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SCREENING AND IDENTIFICATION OF ANTAGONISTIC FUNGI AGAINST TOMATO EARLY BLIGHT AND RESEARCH ON ITS POT-PLANT CONTROL EFFECTS

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Abstract

According to some studies, *Alternaria alternata* has gradually risen to become the dominant pathogen in tomato early blight, which is one of the most widespread and serious diseases caused by *Alternaria* spp. In order to enrich the resources of high-quality biocontrol strains, 8 antagonistic fungus strains with evident inhibition zones were first screened from the soil by dual culture assay, and then the effect of their sterile fermentation broth against *A. alternata* was re-screened by mycelium growth rate method. The results showed that strain L131 functioned well, with a primary screening inhibition zone of 5.54 mm and a re-screening antifungal rate of 66.41%. L131 was identified as *Penicillium citrinum* after morphological characterisitics and molecular identification. In addition, we discovered that the sterile fermentation broth of L131 could inhibit other tested pathogenic fungi. Among them, the inhibition rates of *Sclerotinia sclerotiorum, Rhizoctonia solani, Exserohilum turcicum* and *Fusarium graminearum* were 100%, 96.42%, 88.01% and 83.66%, respectively. Finally, we used the fermentation broth of strain L131 to conduct a pot control effect test, and the control effect was 85.13%.

Key words: *Alternaria alternata*; *Penicillium citrinum*; Antagonistic Fungi.

Introduction

Tomato is the second most consumed vegetable after the potato, and it is widely farmed all over the world (Sallam *et al*., 2021). It is rich in vitamins A, C and lycopene, as well as anti-cancer and heart-protective characteristics, making it popular (Breemen *et al*., 2008; Zhao *et al*., 2017).

Tomato early blight is one of the most widespread and serious plant diseases caused by *Alternaria* spp. (Dong *et al*., 2015). Some studies have shown that *Alternaria alternata* has gradually risen to become the dominant pathogen (Thomma, 2003; Akhtar *et al*., 2004). The disease can occur throughout the entire growth and development stages of tomato plant. It not only inhibits plant growth, but it also directly harms their stems, leaves and fruits. Pathogens can repeatedly infect plants for many times with conidium, and can expand the infection range with external media. If the prevention and control measures are not implemented in a timely manner, the tomato yield can be reduced by 35% to 78% (Vloutoglou & Kalogerakis, 2000; Wang *et al*., 2016), as well as quality, resulting in incalculable losses to the agricultural economy (Liu *et al*., 2013; Li & Song, 2017). The diseased plant rate in general plots is around 30%, while in severe plots it can reach 100% (Chai *et al*., 2014). *Alternaria* species can produce more than 70 secondary toxic metabolites, the most common of which are Alternariol methyl ether (AME), Alternariol (AOH) and Tenuazonic acid (Te A), which can cause health problems in human and animals (Yelko *et al*., 2016).

In production, 10% difenoconazole WG, 80% mancozeb WP and 50% iprodione WP are usually used for chemical control of tomato early blight. Or by adopting disease-resistant varieties, taking reasonable dense planting, timely crop rotation and other measures for agricultural control (Qu *et al*., 2010). However, with the decline in resistance and the ecological problems caused by chemical fungicides, the use of environmentally friendly, hygienic and safe microbial control methods has become an inevitable and promising trend in the future to prevent and control tomato early blight (Yu *et al*., 2005).

Currently, a wide variety of antagonistic microorganisms are used for biological control of plant diseases, including fungi, bacteria and actinomycetes, etc. Antagonistic yeasts have been reported such as *Candida famata*, *Pichia guilliermondii* and *Rhodotorula glutinis* (Yu *et al*., 2010). Antagonistic bacteria include *Bacillus* spp., *Serratia* spp., *Pseudomonas* spp., etc. Besides, actinomycetes are also important biocontrol resources. More than half of the antagonistic actinomycetes are derived from *Streptomycetaceae* spp., like *Streptomycetaceae griseorubens* and *Streptomyces lunalinharesii* (Zhang & Guo, 2019; Zhang *et al*., 2022). Some scholars have also screened antagonistic microorganisms against tomato early blight. Zhou and Peng (2003) have screened out 11 yeasts that have antagonistic effect on tomato early blight (Zhou & Peng, 2003); Ren *et al.* isolated the endophyte yc8 from rape, which could effectively inhibit the spore germination and mycelial growth (Ren *et al*., 2008). Verma *et al*., found that treating tomato seeds with spore suspension of three endophytic actinomycetes strains can antagonize the growth of the pathogen and promote plant growth (Verma *et al.,* 2011).

