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STUDY ON ANTIFUNGAL ACTIVITY OF HYPHAE EXTRACT OF A *STREPTOMYCES* STRAIN HU2014 AGAINST FOUR PHYTOPATHOGENIC FUNGI

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The application of chemical pesticides emerges many disadvantages, so new natural resources of suppressing plant diseases are needed. Actinobacteria are gaining interest in agriculture as biological control agents (BCAs). Streptomyces spp. are part of actinobacteria and are known for producing a large number of active metabolites. In this paper, the antifungal effect of the hyphae methanol extract (HME) of a Streptomyces strain HU2014 on four phytopathogenic fungi was investigated by the growth rate method. A pretest study on different concentrations of the HME was conducted to determine a suitable range of antifungal activity. The result showed that the inhibited effect of the HME against Rhizoctonia solani was better than the other three fungi, reached 100 % with the concentration of 0.5 mg/ml. Based on the above test, the Log concentration-probit regression lines were obtained according to the inhibition rate with different concentrations. The EC_{50} value of the HME against R. solani at 48 h, 72 h, and 96 h were lowest in other fungi respectively. The toxicity regression equations of HME on R. solani was y = 6.9826+1.4028x (Correlation Coefficient r = 0.9783), and the EC_{50} value was 0.0386 mg/ml at 72 h. The toxicity regression equations of HME on Botrytis cinerea was y=5.6627+1.2386x (Correlation Coefficient r = 0.9614), and the EC_{50} value was 0.2917 mg/ml at 72h. The toxicity regression equations of HME on Colletotrichum gloeosporioides was y = 5.3143 + 1.0873x (Correlation Coefficient r=0.9996), and the EC_{50} value was 0.5140 mg/ml at 72 h. The toxicity regression equations of HME on Fusarium graminearum was y = 5.7011 + 2.3280x (Correlation Coefficient r = 0.9869), and the EC_{50} value was 0.5024 mg/ml at 72 h. The Streptomyces HU2014 strain has a significant antifungal effect and may become a new biocontrol agent in agricultural production.

Key words: *Streptomyces, phytopathogenic fungi, antifungal effect, $EC_{50.}$*

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Introduction. Fungal disease is one of the main causes of serious falling yields in modern agriculture (Adesina et al., 2007; Cha et al., 2016; McCulloch et al., 2020). Such as *Rhizoctonia solani* JG Kühn and *Fusarium graminearum* Schwabe are perhaps best known for causing diseases in Gramineae (Breunig et al., 2021; Cubeta et al., 1997), *Botrytis cinereal* Persoon and *Colletotrichum gloeosporioides* (Penz.) Saccardo are two of the most important postharvest fungal pathogens causing significant losses in fresh fruits, vegetables and ornamentals (Chaouachi et al., 2021; Wang et al., 2021). Chemical treatment is the usual method to control fungal disease (Peng et al., 2014). But the long-term use of chemical pesticides has also posed a serious threat to human safety and natural environment protection (Macaulay et al., 2021; Rani et al., 2021).

Biopesticides (in China) refer to the living organisms with pesticide activity or the active substances produced by them, which are used to control diseases, pests and weeds (Deacon et al., 1993; Wei et al., 2008). Biopesticides

have the following advantages (Tian et al., 2007): ① low toxicity and high efficiency; ② strong selectivity (they only have good control effect on target organisms and closely related organisms, but are safe and harmless to non-target organisms such as human and animals; ③ low residue; ④ greatly reduce the use of traditional pesticides without affecting modern agricultural production. Microbial pesticide is a kind of substance with pesticide physiological activity, which is made from microorganism and/or its metabolites. *Streptomyces* can produce a variety of bioactive substances, which play an important role in improving the plant disease resistance (Katz et al., 2016; Tarkka et al., 2008). *Streptomyces* are promising in agriculture as plant-growth-promoting (PGP) bacteria and/or biological control agents (BCAs) (Dias et al., 2017; Viaene et al., 2016). Several *Streptomyces* species have been researched and/or used to control plant diseases (Lu et al., 2016; Patel et al., 2018; Wan et al., 2008; Wu et al., 2019).

In this study, we determined the antifungal activity of the hyphae methanol extract (HME) of the strain HU2014 by growth rate method on *R. solani*, *C. gloeosporioides*, *B. cinerea* and *F. graminearum*. In order to provide scientific basis for finding the antifungal active components and microbial fungicides suitable for crop disease control.

