis cinerea, C. asianum, C. orbiculare, C. scovillei, Exserohilum turcicum, F. graminearum, F. oxysporum, F. proliferatum, F. verticilloides, Phomopsis. arachidicola, S. sclerotiorum,* All the above strains were provided by Plant Disease Institute of Jilin Agricultural University.

Potato dextrose agar (PDA) medium (potato 200 $g \cdot L^{-1}$, glucose 20 $g \cdot L^{-1}$, agar 15 $g \cdot L^{-1}$); Potato dextrose broth (PDB) medium (potato 200 $g \cdot L^{-1}$, glucose 20 $g \cdot L^{-1}$); Optimized fermentation medium (potato 200 $g \cdot L^{-1}$, dextrin 20 $g \cdot L^{-1}$, $(NH4)_2HPO_4$ 2 $g \cdot L^{-1}$, $ZnSO_4 \cdot 7H_2O$ 1 $g \cdot L^{-1}$).

**Isolation, purification and preservation of soil fungi:** A total of 36 soil samples were collected from the vegetable greenhouses of Jilin Agricultural University (located in Changchun, China) and vegetable fields of Huazhong Agricultural University (located in Wuhan, China) by using the five-point sampling method and placed in the refrigerator at 4℃ for spare. The collected soil samples were air-dried and sieved, and 1 g of each soil sample was diluted $10^1$ $10^2$, $10^3$, $10^4$, $10^5$ times with sterile water to make a soil suspension. Using the coating method, the soil suspension was applied to the PDA medium, and single colonies were picked for 2-3 d for purification and culture and preserved (Zhou *et al*., 2020).

**Screening of antagonistic fungi:** Screening of growth potential: On the PDA medium, take three points in a positive triangle 1cm away from the edge of the Petri dish to inoculate the fungus to be tested, and inoculate pepper anthracnose fungi in its center point, place it in 25℃ for culture and observation, and select the strains with fast growth potential of the colonies for further tests.

**Primary screening:** Dual experiments were used to observe whether there were obvious inhibition zones.

**Re-screening:** select the strains with good antagonistic effect in the initial screening, beat two 8mm cakes to access the PD medium, incubate at 25°C and 175 rpm with shaking for 10 d, take the supernatant and centrifuge it at 12000 rpm for 20 min, and then filter it through a 0.45 μm filter to get the aseptic fermentation broth of the fungus to be tested. The fermentation broth and PDA medium were mixed uniformly in the ratio of 1:5, and accessed to pepper anthracnose fungus, set up 3 groups of replicates, and added sterile water as control in the same way. Incubation at 25°C and the diameters of the treatment groups were measured using the crosshatch method to calculate the size of inhibition rate.We calculated the inhibition rate of sterile fermentation broth by using the following formula (Yasmin & Shamsi, 2019).

$$\text{Inhibition rate} = \frac{\text{Colony diameter of control group} - \text{colony diameter of treatment group}}{\text{Colony diameter of control group}} \times 100\%$$

Identification of antagonistic fungi

**Morphological identification:** The antagonistic fungal strains were inoculated on PDA medium and cultured in the dark in a constant temperature incubator at 25℃ for 7 d, and then the colonies were observed for their color, morphological characteristics and the presence or absence of pigment exudation. When the mycelium was mature, the micro-morphological characteristics were observed.

**Molecular biological identification:** Fungal DNA was extracted by CTAB using the fungal universal primer ITS1 (5'-TCC GTA GGT GAA CCT GCG G-3')/ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3'), β-tubulin gene fragment amplification primers bt2a (5'-GGT AAC CAA ATC GGT GCT GCT GCT TTC-3')/bt2b(5'-ACC CTC AGT GTA GTG ACC CTT GGC-3') was subjected to PCR amplification under the following conditions: pre-denaturation at 94°C for 5 min, denaturation for 45 s, annealing at 55°C for 30 s, extension at 72°C for 45 s. A total of 35 cycles were performed, and extension was performed for 5 min at 72°C (Visagie *et al*., 2014). The PCR amplified products were entrusted to Shanghai Bioengineering Company Limited for sequencing, and the sequences obtained from sequencing were sequenced by BLAST sequence homology analysis on NCBI, and the sequences of strains with the highest degree of similarity were selected for comparison using Clustal X. A two-gene phylogenetic evolutionary tree was constructed using the Neighbor-Joining Method with MEGA 7.0 software.