In this experiment, tomato early blight was used as the target disease. We isolated and screened a fungal strain against *A. alternata* from the vegetable planting soil. In addition, we also identified this strain, optimized the fermentation medium, and determined its antifungal effect and control spectrum. This will provide new strain resources for the biological control of tomato early blight.

Material and Methods

Fungal strains and media: *A. alternata, Botrytis cinerea, Colletotrichum capsici, C. gloeosporioides, Exserohilum turcicum, Fusarium graminearum, F. oxysporum, F. proliferatum, Penicillium citrinum*, *Phoma arachidicola, Phomopsis vexans, Phytophthora capsici, Rhizoctonia solani* and *Sclerotinia sclerotiorum.* All the above strains were provided by Plant Disease Institute of Jilin Agricultural University.

Potato dextrose agar (PDA) medium (potato 200 g·L^{-1}, glucose 20 g·L^{-1}, agar 15 g·L^{-1}); Potato dextrose broth (PDB) medium (potato 200 g·L^{-1}, glucose 20 g·L^{-1}); Optimized fermentation medium (potato 200 g·L^{-1}, glucose 20 g·L^{-1}, beef extract 2 g·L^{-1}, NaCl 1 g·L^{-1}).

Isolation, purification and preservation of soil fungi: A total of 35 soil samples were collected from the vegetable greenhouses of Jilin Agricultural University (located in Changchun, China) and vegetable gardens of Hunan Agricultural University (located in Changsha, China) by five-point sampling method. The samples were air-dried, sieved, and serially diluted from 10 to 10^5 times with sterile water to make soil suspensions. The soil suspension was coated on sterile PDA medium, and single colonies were picked for purification and preservation after 2-3 days (Zhou *et al*., 2020).

Screening of antagonistic fungi: Growth potential screening: On the PDA medium, three points were taken 1 cm away from the edge of the petri dish in an equilateral triangle. The soil fungal strains were inoculated at these three points, and *A. alternata* were inoculated at the central point. After culturing at 25ºC, strains with fast growth potential were selected for further experiments.

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

Re-screening: Take two 8mm discs of the strain with good antagonistic effect in the primary screening and put them into PDB medium, and then shaking culture them with at 25ºC and 175 rpm for 7 days. In the next step, the fermentation liquid was centrifuged at 6000 rpm for 15 min, and supernatant was filtered with a 0.45 μm filter to obtain a sterile fermentation broth. We calculated the inhibition rate of sterile fermentation broth by using the following formula (Yasmin & Shamsi, 2019).

$$I = \frac{C - T}{C} \times 100$$

Where, I = Per cent growth inhibition, C = Growth in control, T = Growth in treatment.

Identification of antagonistic fungi

Morphological identification: The antagonistic strain was grown on PDA medium. After culturing in the dark at 25ºC for 48 h, morphological characteristics such as colony diameter, colour of mycelia and presence of exudate, were then checked. When the mycelium matured, its microscopic features such as shape and size of conidiophore and conidium were observed.

Molecular biological identification: DNA was extracted by CTAB method, and PCR amplification was carried out using fungal universal primers ITS1 (5'-TCC GTA GGT GAA CCT GCG G-3') / ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3') and β-tubulin gene fragment amplification primers bt2a (5'-GGT AAC CAA ATC GGT GCT GCT TTC-3') / bt2b (5'-ACC CTC AGT GTA GTG ACC CTT GGC-3'). Thermal cycle programs used for amplification: Initial denaturing-94ºC 5 min; Denaturing-94ºC 45 s; Annealing-55ºC 30 s; Elongation-72ºC 45 s; Final elongation-72ºC 5 min; Cycles-35 (Visagie *et al*., 2014). The PCR amplification products were entrusted to Shanghai Sangon Biotech Co., Ltd. for sequencing. The resulting sequences were subjected to analyze the sequence homology using BLAST (Basic local alignment search tool) in NCBI GenBank, the sequences with the highest similarity were selected. The two-gene phylogenetic tree was constructed using MEGA 7.0 software based on neighbor-joining method.