Materials and methods. *Materials.* The plant pathogenic fungi including *Rhizoctonia solani*, *Botrytis cinerea*, *Colletotrichum gloeosporioides*, *Fusarium graminearum* and the strain HU2014 were afforded by Henan Institute of Science and Technology (HIST).

Methods. Fungi culture and hyphae of the strain HU2014 preparation. The fungi were pre-cultured on potato dextrose agar (PDA) plate at 25°C for about 6 days. The strain HU2014 was pre-cultured on PDA medium at 4°C until required. The hyphae discs were transferred to PDA plate at 25°C for about 8 days. The activated discs were put into sterile GPY broth in 250 ml flasks, incubated at 28°C with shaking at 150 r. min^{-1} for 15 days. The fermentation broth was centrifuged (8000 r·min^{-1}, 4°C) for 15 minutes to separate the supernatants. The hyphae were filtrated through nylon filter, washed with sterile water, and made more drier with filter paper. The filtrated hyphae were lyophilized (Christ ALPHA 1-4 LSC, Germany) to dry using following settings: 72 h, −10°C, 0.5 mbar (primary drying); 24 h, 20°C, 0.01 mbar (final drying) (Grossmann et al., 2018), and stored at 4 °C for experiment.

Extraction of the effective components. The methanol dipping method was employed to extract effective components (Jiao, R. H. et al., 2013; Thabard et al., 2011). After weighting 73.8 g of dried hyphae accurately, it was dissolved with proper methanol and crushed with ultrasonic wave. Methanol was added to 600 ml and the hyphae were soaked for 24h. The fractions of the hyphae were evaporated and then lyophilized (Christ ALPHA 1-4 LSC, Germany) after a Buchner funnel to remove the residue. Then the extract was obtained and stored at 4 °C for experiment.

Determination of the antifungal activity. The antifungal activities of the extracts were determined by growth rate method (Hadacek et al., 2000; Xu, G.-F. et al., 2007). Firstly, we conducted a pretest. The gradient concentration of the extract was 20.0 mg mL^{-1}, 10.0 mg mL^{-1}, 5.0 mg mL^{-1} with sterile water. The above reagents were mixed at ratios of 1:9 (v/v) with melted PDA medium, respectively. That is to say, the tested concentration of the extract was diluted 10 times. Immediately, 10 ml mixed medium was aseptically poured into a sterile 9 cm Petri dish and allowed to solidify. The plug (4 mm in diameter) of each phytopathogenic fungi was separately placed on the center of the plate. Fungal plug was inoculated on pure PDA plate as control. The experiments were conducted three times. And the plates were incubated at 25°C. The treated colony diameter (TCD) and control colony diameter (CCD) was measured at 48 h, 72 h and 96 h, respectively. The formula for the calculation of inhibition rate (I) is as follow: I (%) = [(CCD-4) - (TCD-4)]/(CCD-4)x100%, Where 4: Diameter of the cut fungus (measurement unit: mm).

According to the pretest, we set a series of concentrations of the extracts: 30.0 mg mL^{-1}, 20.0 mg mL^{-1}, 15.0 mg mL^{-1}, 10.0 mg mL^{-1}, 5.0 mg mL^{-1}, 2.5 mg mL^{-1} and 1.25 mg mL^{-1}. The above reagents were mixed at ratios of 1:9 (v/v) with melted PDA medium, respectively. The next test was followed the above. After measuring the inhibition rate, the toxicity regression equations and the EC_{50} value were calculated.

Statistical methods. Statistically significant differences ($p < 0.05$) were evaluated by an analysis of variance (ANOVA) using SPSS version 16.0 (SPSS Inc. Chicago, IL, United States). All data shown are the average value of three biological replicates ± SD.

Results. *The pre-screening of antifungal activities.* It can be drawn from Fig. 1 and Table 1 that different concentrations of the HME had different antifungal effects on the four pathogenic fungi. Three different concentrations of the HME completely inhibited the growth of *R. solani* at 48 h, 72 h and 96 h. the highest inhibition rate was 92.41 % against *B. cinerea* with the concentration of 2 mg/mL at 96 h. Followed by 75 % against *C. gloeosporioide* with the concentration of 2 mg/mL at 72 h, and 88.70 % against *F. graminearum* with the concentration of 2 mg/mL at 72 h. According to the above result, the inhibition rate of the HME against *R. solani* was the best in all that of fungi with the same concentration, and the concentrations of the HME should be decreased against *R. solani* for its completely inhibition at three different concentrations. A series of concentrations of the HME were reset to determine the toxicity curve and the EC_{50} value of the strain HU2014.