**Determination of antifungal spectrum:** Put antagonistic fungus into the optimized fermentation medium and prepare sterile fermentation broth. The PDA mixed with the sterile fermentation broth was used as the treatment group, and the normal PDA medium was used as the control group. Set up three replicates, incubate at 25°C, and then calculate the inhibition rate.

**Pot experiment:** Six groups of treatments were set up according to the water: fermentation solution as 1:0, 1:50, 1:40, 1:30, 1:20, 1:10, and good-growing and uniform-sized chili pepper plants were selected, and at the same time, 10 ml of roots were irrigated and 10 ml of sprays were applied to them, and the onset of disease of the leaves was observed, the area of the lesions was measured and the inhibition rate was calculated after 10 d. The results were summarized as follows.

$$\text{Inhibition rate} = \frac{\text{Leaf spot area of control group} - \text{Leaf spot area of treatment group}}{\text{Leaf spot area of control group}} \times 100\%$$

## Results

**Screening of antagonistic fungi:** We isolated and purified 282 fungal strains from the soil. After preliminary screening, we found that strains C12, C13, C36, H86, A121, A192 and H264 had obvious inhibition zones. In the re-screening, strains C13, C36, A192 and H264 had inhibition rates of more than 60% (Fig. 1; Table 1).

**Table 1. Anti-efficacy of 7 soil fungal strains against *C. scovillei.***

| Antagonistic fungi | Inhibition zone (mm) | Inhibition rate of sterile fermented broth (%) |
|---|---|---|
| C12 | 13.11 ± 0.2253$^{a}$ | 53.78 ± 0.3148$^{e}$ |
| C13 | 2.74 ± 0.1769$^{f}$ | 64.46 ± 0.2207$^{c}$ |
| C36 | 6.04 ± 0.0721$^{b}$ | 78.20 ± 0.2816$^{a}$ |
| H86 | 2.27 ± 0.3064$^{g}$ | 26.34 ± 0.7351$^{g}$ |
| A121 | 5.39 ± 0.1900$^{c}$ | 44.25 ± 0.6413$^{f}$ |
| A192 | 3.16 ± 0.0265$^{e}$ | 61.29 ± 0.1493$^{d}$ |
| H264 | 4.62 ± 0.2629$^{d}$ | 74.22 ± 0.5567$^{b}$ |

Note: Different lowercase letters indicate significant differences of different antagonistic fungi at $p$=0.05 level

By identifying, we excluded the strains that are pathogenic and have been reported to have biocontrol effects, and selected strain A192 for the follow-up study. Microscopic observation of the anthracnose pathogen of chili showed that the normal anthracnose hypha had complete morphology and were uniform in thickness. However, the anthracnose hypha was found to be abnormally branched and deformed after treatment with the bacterial solution, and some of them were slightly enlarged, and their spores were obviously reduced, and some of them appeared to be ruptured. This indicates that there are bacteriostatic substances in the fermentation broth of strain A192, which destroys the mycelium and spores of *C. scovillei*, thus inhibiting its growth.

**Identification of antagonistic fungi:** Strain A192 had a velvety texture on the front side of the colony on PDA, with a flatter surface, a thicker central area, fewer conidial structures, a bluish-gray conidial surface, and a white mycelium with a small amount of pigmentation exuding from it; the reverse side of the colony was yellowish, with ruffles at the edges. The colony morphology is illustrated in (Fig. 3, a & b).