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 25ºC, and then calculate the inhibition rate.

Pot experiment: Set 6 treatments according to water: fermentation broth at 1:0, 1:50, 1:40, 1:30, 1:20, 1:10. Select tomato plants with good growth and the same size, irrigate the root with 10 mL and spray the leaf with 10 mL at the same time. After 10 days, the diseased condition of leaves was observed, the area of lesions was measured and the inhibition rate was calculated.

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

Results

Screening of antagonistic fungi: We isolated and purified 173 fungal strains from the soil. After preliminary screening, we found that strains 22+, L131, 21, H31, 8, H28, X32 and H5 had obvious inhibition zones. In the re-screening, strains 22+ and L131 had inhibition rates of more than 65% (Fig. 1; Table 1).

By preliminary identification, strain 22+ is likely to be *Penicillium aurantiogriseum*. The biological control research about it is relatively mature, so 22+ was not selected but L131 was selected for follow-up research.

Table 1. Anti-efficacy of 8 soil fungal strains against *A. alternate*.

Soil fungal strains	Inhibition zone (mm)	Inhibition rate of sterile fermentation broth (%)
22+	**7.04 ± 0.19^{a}**	**97.66 ± 2.5425^{a}**
L131	**5.54 ± 0.33^{b}**	**66.41 ± 2.2634^{b}**
21	2.59 ± 0.28^{c}	64.22 ± 1.0651^{bc}
H31	5.96 ± 1.51^{ab}	62.10 ± 1.1646^{c}
8	1.69 ± 0.34^{c}	56.50 ± 1.2900^{d}
H28	2.01 ± 0.19^{c}	53.61 ± 1.5234^{e}
X32	2.28 ± 0.33^{c}	20.90 ± 3.1965^{f}
H5	2.65 ± 0.69^{c}	8.89 ± 2.1215^{g}

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

The microscopic observation of *A. alternata* showed that the normal hypha and conidium had complete morphology and uniform thickness. After treatment with the fermentation broth, the hyphae of *A. alternata* were deformed, bent, and partially enlarged, and the conidia were also ruptured (Fig. 2). It indicated that there were antifungal substances in the fermentation broth of strain L131, which destroyed the hyphae and conidia of *A. alternata*, thereby inhibiting its growth.

Identification of antagonistic fungi: Morphological identification: colony on PDA is velvety, surface flat, center slightly raised, abundant sporulation, conidial area deep green, mycelium white, exudate clear to light yellow; reverse orange or yellow, edge color light (Fig. 3-a, b).

Conidiophores commonly borne from mycelium mat; stipes smooth, 80-250 × 2.5-3.0 μm; broom-shaped branch usually biverticillate or terverticillate; metulae in whorls of 3-4, 10.0-16.0 × 2.2-3.0 μm; phialides in whorls of 6-10, ampulliform, 7.5-9.0 × 2.0-2.5 μm; conidia globose to subglobose, 2.5–3.0 μm, with smooth walls (Fig. 3-c, d). It is consistent with Kong Huazhong's description of *Penicillium citrinum* (Kong, 2007).

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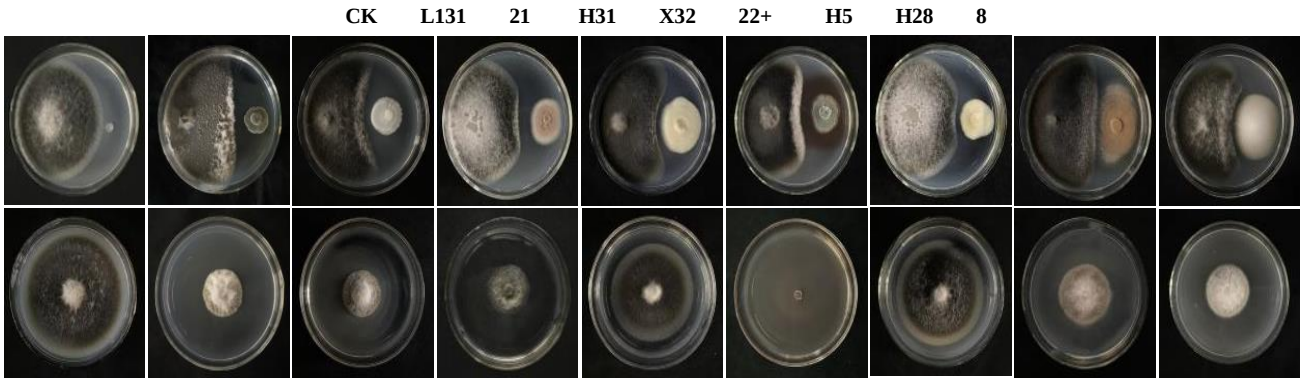

Fig. 1. Antifungal effects of primary screening and re-screening of 8 soil fungal strains.

