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Full Length Article

Biocontrol Potential of *Acinetobacter johnsonii* ZY86 against Gray Mold by *Botrytis cinerea*

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Abstract

To resolve the problem of pesticide resistance in strawberry gray mold *Botrytis cinerea* and to improve the effectiveness of disease preventive measures by developing biological pesticides, a bacterial strain was identified using plate confrontation culture method, morphological characteristics, physiological and biochemical analyses, and 16S rDNA gene sequence analysis. The antifungal components of the antagonistic bacterial strain were analyzed by GC-MS, and their *in vitro* control effects were evaluated. One hundred bacterial strains were isolated from the strawberry rhizosphere soil, of which one showed superior antagonistic effect, accounting for 1% of all of the strains, and it was designated as strain ZY86. Further investigation indicated that strain ZY86 exhibited antifungal effects not only against *B. cinerea* but also against *Rhizoctonia solani*, *Magnaporthe grisea*, *Colletotrichum capsici*, *Phytophthora capsici* and *Sclerotinia sclerotiorum*. Strain ZY86 (GenBank Accession number MK111627) was highly similar to model strain *Acinetobacter johnsonii* (ATCC17909), with a homologous identity of 99%, and thus, strain ZY86 was identified as *A. johnsonii*. Analyses of the antifungal components showed that the active components produced by strain ZY86 were primarily amines, accounting for 20.80% of the total active components, which were followed, in order, by ketones, acids, esters, alcohols, peptides, hydrocarbons, alkanes, aldehydes, and alkenes in terms of proportion. *In vitro* testing showed that the control effect of strain ZY86 on strawberry gray mold was 84.14%, and higher the concentration, the better the control effect. In addition, strain ZY86 imparted a strong control effect against strawberry gray mold, and therefore, it is a promising biocontrol agent for agricultural production.

Keywords: Antagonistic bacteria; Active components; *Botrytis cinerea*; Control effect; Identification

Introduction

Strawberry (*Fragaria* × *ananassa* Duch.) is a perennial herb belonging to the Rosaceae family. Its fruit is of very high nutritional value, bright color and tasty, has become a novelty, excellent health food for consumers. Strawberry is one species of economic plant, with the characteristics of rapid bearing, high-production and strong adaptability (Zhang *et al*., 2015a). Currently, strawberry is at the top of global small berry production, with an annual global production of over 7.2 million tons and cultivation acreage of over 300,000 hm^2 (FAOSTAT, 2015). In the past 20 years, the strawberry cultivation area in other countries has stabilized, whereas that in China has dramatically and continuously increased. Strawberry cultivation methods have also changed from a single open cultivation mode to the combination of open cultivation and facility cultivation, making strawberry production the pillar industry of many regions in China (Yin and Larson, 2009; Zhang and Zhong, 2018).

During growth, strawberries are affected by various factors, such as the temperature, humidity, light, nutrients, and pests, such as the strawberry gray mold, which is known as one of the most devastating fungal plant diseases around the world. Strawberry gray mold is caused by *B. cinerea*, which is widely distributed throughout the primary strawberry-producing areas around the world, including China, and it has been more severe in southern China than in northern China (Li *et al*., 2007; Chen *et al*., 2011; Chen *et al*., 2018). During the strawberry flowering period, under low temperature and high humidity conditions, *B. cinerea* infects the flower calyxes, fruits, and leaves of strawberry plants, but not the roots and stems. Strawberry gray mold is

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common in both greenhouse and open field cultivations and results in yield losses of 20–50% or higher in severe cases (Cordova *et al*., 2017).

In practice, the prevention and control of strawberry gray mold primarily include agricultural, chemical, and biological approaches. Commonly practiced agricultural control measures include planting disease-resistant varieties and covering the field with mulch film (Evenhuis and Wanten, 2006; Bestfleisch *et al.*, 2015; Feliziani and Romanazzi, 2016), improving the soil nutrients conditions (Novotny *et al*., 2017), cultivation methods (Holger, 2000), irrigation methods (Seong *et al*., 1993), and soil management (Jenkins, 1968). However, these measures are time consuming and slow to take effect. Chemical control has the advantages of low cost, fast response, and good control effects, and it has always been an important means to control strawberry gray mold. Current chemical agents commonly used to control strawberry gray mold include procymidone, iprodione, benomyl, diethofencarb, carbendazim, azoxystrobin, pyrimethanil, and thiabendazole; however, their long-term application has polluted the environment as well as resulted in the development of pesticide-resistant pathogens (Washington *et al*., 1992; Yourman and Jeffers, 1999; Elad *et al*., 2010; Pan *et al*., 2013; Grabke *et al*., 2014; Yin *et al*., 2015; Zhang *et al*., 2016a; Lopes *et al*., 2017). In some areas, *B. cinerea* has developed dual agent and even multi-agent resistance, and in some cases, this fungus is resistant to new chemicals that have just been introduced to the market. Recent studies have shown that gray mold is resistant to fludioxonil (Fernandez-Ortuno *et al*., 2014), but sensitive to cyprodinil (Errampalli, 2014). Amiri *et al.* (2014) revealed that *B. cinerea* is at risk of becoming resistant to fluopyram, fluxapyroxad, and penthiopyrad, and thus novel strategies for strawberry gray mold control must be developed. The application of biological control can prevent environmental pollution, mitigate chemical resistance in pathogens, and improve disease prevention effectiveness, and it is highly efficient, has low toxicity, and is environmentally friendly. The use of antagonistic bacteria to control plant diseases is an important part of biological control.