Conidiophores occurring in aerial mycelium, conidiophore 10-50 × 1.6-2.8 μm, smooth, broomlike branches conspicuously borne in a single whorl, occasional pedunculated basal branches; vase peduncles 5-9 in each whorl, 5.0-7.0 × 1.8-2.5 μm, short, vase-shaped, peduncles with short necks. Conidia appearing spherical or subglobose, or subovoid, 2.0-2.5 μm, wall conspicuously spiny and rough; conidial chains short. Lax and irregular. Microscopic morphology is shown in (Fig. 3, c and d). It is more consistent with the description of *Penicillium* confined in the genus *Penicillium* and its related sexual-type genera in the Chinese Fungi (Kong, 2007).

**Molecular biological identification:** The obtained sequences were subjected to Blast comparison on NCBI, and it was found that both the ITS amplified sequence and the BenA gene primer amplified sequence of strain A192 were 100% homologous to *Penicillium restrictum*. Using MEGA 7.0 to construct the phylogenetic tree, *Rhizopus stolonifer* was selected as the outgroup, and the antagonist strain A192 was obviously classified in the same clade with *Penicillium restrictum*, which assisted in proving that strain A192 was *Penicillium restrictum* from the molecular point of view. The phylogenetic tree construction is shown in (Fig. 4).

**Determination of antifungal spectrum:** The inhibitory effect of the fermentation broth of the antagonist strain A192 on 13 pathogens showed that: fermented in the optimized liquid medium, the fermentation broth of strain A192 inhibited the anthracnose fungi of pepper by 85.56%, and inhibited the rest of the 12 pathogens to a certain extent, and the inhibitory effect on the four pathogens, namely, *S. sclerotiorum, F. oxysporum, A. lternata, P. arachidicola* was relatively high. The inhibition rates were 80.18%, 79.11%, 77.35% and 71.67% respectively. The inhibition rate of the other nine pathogens ranged from 58.23-68.45%. It can be seen that strain A192 has a wide spectrum of fungal inhibition and has a certain inhibitory effect on a variety of pathogenic fungi. The magnitude of fungal inhibition rate is shown in (Table 2).

**Control effect of pot experiment:** The control group showed dark brown whorled spots on the leaves of chili peppers with a large spot area. The leaf spots in the treatment group were smaller in size and slightly sunken. The control effect could reach more than 80% when the fresh water: fermentation solution was 1:30 (Table 3), indicating that the fermentation solution of strain A192 had a better effect on the control of anthracnose of pepper.

The infestation of pepper plants by anthracnose fungi after treatment with different percentages of A192 fungi fermentation solution is shown in (Fig. 6).

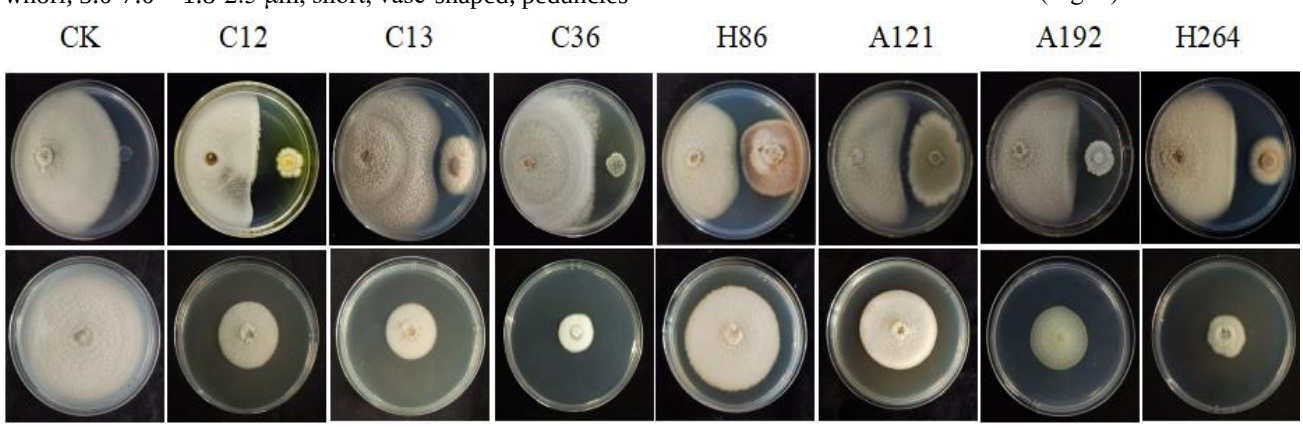


Fig. 1. Antifungal effects of primary screening and secondary screening of 7 soil fungal strains.