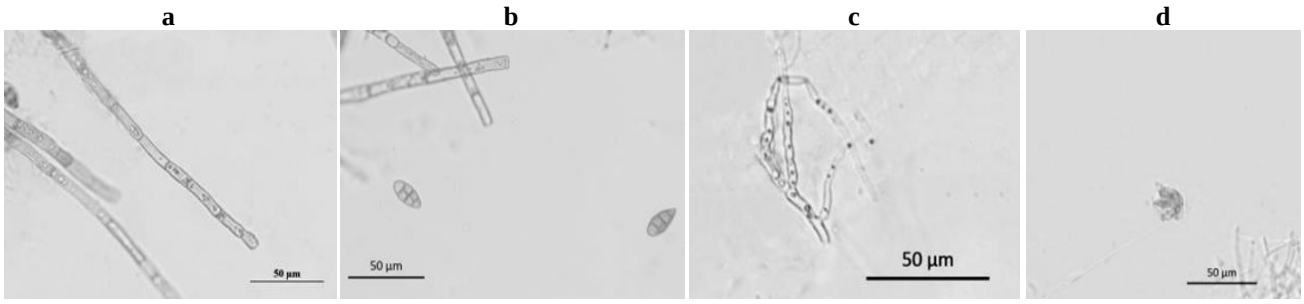

Fig. 2. Inhibitory effect of fermentation broth of strain L131 on *A. alternata* under microscope
a: normal hyphae; b: normal conidia; c: treatment group hyphae; d: treatment group conidia

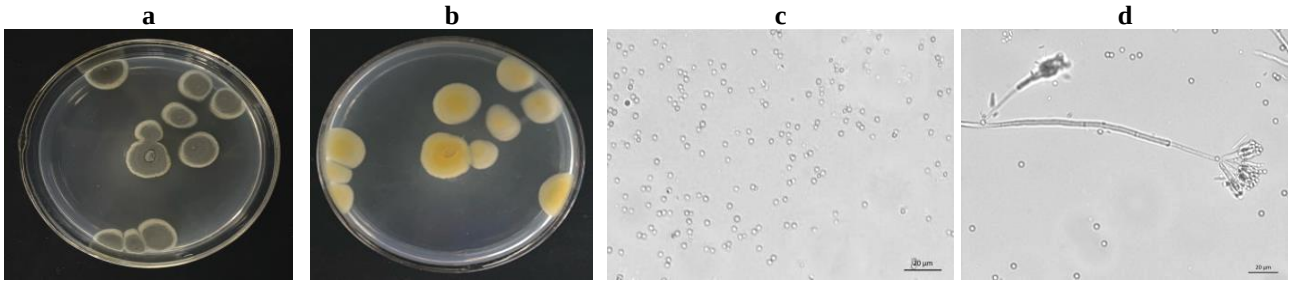

Fig. 3. Colony morphology and microscopic morphology of strain L131
a: Obverse of colonies; b: Reverse of colonies; c: Conidia; d: Conidiophores; Scale bars: 20 μm.

Molecular biological identification: The obtained ITS amplified sequence and β-tubulin gene amplified sequence both showed 100% similarity with *Penicillium citrinum.* A phylogenetic tree was constructed by selecting *Aspergillus terreus* as the outgroup, and it showed that the strain L131 was clearly divided into the same clade as *Penicillium citrinum* (Fig. 4). From the molecular point of view, it is assisted to prove that the strain L131 is *Penicillium citrinum.*

Determination of antifungal spectrum: We screened out the optimal carbon source, nitrogen source and inorganic salt through orthogonal experiments, and produced an optimized fermentation medium. Fermented in the optimized fermentation medium, the inhibition rate of the fermentation broth of L131 strain on *A. alternata* was 75.12%, and it also had a certain degree of inhibitory effect on the other 12 pathogens. The inhibition rates of 4 pathogens including *S. sclerotiorum*, *R. solani*, *E. turcicum*, and *F. graminearum* were relatively high, which were 100%, 96.42%, 88.01% and 83.66%, respectively. The antifungal rates against the other 9 pathogens were at the range of 20.46-78.84%. It can be known that strain L131 has a broad antifungal spectrum and has a certain antifungal effect on a variety of pathogenic fungi (Table 2; Fig. 5).