Currently, there are more than 30 antagonistic bacterial strains that can be used to control strawberry gray mold, which include *Bacillus, Pseudomonas, Lactobacillus*, and the halophilic bacteria *Kurthia sibirica*, and have been registered or mass-produced (Liu, 1993; Essghaier *et al*., 2010; Johan and Ibrahim, 2012; Zamani-Zadeh *et al*., 2013; Shternshis *et al.*, 2015). However, the application of antagonistic bacteria to control plant diseases is hampered by several issues. For instance, no *in vivo* ecological studies on antagonistic bacteria, particularly quantitative and qualitative studies on the antagonism of indigenous microorganisms, have been conducted to date. In addition, currently available antagonistic bacterial products have a short shelf life, most of their active components are unknown, and some bacterial strains are prone to degeneration, and even with rejuvenation, their control effects remain unsatisfactory (Zhang *et al*., 2000; Qiu, 2010). Therefore, there is a need to identify novel antagonistic strains. In this study, an antagonistic bacterial strain from strawberry rhizosphere soil was isolated, its antifungal spectrum was characterized, and morphological observations and physiological and biochemical assays as well as 16S rDNA sequence analysis were conducted to identify the species of the antifungal strain. The antifungal components of strain ZY86 were identified, the *in vitro* control effect of the antifungal components on *B. cinerea* was clarified to evaluate ZY86's bio-pesticide potential, and to provide more scientific data for further prevention and control measures of *B. cinerea*.

Materials and Methods

Test Strain

B. cinerea was isolated from the strawberry cultivation greenhouse at the teaching farm of Hunan Agricultural University (Changsha City, Hunan Province, China). Strain ZY86 was isolated from strawberry rhizosphere soil in the Laboratory of Pesticides at Hunan Agricultural University.

Screening of Strain ZY86

Bacterial strains were isolated from strawberry rhizosphere soil using the dilution isolation method of Zhang *et al*. (2015b). Antagonistic bacterial strains were screened using the dual culture inhibition method (Zhang *et al*., 2016b).

Observation of Colony Characteristics

Strain ZY86 was inoculated onto various media, including PDA (200 g potato, 20 g glucose, 18 g agar, 1 L H_2O), NA (3 g beef extract, 10 g peptone, 5 g NaCl, 1 L H_2O, pH 7.2–7.4) and GSA [Gauze's Synthetic Medium No. 1 (GSYH): 20 g soluble starch, 0.5 g NaCl, 1 g KNO_3, 0.5 g $K_2HPO_4 \cdot 3H_2O$, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.01 g $FeSO_4 \cdot 7H_2O$, 15–25 g agar, and 1 L H_2O, pH 7.4–7.6]. Colony characteristics, such as size, shape, edge, surface features, topographic shape, surface glaze, texture, color, transparency, ease of picking, binding to the medium, pigmentation, the consistency of color on both sides of the colony, and other traits were assessed (Zhao and He, 2002).

Identification of Strain ZY86

The morphological, physiological, and biochemical characteristics of strain ZY86 were analyzed according to Dong and Cai (2001). Molecular identification and phylogenetic reconstruction were performed according to Zhang *et al.* (2015b).

Preparation of Antifungal Compound from Strain ZY86

A 24-h culture suspension of strain ZY86 was inoculated into 400 mL of NB liquid medium (or NB for short; 3 g beef extract, 10 g peptone, 5 g NaCl, and 1 L H_2O, pH 7.4–7.6) at an inoculation rate of 3% in a 1-L flask and cultured on a shaker (160 rpm) for 72 h at 28°C. The culture broth was then centrifuged at 12,000 rpm for 5 min, and the supernatant was passed through a 0.22-μm filter. The filtrate was mixed with two volumes of ethyl acetate and extracted with a separatory funnel, and the upper layer was retained and rotavapored at 40°C to obtain a paste containing the active components, which was stored at 4°C.