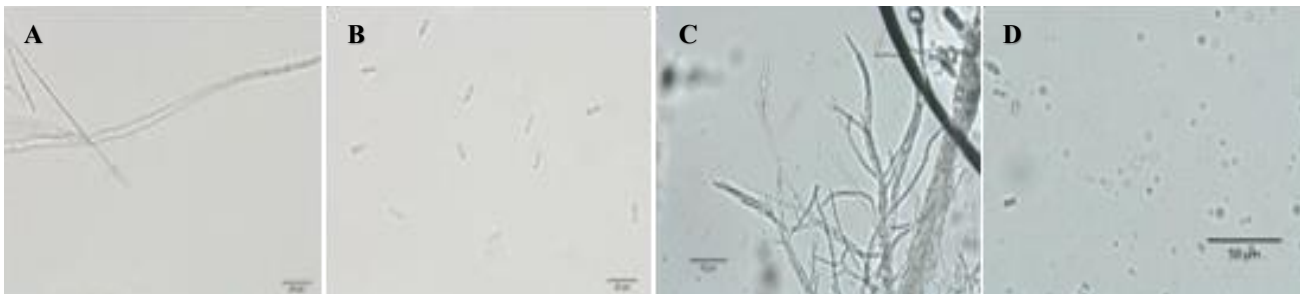


Fig. 2. Inhibitory effect of fermentation broth of strainA192 on *C. scovillei* under microscope.
a: Normal hyphae; b: Normal conidia; c: Treatment group hyphae; d: Treatment group conidia

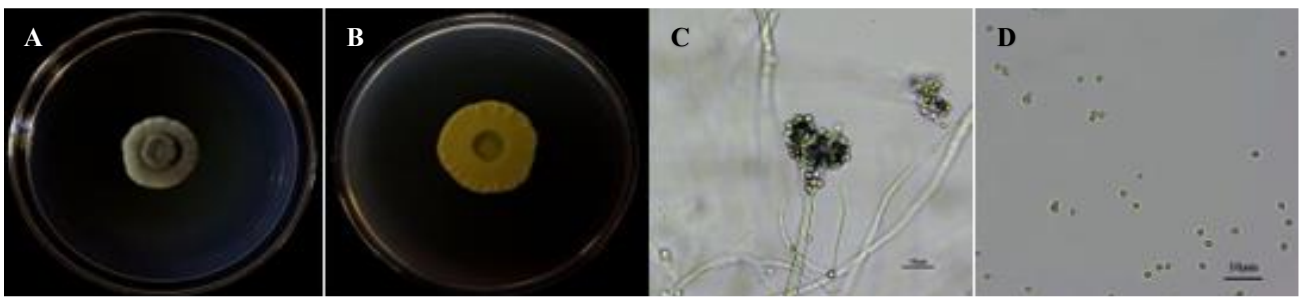


Fig. 3. Colony morphology and microscopic morphology of strain A192.
a: Positive picture of strain b: Reverse picture of strain; c: Conidiophore and conidia d: Conidia