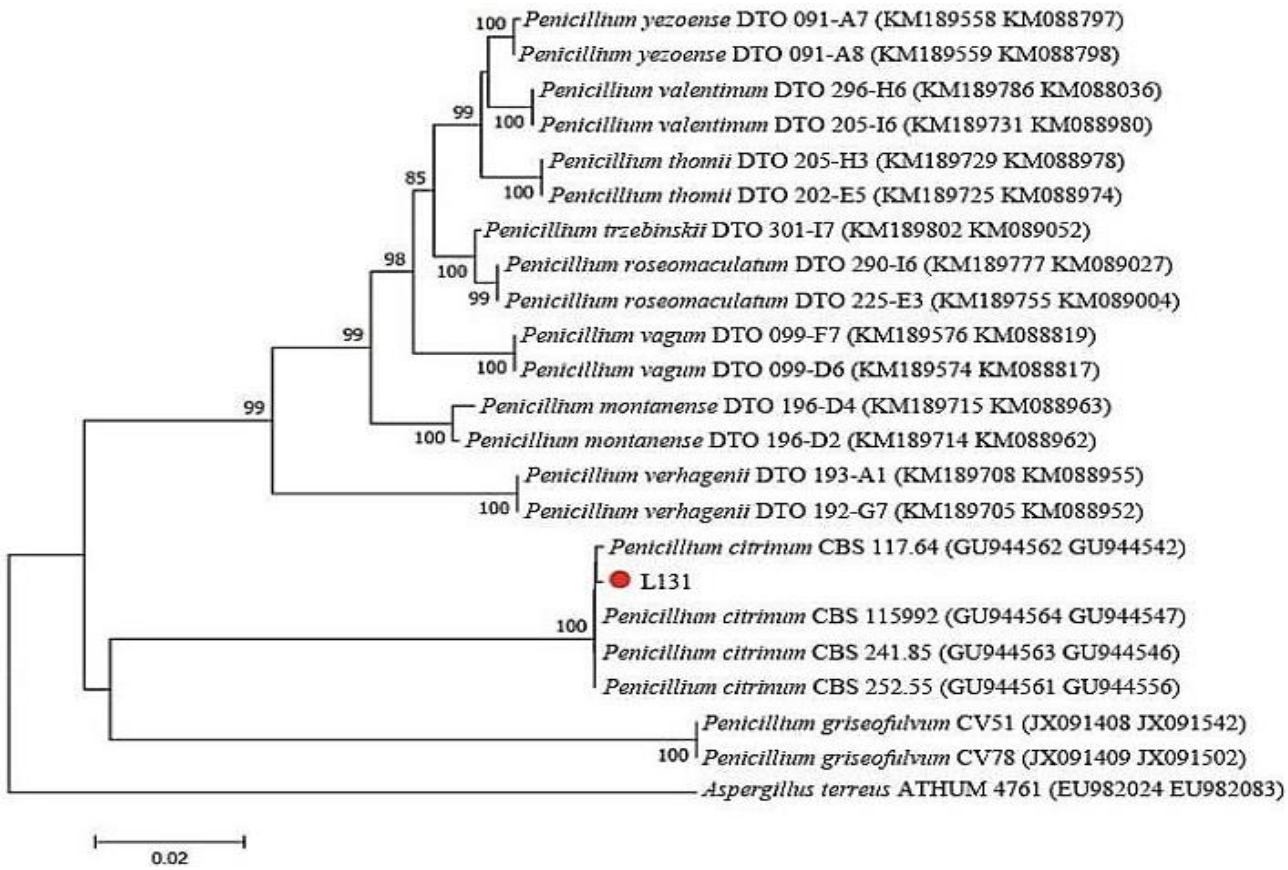

Fig. 4. Phylogenetic tree.