GC-MS Detection Conditions

The active components were analyzed by GC-MS using the following conditions: column: Rxi-50 column (30 m × 0.25 mm ID × 0.25 μm df); carrier gas: high-purity helium; carrier gas flow rate: 1.0 mL min^{-1}; split ratio: 5; gasification temperature: 300°C; temperature program: the initial temperature was set at 80°C and maintained for 1 min, and then the temperature was raised to 120°C at a heating rate of 10°C/min, immediately raised to 200°C at a heating rate of 20°C min and maintained for 1 min, then raised to 240°C at a heating rate of 10°C min^{-1} and maintained for 2.0 min, and finally to 280°C at a heating rate of 20°C min^{-1} and maintained for 10.0 min; injection volume: 1 μL; solvent delay time: 3.6 min; ion source: EI source; electron energy: 70 eV; ion source temperature: 230°C; interface temperature: 280°C; mass scanning range: m/z 33–600; and mass spectrometry standard libraries: NIST05, NIST05s, and WILEY7.

Indoor Virulence and *in vitro* Control Effect of Strain ZY86 on *B. cinerea*

The antifungal preparation was dissolved in acetone, which was then diluted with sterile water into solutions of various concentrations (30, 60, 120, 240 and 480 μg mL^{-1}). The antifungal solution was then mixed with melted PDA medium (45°C) at a 1:9 ratio and plated onto sterile Petri dishes measuring 9 cm in diameter, and the control plates were made with water instead of the antifungal solution. After the gel solidified, one strawberry gray mold fungus disc that was made from the 48-h fungal culture using a puncher was inoculated onto the antifungal solution containing medium, with the mycelial side of the fungus disc facing downward and three replicates per concentration and was cultured in the dark for 48 h at 22°C. Then, the diameters of the colonies in each treatment were measured to calculate the inhibition rate (IR) of each treatment on the test strain using the following equation (Xiang *et al*., 2018):

$$IR(\%) = \frac{CDC - CDT}{CDC - CDFDC} \times 100$$

Where, IR is the inhibition rate; CDC is the colony diameter of control; CDT is the colony diameter of each treatment; and CDFDC is the colony diameter of fungal disc cake.

The antifungal preparation from the culture broth of strain ZY86 was dissolved in an appropriate amount of acetone and then diluted with sterile water to obtain a stock solution of 500 μg mL^{-1}. Five treatments, *i.e.*, 30, 60, 120, 240 and 480 μg mL^{-1}, together with a water control, were designed. The above antifungal preparations were evenly applied to strawberry fruits of the same size and air-dried, and then the samples were inoculated with strawberry gray mold discs at three replicates per treatment, using water as control. The treated strawberry fruits were then cultured in the dark at 20–23°C for 48 h, and the colony diameters were measured using the cross method, which were then used to calculate the control effect (CE) using the following equation:

$$CE = \frac{CDC - CDT}{CDC - CDFDC} \times 100$$

Where CDC is the colony diameter of the control; CDT is the colony diameter of each treatment; and CDFDC is the colony diameter of fungal disc cake.

Data Analysis

ANOVA was performed on the data collected in this study using S.P.S.S. 11.5 statistical analysis software, and differences between treatments were determined using Duncan's new complex range method.

Results

Screening of Strain ZY86

A total of 100 bacterial strains were isolated from the strawberry rhizosphere soil, of which one showed antagonistic activity against *B. cinerea*, accounting for 1% of the total isolates. Strain ZY86 demonstrated the best antagonistic effect (Fig. 1a), while the other strains exhibited poor antagonistic effects. Subsequent assays revealed that in addition to *B. cinerea*, strain ZY86 also had antifungal effects against *Rhizoctonia solani*, *Magnaporthe grisea*, *Colletotrichum capsici*, *Phytophthora capsici* and *Sclerotinia sclerotiorum*, indicating a broad antifungal spectrum (Fig. 1b-f) and suggesting that this strain has a wide application range and thus a significant potential for further development.