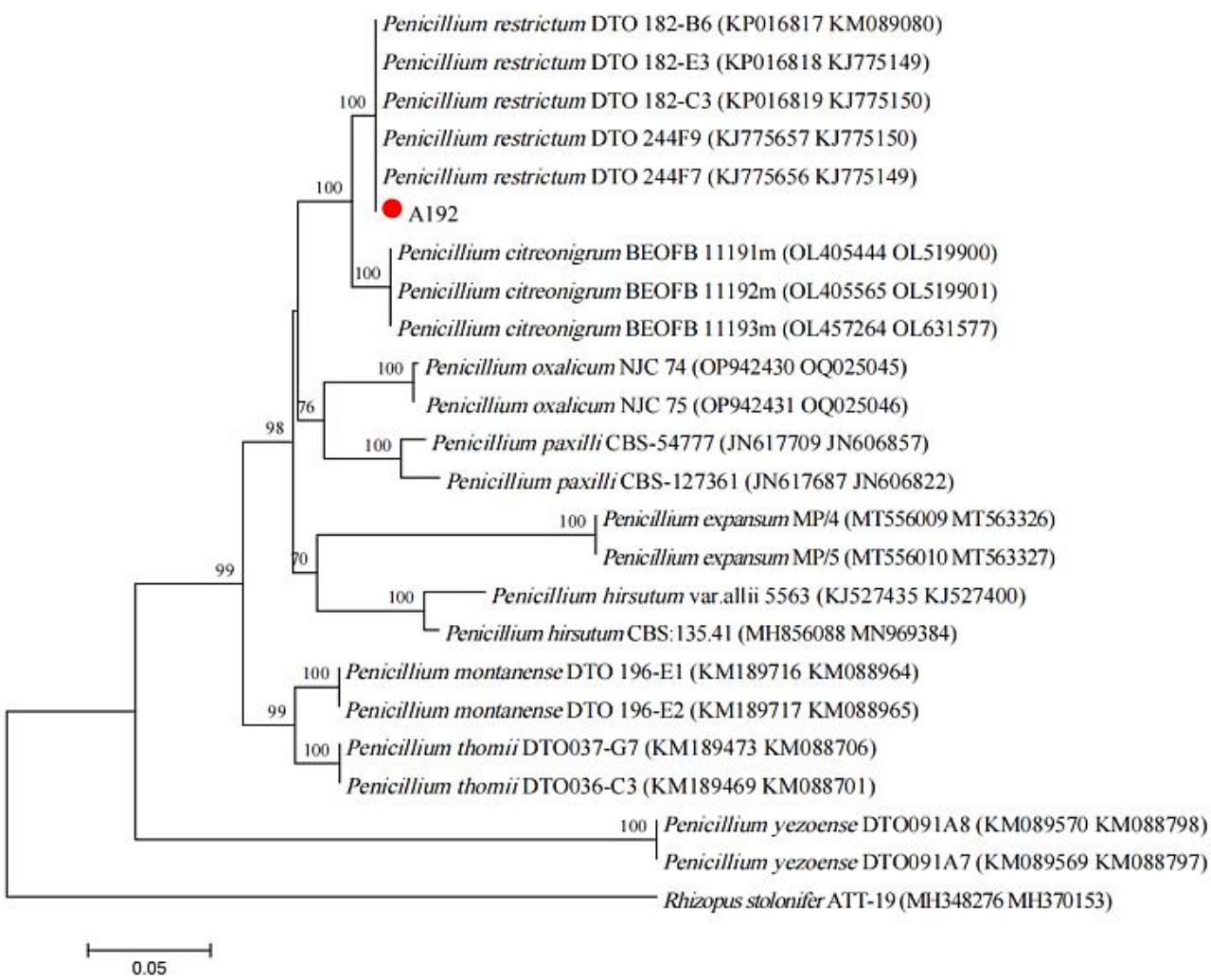


Fig. 4. Phylogenetic tree.