3.2 The EC_{50} curve analysis. The 50 % effective concentration (EC_{50}) values were deduced from log probit analysis at 48 h,72 h and 96 h (Jiang et al., 2004). From Table 2, the results showed that there was a high correlation between the concentration of the HME and the inhibition rate of fungal growth. The EC_{50} value of the HME against *R. solani always* was the lowest in all that of fungi at three sampling times, for 48 h, 72 h and 96 h showed 0.0284 mg/mL, 0.0386 mg/mL and 0.1040 mg/mL respectively. and that against *B. cinerea* were 0.3000 mg/mL, 0.2917 mg/mL and 0.3560 mg/mL respectively. Interestingly, that of the HME against *C. gloeosporioides* did not change significantly with concentration, varied from 0.5140 mg/ mL to 0.5520 mg/ mL. The EC_{50} value of the HME against *F. graminearum* were 0.3200 mg/mL, 0.5024 mg/mL and 0.5311 mg/mL respectively.

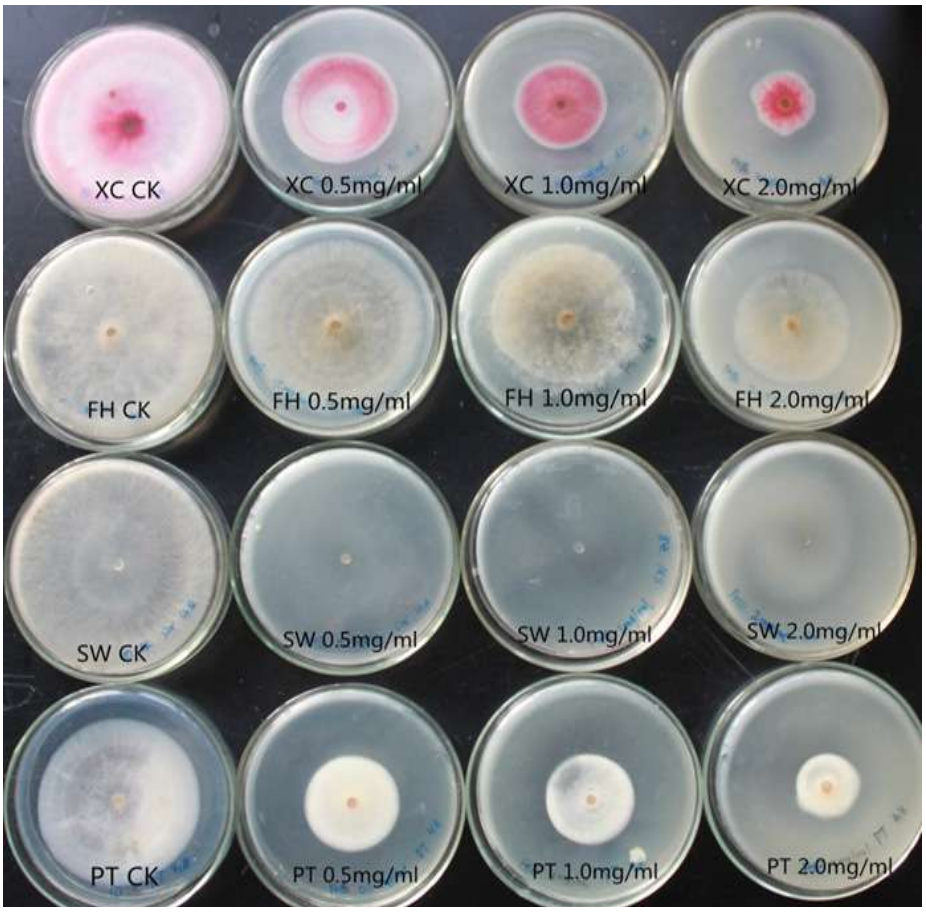

Fig. 1. The antifungal activities of the HME at three concentrations against four pathogenic fungi. Where XC: *F. graminearum*; FH: *B. cinerea*; SW: *R. solani*; PT: *C. gloeosporioide*