Morphological Characterization of Strain ZY86

Strain ZY86 is rod-shaped, gram-negative, without flagella, and non-sporulating. On PDA medium, the strain rapidly proliferated, and the colonies were raised, grayish-white in color and round, with uniform margins, unglazed, and opaque (Fig. 2a). On NA medium, the strain grew relatively slowly, and the colonies were round, raised, with a uniform

Table 1: Physiological and biochemical characteristics of antagonistic strain ZY86

Characteristics	ZY86
Oxidase	-
Peroxidase	+
Citric acid test	+
Methylred reaction	-
V-P reaction	-
Nitrate reduction	-
Gluten hydrolysis	-
Starch hydrolysis	-
Sugar fermentation test	-
Urease test	-

Note: "+" Positive; "–" Negative

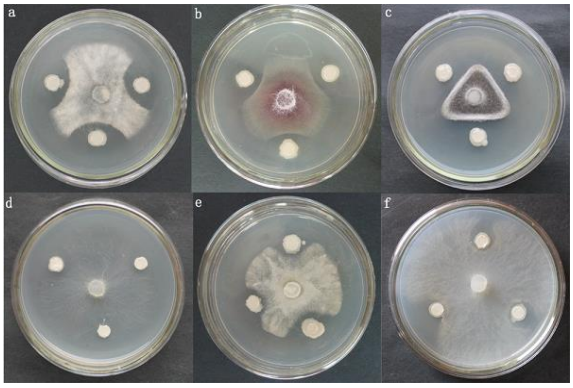

Fig. 1: Strain ZY86 inhibition of different plant pathogenic fungi (**a**: *Botrytis cinerea*, 48 h; **b**: *Colletotrichum gloeosporioides*, 48 h; **c**: *Pyricularia grisea*, 48 h; **d**: *Rhizoctonia solani*, 48 h; **e**: *Phytophthora capsici*, 48 h; **f:** *Sclerotinia sclerotiorum*, 72 h)

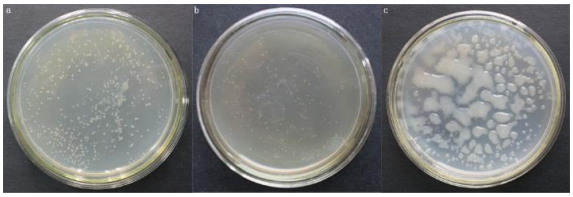

Fig. 2: Colonial morphology of strain ZY86 on different media (**a**: PDA, 48 h; **b**: NA, 48 h; and **c**: GSYH, 96 h)

margin, unglazed, and opaque (Fig. 2b). On GSYH medium, the strain grew very slowly, and the colonies were raised, viscous, milky white in color, glazed, and opaque (Fig. 2c).

Physiological and Biochemical Characterization of Strain ZY86

Table 1 shows that strain ZY86 generated negative results for the theoxidase and methyl red, Voges-Proskauer (VP), nitrate reduction, gelatine liquefaction, starch hydrolysis, glycolysis, and urease tests. It was positive for the catalase and citric acid tests. Strain ZY86 was identical to model strain ATCC 17909 in relation to most physiological and biochemical indicators, with only a few exceptions, which are likely due to different sources of the strains.

Analysis of the16S rDNA Gene Sequence of Strain ZY86

The genomic DNA of strain ZY86 was extracted and used as a template to amplify the 16S rDNA gene, and the PCR amplicon was subjected to agarose gel electrophoresis, which revealed that a gene fragment size was roughly 1,400 bp (Fig. 3). Further sequencing confirmed that the size of the 16S rDNA sequence of strain ZY86 was 1,431 bp (GenBank Accession number MK111627), which was coincided with the results of electrophoresis. Fig. 4 shows that strain ZY86 belongs to the *Acinetobacter* sp. Cluster, and is most closely related to *A. johnsonii* (ATCC 17909). Phylogenetic analysis using 16S rRNA sequencing, combined with the morphological and culture characteristics and physiological and biochemical analysis results, indicated that strain ZY86 is *A. johnsonii*.

Analysis of the Antifungal Components of Strain ZY86

The antifungal components of strain ZY86 were analyzed using GC-MS, and a total ionization chromatogram was obtained. The mass proportion of each component out of the total components was calculated using peak area normalization (Fig. 5). According to the criteria in which the similarity is higher than 80% and the content is higher than 0.15% of the total, a total of 50 compounds were detected, of which 47 were identified, accounting for 94% of the total components (Table 2). The antifungal components of strain ZY86 were primarily amines, ketones, acids, esters, alcohols, aldehydes, alkanes, alkenes, and hydrocarbons, of which the amines predominated, accounting for 20.80% of the total active components, followed by ketones (20.31%) and acids (18.15%). Esters and alcohols accounted for 13.87 and 6.27%, respectively, of the total active components, and peptides and hydrocarbons accounted for 10.12 and 5.07%, respectively, of the total active components, and the rest were in the following descending order in terms of content: alkanes, aldehydes, and alkenes.