Table 2. Inhibition rate of sterile fermentation broth of strain L131 against 13 pathogens.

Pathogens	Average colony diameter (mm)		Inhibition rate (%)
	Test	CK	
Alternaria alternata	27.16	85.00	75.12 ± 0.1624^{f}
Sclerotinia sclerotiorum	8.00	85.00	100.00 ± 0.0000^{a}
Rhizoctonia solani	10.76	85.00	96.42 ± 0.5338^{b}
Exserohilum turcicum	17.23	85.00	88.01 ± 0.7945^{c}
Fusarium graminearum	20.58	85.00	83.66 ± 0.4541^{d}
Fusarium oxysporum	24.29	85.00	78.84 ± 0.4351^{e}
Colletotrichum gloeosporioides	23.83	78.00	77.38 ± 0.3018^{ef}
Phomopsis vexans	26.85	85.00	75.52 ± 1.1892^{f}
Fusarium proliferatum	29.87	85.00	71.60 ± 0.5111^{g}
Colletotrichum capsici	31.40	80.00	67.50 ± 1.8560^{h}
Phytophthora capsici	31.69	70.00	61.79 ± 1.0089^{i}
Botrytis cinerea	44.90	82.00	50.14 ± 5.8605^{j}
Phoma arachidicola	47.37	57.50	20.46 ± 0.4242^{k}

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

Fig. 5. Inhibitory effects of fermentation broth of strain L131 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: *S. sclerotiorum*; b: *R. solani*; c: *E. turcicum*; d: *F. graminearum*; e: *F. oxysporum*; f: *C. gloeosporioides*; g: *P. vexans*; h: *A. alternata*; i: *F. proliferatum*; j: *C. capsici*; k: *P. capsici*; l: *B. cinerea*; m: *P. arachidicola*