**Table 2. Inhibition of fermentation broth of strain A192 against 13 kinds of common pathogens.**

| Pathogens | Average colony diameter (mm) | | Inhibition rate (%) |
|---|---|---|---|
| | test | CK | |
| *Colletotrichum scovillei* | 18.61 ± 0.0586$^{a}$ | 81.56 ± 0.1752$^{b}$ | 85.56 ± 0.0794$^{a}$ |
| *Sclerotinia sclerotiorum* | 23.13 ± 0.8723$^{ab}$ | 84.59 ± 0.3646$^{a}$ | 80.18 ± 1.1399$^{b}$ |
| *Colletotrichum orbiculare* | 30.64 ± 1.0004$^{abcd}$ | 70.31 ± 0.6806$^{c}$ | 63.67 ± 1.6058$^{fg}$ |
| *Exserohilum turcicum* | 32.16 ± 0.6500$^{bcd}$ | 84.58 ± 0.3124$^{a}$ | 68.45 ± 0.8492$^{e}$ |
| *Fusarium graminearum* | 37.48 ± 1.2450$^{cd}$ | 84.58 ± 0.4937$^{a}$ | 61.50 ± 1.6269$^{g}$ |
| *Fusarium oxysporum* | 23.97 ± 1.4833$^{ab}$ | 84.44 ± 0.1950$^{a}$ | 79.11 ± 1.9394$^{bc}$ |
| *Colletotrichum asianum* | 39.28 ± 0.4885$^{d}$ | 84.93 ± 0.0351$^{a}$ | 59.34 ± 0.6358$^{h}$ |
| *Fusarium annulatum* | 40.00 ± 0.7912$^{d}$ | 84.61 ± 0.2838$^{a}$ | 58.23 ± 1.0039$^{h}$ |
| *Fusarium proliferatum* | 36.61 ± 1.0306$^{bcd}$ | 84.67 ± 0.3879$^{a}$ | 62.68 ± 1.3427$^{fg}$ |
| *Alternaria lternata* | 25.32 ± 0.6730$^{abc}$ | 84.50 ± 0.3208$^{a}$ | 77.35 ± 0.8790$^{c}$ |
| *Alternaria longipes* | 35.02 ± 0.7753$^{bcd}$ | 84.46 ± 0.3262$^{a}$ | 64.67 ± 1.0103$^{f}$ |
| *Botrytis cinerea* | 33.40 ± 1.3661$^{bcd}$ | 84.63 ± 0.2920$^{a}$ | 66.86 ± 1.7814$^{e}$ |
| *Phoma arachidicola* | 25.75 ± 0.8806$^{abc}$ | 70.67 ± 0.6213$^{c}$ | 71.67 ± 1.4062$^{d}$ |

Note: Different lower-case letters indicated significant differences of different antagonistic fungi at $p$=0.05 level