Indoor Toxicity and *in vitro* Control Effects of Strain ZY86 against *B. cinerea*

Table 3 shows that the concentrations of the antifungal compounds from strain ZY86 were proportional to the observed antifungal effect; the higher the concentration, the greater the antifungal effect. At a concentration of 480 μg mL^{-1}, the antifungal effect was the highest (91.57%), which was 2.19 times that of the antifungal effect at the lowest concentration. The virulence regression equation of antifungal compounds from strain ZY86 against *B. cinerea* was Y =1.2683x + 2.9167, with a correlation coefficient of 0.9962, and the EC_{50} was 43.91 μg mL^{-1}. The ANOVA result showed that the inhibitory effects from different concentrations of the antifungal compound from strain ZY86 against *B. cinerea* were significantly

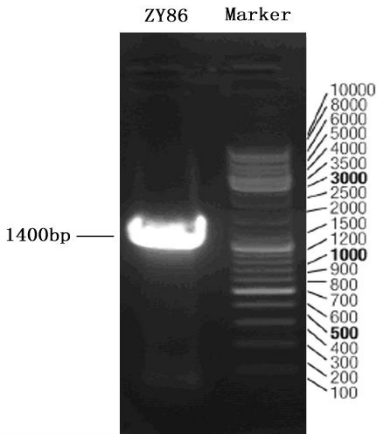

Fig. 3: Electrophoresis of strain ZY86 16S rDNA

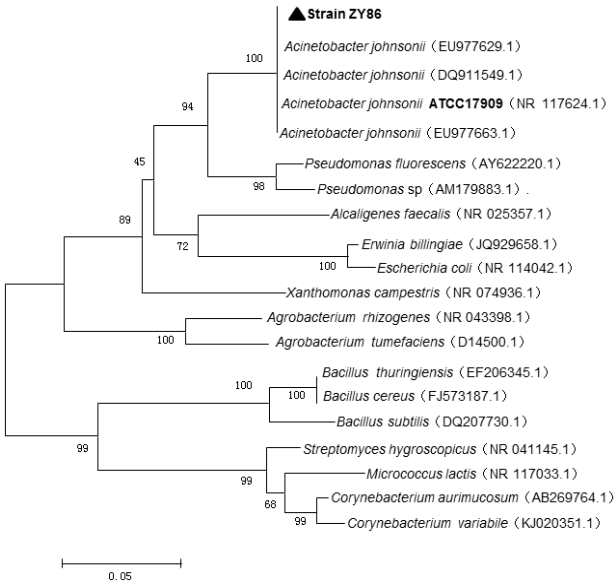

Fig. 4: Phylogenetic reconstruction of strain ZY86

different ($P < 0.05$).

Table 4 shows that the antifungal compound from strain ZY86 had good control effects against *B. cinerea*. The higher the concentration of the antifungal compounds from strain ZY86, the greater the antifungal effect. At a concentration of 480 μg mL^{-1}, the *in vitro* control effect was 84.14%, which was 3.36 times the effect at the lowest concentration (Fig. 6). The ANOVA result showed that the inhibitory effects from different concentrations of the antifungal compound from strain ZY86 against *B. cinerea* were significantly different ($P < 0.05$), indicating that the antifungal compound from strain ZY86 can be used as a pesticide candidate for controlling strawberry gray mold.

Discussion

Strawberry is an important fruit that is frequently infected by strawberry gray mold during its growth, thereby affecting its yield and quality. In recent years, with the expansion of strawberry planting, changes in climatic conditions, farming methods, and environmental conditions, the prevalence of strawberry gray mold has significantly increased. The use of antagonistic bacteria to control strawberry gray mold prevents the negative problems caused by chemical agents, and it is also a highly effective, low in toxicity, and environmentally friendly and is thus an important method for the control of strawberry gray mold, with great developmental potential (Donmez *et al*., 2011).