Table 1

The antifungal activities of the HME against four pathogenic fungi

Tested fungi	Inhibition rate (%)								
	48 h			72 h			96 h		
	2 mg/mL	1 mg/mL	0.5 mg/mL	2 mg/mL	1 mg/mL	0.5 mg/mL	2 mg/mL	1 mg/mL	0.5 mg/mL
R. solani	100±0^{a*}	100±0^{a}	100±0^{a}	100±0^{a}	100±0^{a}	100±0^{a}	100±0^{a}	100±0^{a}	100±0^{a}
B. cinerea	86.49±4.68^{b}	66.27±4.17^{b}	43.66±4.88^{c}	92.09±4.27^{b}	67.33±2.57^{b}	69.90±9.35^{b}	92.41±1.58^{b}	69.58±1.04^{b}	69.91±6.91^{b}
C. gloeosporioide	71.74±3.77^{c}	63.24±2.55^{b}	62.71±2.94^{b}	75.00±3.57^{c}	65.42±4.28^{b}	66.33±3.06^{b}	68.29±0^{c}	58.50±5.14^{b}	56.94±2.41^{c}
F. graminearum	71.43±3.53^{c}	57.14±8.57^{b}	42.11±4.56^{c}	88.70±1.51^{b}	59.57±3.69^{c}	53.00±3.46^{c}	86.49±1.87^{d}	68.39±3.59^{b}	60.45±5.18^{c}

*Where C: concentration; The experiment was in 3 replicates. * mean values followed by different letters in each column are significantly different (P<0.05).*

Table 2

The toxicity regression line of the HME against four fungi

Time	Fungi	Equation	EC_{50}(mg/ mL)	SD	F value	R	P value
48h	*R. solani*	y=6.9613+1.2686x	0.0284	0.1853	46.8677	0.9695	0.0039
	B. cinerea	y=5.7989+1.5281x	0.3000	0.1347	128.6081	0.9848	0.0003
	*C. gloeosporioides*on	y=5.327+1.2635x	0.5511	0.1185	113.7514	0.9871	0.0018
	F. graminearum	y=5.8095+1.6403x	0.3210	0.2749	35.5939	0.9603	0.0094
72h	*R. solani*	y=6.9826+1.4028x	0.0386	0.2235	39.3903	0.9783	0.0033
	B. cinerea	y=5.6627+1.2386x	0.2917	0.1772	48.8687	0.9614	0.0022
	*C. gloeosporioides*on	y=5.3143+1.0873x	0.5140	0.0573	360.1898	0.9959	0.0003
	F. graminearum	y=5.7011+2.3280x	0.5024	0.1083	639.0019	0.9996	0.0096
96h	*R. solani*	y=6.8632+1.8956x	0.1040	0.1552	149.1955	0.9869	0.0003
	B. cinerea	y=5.3193+0.7135x	0.3560	0.0254	788.2814	0.9981	0.0001
	*C. gloeosporioides*on	y=5.2563+0.9941x	0.5520	0.1039	91.4727	0.9840	0.0024
	F. graminearum	y=5.6953+2.5297x	0.5311	0.0913	767.8400	0.9993	0.0230

Where SD: Standard deviation; R: r coefficient

We can draw a conclusion that the HME had the highest antifungal activity on *R. solani* and its EC_{50} value at 48 h and 72 h just were closed to one percent of that against other tested fungi. This was an exciting finding for further research.

Discussion. It is a well-known fact that *Streptomyces sp.* produces active metabolites that can inhibit the growth of phytopathogens (Adesina et al., 2007; Katz et al., 2016; Patel et al., 2018; Wu et al., 2019). The EC_{50} value is commonly used to evaluate drug potency and sensitivity of plant pathogens(Li, J. L. et al., 2015; Li, M. et al., 2015; Liang et al., 2015). Some reports about the compounds had excellent inhibition activities through the comparison with the EC_{50} value of commercialized fungicides (Hu, H. R. et al., 2020; Hu, M. J. et al., 2013; Xu, S. Q. et al., 2019; Yang et al., 2020). In this study, bioassay results in vitro indicated that the HME exhibited strong activity against *R. solani*, *B. cinerea and F. graminearum*. The anti- *R. solani* EC_{50} values were 0.0284 mg/ mL at 48 h, followed by the EC_{50} values of anti- *B. cinerea* indicating 0.3000 mg/ mL at 48 h and 0.2917 mg/mL at 72 h respectively. The values had a certain gap between the HME and the commonly used fungicides, such as carbendazim (EC_{50}=0.43 mu g/ mL) (Jiao, J. et al., 2021) and fluopyram (EC_{50}=0.244 mg/L) (Yan et al., 2020). We know, the HME in this study was not the pure compound. Although the antifungal activity of the HME of the strain HU2014 was determined in this study, it is not sure which substance had antifungal effect on the four tested fungi, and it needs to further purify and verify the antifungal activity. This study is only a preliminary exploration and the mechanism of action needs further study.