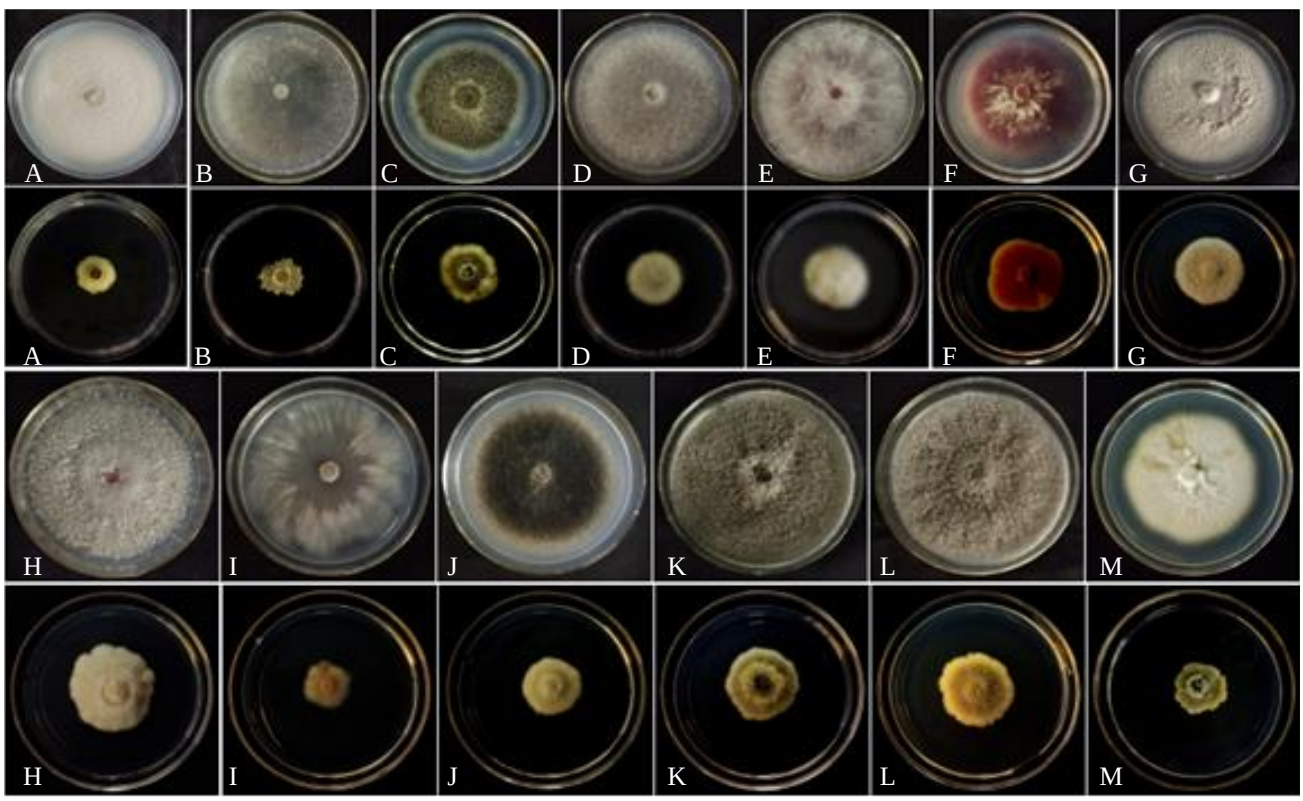


Fig. 5. Inhibitory effects of fermentation broth of strain A192 against 13 pathogens.

**Note:** The first row and the third row are the control groups, the second row and the fourth row are the treatment groups. a: *C. scovillei*; b: *S. sclerotiorum*; c: *C. orbiculare*; d: *E. turcicum*; e: *F. graminearum*; f: *F.oxysporum*; g: *C. asianum*; h: *F. annulatum*; i: *F. proliferatum*; j: *A. lternata*; k: *A. longipes*; l: *B. cinerea*; m: *P. arachidicola*

## Discussions

*Colletotrichum scoville* is highly pathogenic to chili peppers and has a wide host range, which can infect a wide range of plants, making its disease control challenging. Currently, the main effective chemical agents for controling of *C. scoville* include 75% oxime-pentaconazole WDG, 10% phenyl ether metronidazole WDG, and 250 g/L piconazole ethermectate SC (Zhou *et al*., 2023). It is essential to note that the amount of chemical agents used will inevitably increase with the aggravation of the disease leading to the accumulation of pesticide residues in the long run which can easily cause adverse effects on both the environment and human health. In the long run, the accumulation of pesticide residues may cause adverse effects on the environment and human health. What is more, the pathogens may easily develop resistance, which will lead to the aggravation of the disease in a vicious circle.

Nature is a treasure trove of resources and the use of microorganisms to control plant diseases is a hygienic and safe means of green control without burdening the natural environment. The microorganisms that have been studied and found to have good antagonistic effects against *C. scovillei* are predominantly bacteria. In 2023, Mohamed *et*

*al*. screened a strain of bacterium P39 based on taxonomy that was able to phagocytose and digest the mycelium of *C. scovillei* converting it into bacterial biomass (Mohamed *et al*., 2023); Additionally, Yuliar found a strain of *Bacillus subtilis* ATCC 21556 that was capable of controlling postharvest anthracnose in peppers (Yuliar, 2020).