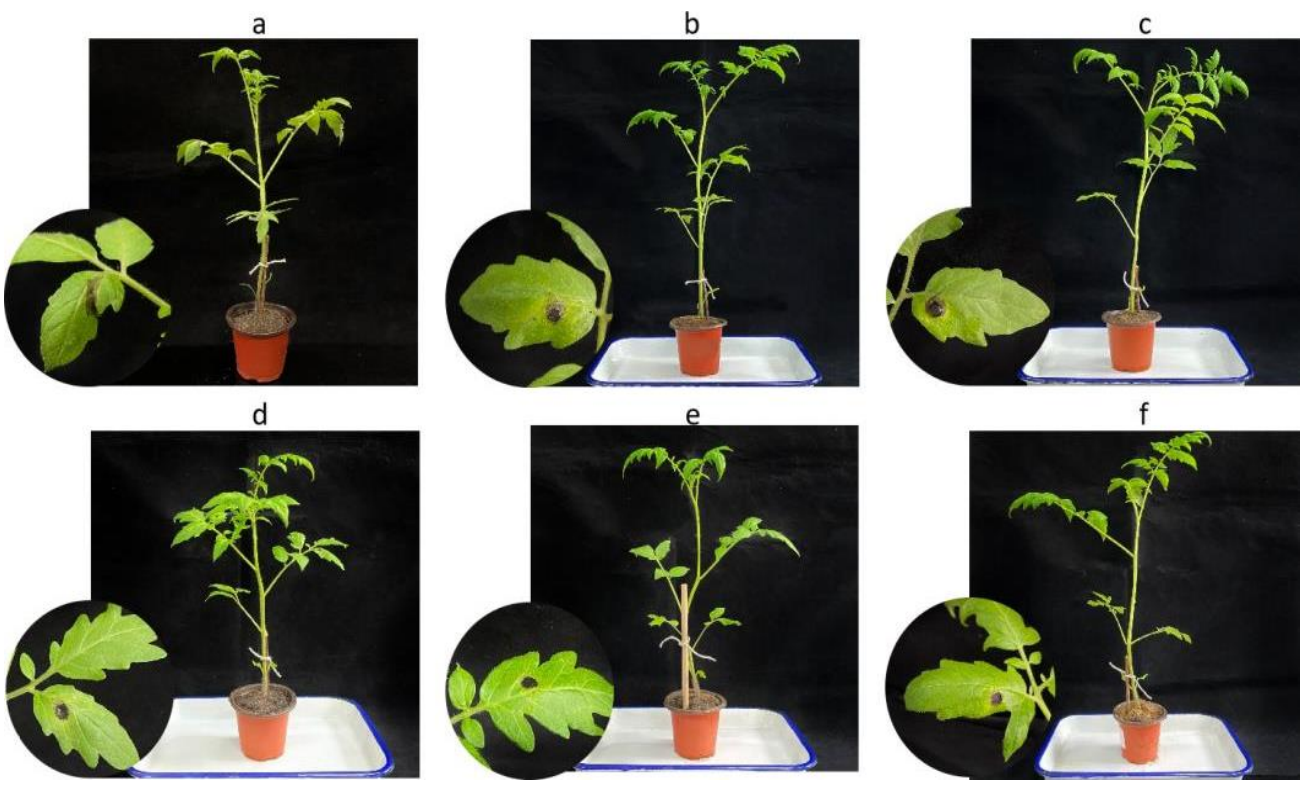

Fig. 6. Potted control effect of fermentation broth of strain L131 on tomato early blight.
Note: a: Water; b: Fermentation broth: Water=1:50; c: Fermentation broth: Water=1:40; d: Fermentation broth: Water=1:30; e: Fermentation broth: Water=1:20; f: Fermentation broth: Water=1:10

Table 3. Potted control effect of fermentation broth of strain L131 on Tomato early blight.

Fermentation broth : Water	0 : 1	1 : 50	1 : 40	1 : 30	1 : 20	1 : 10
Leaf spot area / cm^2	2.2308	1.766	1.1304	0.4416	0.3847	0.3317
Inhibition rate / %	0.00	20.84	49.33	80.20	82.76	85.13

Control effect of pot experiment: In the control group, tomato leaves appeared dark brown ringspots, which were larger in length and obviously concave. In the experimental group, the leaf lesions were significantly reduced and slightly concave. The fermentation broth of strain L131 can significantly reduce the incidence of tomato early blight, and the control effect of high concentration reaches 80% (Table 3), indicating that the fermentation broth of strain L131 has a good control effect on *A. alternata*.

Discussions

Many rhizosphere microorganisms are capable of inhibiting the growth and reproduction of pathogenic microorganisms. They can secrete metabolites that are harmful to some pathogenic microorganisms, or reproduce rapidly and massively to compete for nutrients and ecological niches, or parasitize pathogens to obtain nutrients (Prasad *et al*., 2020).

In this study, 173 fungal strains were isolated and purified from 35 soil samples. L131 was more effective against *A. alternata*, and after morphological identification and molecular identification, it was identified as *P. citrinum*. The inhibition zone of primary screening was 5.54 mm, and the antifungal rate of re-screening was 66.41%. It not only has good antifungal activity against *A. alternata*, but also can inhibit common pathogenic fungi such as *S. sclerotiorum, R. solani, E. turcicum, F. graminearum* etc., with wide antifungal range and a promising prospect.

Penicillium spp., are widely distributed in nature, with rapid propagation and strong sporulation ability. Its medium raw materials have the advantages of wide sources and low prices. Domestic and foreign studies have found many *Penicillium* spp. with biocontrol potential. For example, *P. lilacinus* can significantly reduce the parasitic rate and hatch rate of *Meloidogyne* spp. parasitized in tomatoes and cucumbers (Nie *et al.*, 2016; Wang, 2016); *P. oxalicum* can be used to control tomato *Fusarium* wilt, tomato gray mold and tomato late blight (Larena *et al.*, 2010); the fermentation broth of *P. griseofulvum* is able to inhibit the seed germination and the germ tube growth of *Orobanche aegyptiaca* (Chen *et al.*, 2019). Studies on *P. citrinum* have shown more focus on it as an important source of oil and nuclease P1, while its metabolites have anticancer effects (Luo *et al.*, 2020; Li *et al.*, 2021; Ni *et al.*, 2019). Furthermore, Hou *et al.* found that *P. citrinum* had an inhibitory effect on *Candida albicans*, *Escherichia coli* and *Xanthomonas oryzae* (Hou *et al.*, 2021). However, the research on using *P. citrinum* to control vegetable fungal diseases, especially tomato early blight, is very limited.

The *P. citrinum* L131 screened in this paper enriched the fungal resources of vegetable fungal diseases, and provided data support for the later exploration of its mechanism of biological control and isolation of inhibitory metabolites.

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