Conclusions. We found a streptomyces strain HU2014 and study the antifungal activity of the HME of the strain with different concentrations against *R. solani, B. cinerea, C. gloeosporioides* and *F. graminearum*. The results showed that in a certain concentration range, the antifungal effect of the HME on the hyphae growth of four pathogenic fungi was highly significant correlations with concentration. According to the inhibition rate, EC_{50} and related parameters, the order of antifungal effect of the HME against four pathogenic fungi was as follows: *R. solani* > *B. cinerea* > *F. graminearum* > *C. gloeosporioides*. Especially, the EC_{50} value of the HME against *R. solani* was significantly lower than that of other three fungi. The results showed that the HME of the strain HU2014 had outstanding antifungal activity on *R. solani*, and would be a choice for biocontrol agents.

With the improvement of people's quality of life and the gradual strengthening of environmental awareness, this kind of microbial pesticide would attract attention of the researchers and planters. Therefore, it is necessary to further purify the components and strengthen the pharmacological research on the antifungal effect of the HME of the strain HU2014, increase its value in agricultural production, and create greater economic benefits for human society.

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Дослідження протигрибної активності екстракту гіф штаму Streptomyces HU2014 щодо чотирьох фітопатогенних грибів

Застосування хімічних пестицидів має багато недоліків, тому необхідні нові природні ресурси для регулювання розвитку хвороб рослин. Актинобактерії набувають інтересу для сільського господарства як агенти біологічної боротьби. Streptomyces spp. є частиною актинобактерій і відомі продукуванням великої кількості активних метаболітів. У цій роботі методом вимірювання швидкості росту досліджено протигрибну дію метанолового екстракту гіф (МЕГ) штаму Streptomyces HU2014 на чотири фітопатогенні гриби. Для визначення відповідного

діапазону протигрибної активності було проведено попереднє тестування з різними концентраціями МЕГ. Результати показали, що ефект інгібування Rhizoctonia solani був кращим, ніж трьох інших грибів, і склав 100 % з концентрацією 0,5 мг/мл. На основі вищезазначеного тесту були отримані лінії регресії концентрації Log-пробіту відповідно до швидкості інгібування з різними концентраціями. Значення (50 % -відсоткової ефективної концентрації) EC_{50} МЕГ до R. solani через 48 годин, 72 години та 96 годин було найнижчим порівняно з іншими грибами. Рівняння регресії токсичності МЕГ на R. solani склало y = 6,9826 + 1,4028x (коефіцієнт кореляції r = 0,9783), а значення EC_{50} становило 0,0386 мг/мл через 72 години. Рівняння регресії токсичності МЕГ на Botrytis cinerea становило y = 5,6627 + 1,2386x (коефіцієнт кореляції r = 0,9614), а значення EC_{50} становило 0,2917 мг/мл через 72 години. Рівняння регресії токсичності МЕГ на Colletotrichum gloeosporioides склало y = 5,3143+1,0873x (коефіцієнт кореляції r = 0,9996), а значення EC_{50} становило 0,5140 мг/мл через 72 години. Рівняння регресії токсичності МЕГ на Fusarium graminearum склало y = 5,7011 + 2,3280x (коефіцієнт кореляції r = 0,9869), а значення EC_{50} становило 0,5024 мг/мл через 72 години. Штам Streptomyces HU2014 має значний протигрибний ефект і може стати новим агентом біоконтролю у сільськогосподарському виробництві.

***Ключові слова**: стрептоміцети, фітопатогенні гриби, протигрибна дія, EC_{50}.*