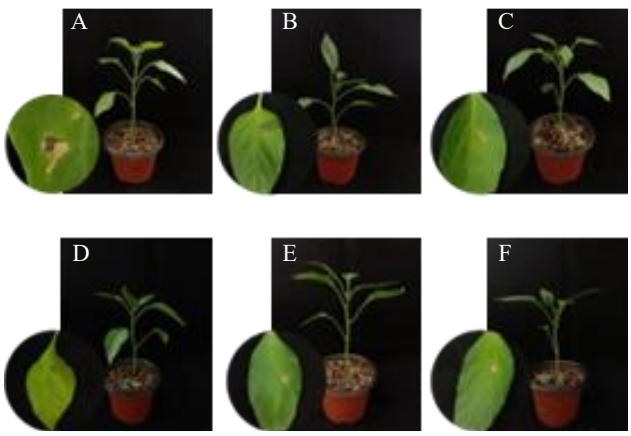


Fig. 6. Potted control effect of fermentation broth of strain A192 against *C. scovillei.*

**Note:** a: Water; b: Water: Fermentation broth=1:50; c: Water: Fermentation broth = 1:40; d: Water: Fermentationbroth = 1:30; e: Water: Fermentation broth = 1:20; f: Water: Fermentation broth = 1:10

**Table 3. Potted control effect of fermentation broth of strain A192 against *C. scovillei*.**

| Code | Fermentation broth: Water | Leaf spot area ($cm^2$) | Inhibition rate (%) |
|---|---|---|---|
| A | 0:1 | $1.0836 \pm 0.0364^{a}$ | — |
| B | 1:50 | $0.5183 \pm 0.0160^{b}$ | $52.17 \pm 1.4750^{e}$ |
| C | 1:40 | $0.2518 \pm 0.0121^{c}$ | $76.76 \pm 1.1155^{d}$ |
| D | 1:30 | $0.1845 \pm 0.0102^{d}$ | $82.97 \pm 0.9456^{c}$ |
| E | 1:20 | $0.1251 \pm 0.0125^{e}$ | $88.46 \pm 1.1500^{b}$ |
| F | 1:10 | $0.0724 \pm 0.0029^{f}$ | $93.32 \pm 0.2686^{a}$ |

*Penicillium*, as a widespread fungus in nature, exhibits strong ability to adapt to diverse environments and holds significant potential for biocontrol purposes. Currently known *Penicilliums* spp., used for biocontrol mainly include *Penicillium purpureum*, *P. oxalicum*, etc. (Zhao *et al*., 2019; Zhang *et al*., 2016). During the screening phase of this study, in addition to A192, C36 and H264, which are also *Penicillium* species, displayed promising antagonistic effects against *C. scoville*, which need to be further verified in the subsequent experiments.

In this study, 282 strains of fungi were isolated and purified from 36 soil samples, A strain of antagonistic fungus A192, with superior inhibitory effects on the anthracnose pathogen of chili pepper, was screened by using the plate standoff method and the fungal plate method containing toxic medium, using chili pepper anthracnose pathogen as the indicator fungus. Through morphological observation, molecular biological identification, and construction of a phylogenetic tree, the strain A192 was identified as Penicillium restrictum. The antagonistic inhibition zone of A192 against anthracnose of chili pepper measured 3.16 mm, and the inhibition rate of the fermentation broth against early blight was 61.29%. In addition to the good antimicrobial activity against *C. scovillei*, A192 demonstrated inhibitory effects against common pathogenic fungi such as *S. sclerotiorum*, *F. oxysporum*, *A. lternata*, *P. arachidicola* and other common pathogens, with a wider range of bacterial inhibition and promising application prospects.

On the basis of this study, the composition of the fermentation medium of the strain, such as carbon source, nitrogen source, inorganic salt, as well as the fermentation conditions of the strain, such as temperature, pH, rotational speed, and number of days, will be optimized. Furthermore, a comprehensive investigation into its secondary metabolites will be conducted, so as to explore its biocontrol mechanism. At the same time, field trials will be conducted to further verify the biocontrol effect of strain A192 on anthracnose of chili peppers, providing theoretical reference for the practical application in agricultural production.

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