The strain ZY86, which was later identified as a strain of *A. johnsonii*, and showed an antagonistic effect against *B. cinerea*, thereby representing a new antifungal resource. Due to its broad nutrient use and ease of culture and propagation, strain ZY86 has great potential for development into a biological pesticide. Previous studies have shown that *Acinetobacter* has broad application prospects in industry, environmental protection, and agriculture. *Acinetobacter* can produce active enzymes for industrial uses. Qi *et al*. (2009) showed that *A. johnsonii* LP28 can produce lipase, which can be adapted for industrial uses, such as washing, fat hydrolysis, and wastewater treatment. Bonting *et al*. (1999) found that *Acinetobacter* can produce pyrophosphatase that degrades pyrophosphate. Grangeasse *et al*. (2010) found that *Acinetobacter* produces tyrosine kinase, which catalyzes the phosphorylation of various protein substrates with tyrosine residues and plays an important role in cell growth, proliferation, and differentiation. *Acinetobacter* also degrades toxins and heavy metals (Jiang *et al*., 2018) showed that *A. johnsonii* can efficiently degrade phenanthrene and naphthalene. *Acinetobacter* has also been shown to efficiently degrade benzene (Hoyle *et al*., 1995). Boswell *et al*. (1999) demonstrated that *Acinetobacter* is capable of degrading heavy metals. Lian *et al*. (2009) showed that *A. johnsonii* has a phosphorus removal rate of 68.88%, and thus can be used as a water purification agent. Moreover, in agriculture, *Acinetobacter* has been shown to have a high application value. Wang *et al*. (2012) showed that *Acinetobacter* sp. CW17 can degrade the herbicide pyrazosulfuron-ethyl. Xie *et al*. (2009) found that *A. johnsonii* MA19 can degrade the insecticide malathion. Tian *et al*. (2016) demonstrated that *A. johnsonii* MB44 has nematicidal activity. Shi *et al*. (2011) have shown that endophytic *A. johnsonii* strain 3-1 can promote the growth of beets and increase N, P, K, and microbial content, thus indicating broad applications to agriculture. However, despite findings that *Acinetobacter* plays specific functions in enzyme production, degradation, and growth promotion, there are still many problems to address. Investigations on the detailed mechanisms and pathways of degradation by *Acinetobacter*, the discovery of key enzymes, the establishment of genetic tools, and the construction of genetic engineering bacteria are thus warranted.

Table 2: Active components of strain ZY86 and their relative contents

Peak number	t/min	Compound	Molecular formula	Relative molecular mass (g/mol)	Peak area percentage (A%)
1	4.58	Isovaleramide	$C_5H_{11}NO$	101	0.28
2	5.13	Caprylic acid	$C_8H_{16}O_2$	144	0.62
3	6.86	Piperazine, 1-(4-acetylphenyl)-4-methylsulfonyl	$C_{13}H_{18}N_2O_3S$	282	0.24
4	7.07	Diethanolamine lauric acid	$C_{16}H_{33}NO_3$	287	4.64
5	7.21	Hexadecanal	$C_{16}H_{32}O$	240	0.25
6	7.29	2-Piperidone	C_5H_9NO	99	1.12
7	7.37	Piperidone	C_5H_9NO	99	0.51
8	7.93	3-Phenylpropionic acid	$C_9H_{10}O_2$	150	0.97
9	8.5 7	2,4-Imidazolidinedione, 3-methyl	$C_4H_6N_2O_2$	114	3.57
10	9.1 1	Trans-3-dodecenoic acid	$C_{12}H_{22}O_2$	198	3.85
11	9.73	P-hydroxyphenylethanol	$C_8H_{10}O_2$	138	0.35
12	10.2 0	Enoxadione	$C_8H_{12}O_2$	140	0.44
13	10.6 6	Orthotetradecyl carbonate	$C_9H_{18}FO_2P$	208	0.17
14	11.03	Myristic acid	$C_{14}H_{28}O_2$	228	0.3
15	13.3 9	1-Diketo-2,4-diazepane	$C_5H_8N_2O_2$	128	0.33
16	13.5 2	Palmitic acid	$C_{16}H_{32}O_2$	256	1.62
17	14.1 4	Diisobutyl phthalate	$C_{16}H_{22}O_4$	278	1.43
18	14.71	DL-alanyl-L-leucine	$C_9H_{18}N_2O_3$	202	2.9 0
19	14.96	N-dodecyl-1,3-propanediamine	$C_{15}H_{34}N_2$	242	1.16
20	15.17	Anti-oleic acid	$C_{18}H_{32}O_2$	282	0.29
21	15.32	(1S,2S)-2-Aminocyclohexanecarboxylic acid	$C_7H_{13}NO_2$	143	2.33
22	15.46	2,6-Piperazinedione, 1-(5-hydroxyphenyl)	$C_{10}H_{17}NO_3$	199	0.48
23	15.8 2	Dibutyl phthalate	$C_{16}H_{22}O_4$	278	6.35
24	15. 89	Cyclic (glycyl-L-leucyl)	$C_8H_{14}N_2O_2$	170	2.04
25	15.9 4	4-Methyl S-2-(diisopropylamine)ethyl	$C_{17}H_{38}NO_2PS$	351	0.68
26	15.99	3,6-Bis(1-methylethylpiperazinedione)	$C_{10}H_{18}N_2O_2$	198	0.23
27	16.12	Proline cyclic peptide	$C_{11}H_{18}N_2O_2$	210	5.48
28	16.60	Undecyl alcohol	$C_{11}H_{24}O$	172	5.92
29	16.6 7	Propionate	$C_{13}H_{26}O_2$	214	0.63
30	16.76	-	$C_{14}H_{28}N_2O_2$	256	0.78
31	17.0 4	Proline cyclic peptide	$C_{11}H_{18}N_2O_2$	210	4.64
32	17.2 1	Cyclic (L-prolyl-L-leucyl)	$C_{11}H_{18}N_2O_2$	210	11.55
33	17.3 6	-	$C_{10}H_{21}BO_2$	184	2.08
34	17.45	-	$C_{18}H_{25}CLN_2O$	320	1.18
35	17.8 9	Isooctyl acrylate	$C_{11}H_{20}N_{20}$	196	0.29
36	17.9 1	Propetamide	$C_{14}H_{22}N_2O_2$	250	0.39
37	18.20	Diisohexyl adipate	$C_{22}H_{42}O_4$	370	0.74
38	18.29	N-tetradecane	$C_{34}H_{70}$	186	0.21
39	19.5 1	L-nitroarginine	$C_6H_{13}N_5O_4$	219	0.63
40	18.9 1	Stearamide	$C_{18}H_{37}NO$	283	0.43
41	18.95	Oleamide	$C_{18}H_{35}NO$	281	1.39
42	19.84	2,5-Piperazinedione	$C_{12}H_{22}N_2O_2$	226	12.35
43	19.96	L-leucyl-D-leucine, dihydrate	$C_{12}H_{24}N_2O_3$	226	1.39
44	20.18	Dioctyl phthalate	$C_{24}H_{38}O_4$	390	4.43
45	20.6 9	Benzyl piperazine dione-2,5-3-	$C_{11}H_{12}N_2O_2$	204	1.61
46	21.1 4	Cyclic (L-leucyl-L-phenylalanyl)	$C_{15}H_{20}N_2O_2$	260	0.45
47	21.3 1	N-ethyl-N-3-((3-dimethylamino-1-oxo-2-propenyl) phenyl)acetamide	$C_{15}H_{20}N_2O_2$	260	0.17
48	21.43	Squalene	$C_{30}H_{50}$	410	0.18
49	22.11	Dihydroergotamine	$C_{33}H_{37}N_5O_5$	583	0.86
50	22.49	Dihydroergotine	$C_{35}H_{41}N_5O_5$	611	5.07

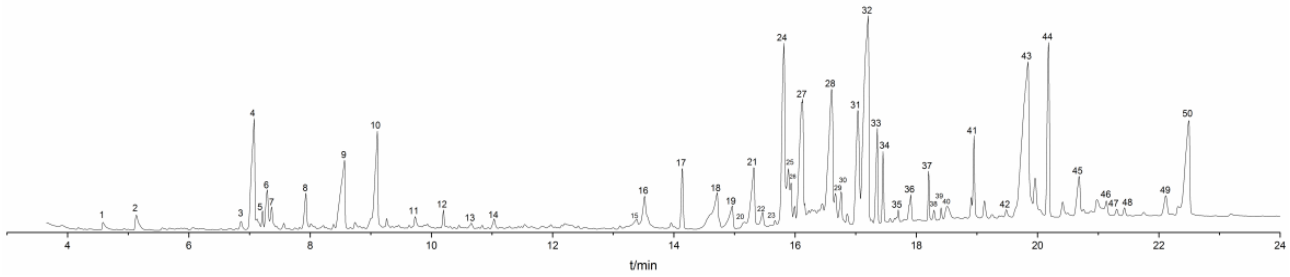

Fig. 5: Total ionization chromatogram of ethyl acetate extraction from strain ZY86

Table 3: Inhibition of *Botrytis cinerea* by the active components from antifungal strain ZY86

Treatment (μg mL^{-1})	Colony average diameter (cm)	Inhibition (%)
30	2.50 ± 0.10^{b}	41.86 ± 2.87^{e}
60	1.93 ± 0.15^{c}	58.33 ± 2.60^{d}
120	1.53 ± 0.06^{d}	69.96 ± 0.75^{c}
240	1.17 ± 0.06^{e}	80.62 ± 1.12^{b}
480	0.79 ± 0.01^{f}	91.57 ± 0.57^{a}
CK	3.94 ± 0.15^{a}	

CK is the control. Value ± SD followed by different letters indicate significantly different scores in the same phase according to Duncan's multiple range tests at a $P < 0.05$ significance level

Table 4: Control effects of the active components from antifungal strain ZY86 against strawberry gray mold (fruit)

Concentration (μg mL^{-1})	Lesion average diameter (cm)	Inhibition (%)
30	3.30 ± 0.08^{b}	25.07 ± 3.12^{e}
60	3.12 ± 0.01^{c}	30.35 ± 0.77^{d}
120	2.50 ± 0.08^{d}	48.13 ± 3.68^{c}
240	1.90 ± 0.08^{e}	65.42 ± 3.42^{b}
480	1.25 ± 0.04^{f}	84.14 ± 1.22^{a}
CK	4.17 ± 0.05^{a}	

CK is the control. Value ± SD followed by different letters indicate significantly different scores in the same phase according to Duncan's multiple range tests at the $P < 0.05$ significance level

Fig. 6: Control effects of the active substance from antifungal strain ZY86 against strawberry gray mold (fruit).
Note: A: CK; B: 30 μg mL^{-1}; C:60 μg mL^{-1}; D: 120 μg mL^{-1}; E: 240 μg mL^{-1}; and F: 480 μg mL^{-1}

Using GC-MS analysis, the present study has determined that the antifungal compounds produced by *A. johnsonii* ZY86 are primarily amines, ketones, and acids, which agree with the findings of previous studies. Li and Shao (2008) showed that *Acinetobacter* can produce emulsifiers, of which the primary components are active peptides and glycosyl esters. Navonvenezia *et al*. (1995) found that *A. radioresistens* KA53 can produce bioemulsifiers, although they did not identify its active components. Here, this study characterized the active components of *Acinetobacter*. However, regarding various questions, *e.g*., the identity of the specific compounds, their molecular weights and structural formulas, and whether these are novel or reported compounds, require confirmation through the isolation and purification of pure compounds. Their structural elucidation should be performed through various spectral techniques to account for their mass spectrum, hydrogen spectrum, and carbon spectrum. This research aimed to identify major compounds with novel structures.

Further investigation using an *in vitro* assay indicated that the control effect of the antifungal compounds from strain ZY86 was 84.14%, which is higher than that of procymidone (Zhang *et al*., 2015c) and that of boscalid (Qiu *et al*., 2018) on strawberry gray mold. Due to the ease in the preparation of active compounds as well as its low cost, safety, environmental friendliness, and good disease control effectiveness, strain ZY86 has broad application prospects.

Moreover, although this study has made some discoveries, additional follow-up investigations are still needed. This study confirmed the antifungal activity of the compounds produced by strain ZY86 against *B. cinerea*, but the genes and the biosynthetic pathway of these compounds remain unclear. Therefore, to improve the yield of the antifungal compound, it is necessary to apply genomics technologies to clarify the biosynthetic genes and regulatory pathway of the active components, which would be very helpful in obtaining a high-yield strain. Certainly, the fermentation of strain ZY86, e.g., conditions, temperature, light, dissolved oxygen, pH, shaking speed, and fermentation volume, should also be established in future investigations, and the response surface method can be adopted to optimize the fermentation parameters to increase the active components yields of strain ZY86.

Currently, the method for preventing disease through biocontrol bacteria primarily employs living preparations or its metabolites. The use of living preparations to prevent diseases is largely influenced by the environment. Bacterial viability, safety, and colonization determine disease prevention outcomes. *A. johnsonii* was found to be a conditional pathogen, and it can cause disease in fish and thus poses safety risks. This study did not evaluate the safety of strain ZY86 *in vivo*, and thus its safety to humans and animals requires investigation. Therefore, it is recommended that future efforts in developing biological pesticides should first investigate its active metabolites and explore major compounds with novel structures.

Conclusion

This study confirmed the antagonistic effect of strain ZY86, identified as *A. johnsonii*, against *B. cinerea*, and the strain had abroad spectrum of activity in antifungal. 50 actifungal components of strain ZY86 against *B. cinerea* had been identified, and the main constituents were amines, ketones,

acids, esters, alcohols, aldehydes, alkanes, alkenes, and hydrocarbons. *In vitro* experimentation revealed that the control effect of strain ZY8 against strawberry gray mold was 84.14%. Therefore, strain ZY86 may be potentially utilized as a biological agent for agricultural production.

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