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Thesis

BIOCONTROL MECHANISMS OF *STREPTOMYCES* SP. HU2014 AGAINST *RHIZOCTONIA* SPP. CAUSED WHEAT SHARP EYESPOT

Specialty 202 "Plant protection and quarantine".

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The dissertation contains the results of own research. The use of ideas, results and texts of other authors are linked to the corresponding source.

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ANNOTATION

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Wheat is the important source of global food and its yield is affected by several main factors, such as diseases, pests, weeds and climates. Wheat sharp eyespot (WSE) is a serious disease caused by the phytopathogens *Rhizoctonia cerealis* and *R. solani*. *R. cerealis* has a higher pathogenicity than *R. solani* and is the main pathogen of WSE in China. Currently, the principal method of controlling this disease is by the use of synthetic chemical fungicides. In order to develop sustainable agriculture and protect the ecological environment, biocontrol approach is a possible alternative, which uses beneficial microorganisms to control pathogens and improve plant health. *Streptomyces* spp. belong to these microbes and are researched in the plant protection field. Some species in the genus *Streptomyces* have been identified as potential biocontrol agents against phytopathogens. However, reports on using *Streptomyces* spp. against *R. cerealis* are lacking. A new strain of *Streptomyces* sp. HU2014 was investigated to gain insight into the determinants of its utility as a potential biocontrol agent. The main directions of this study were to evaluate the antifungal mechanisms of HU2014 against WSE and the ability to promote plant growth.

The dissertation work provides a theoretical foundation and practical solution for screening biocontrol in agriculture.

Previous work showed that the strain HU2014 had strong antifungal activities against phytopathogens. To further study this strain, Genome sequencing and average nucleotide identity (ANI) calculation of it were established using the Oxford Nanopore Technologies standard protocol and the J Species WS Online Service. The genome size of HU2014 was 8,170,612 bp and the values of ANIb and ANIm were 93.30% and 94.01% respectively. The result indicated that HU2014 is a novel *Streptomyces* species most closely related to *Streptomyces albireticuli*. The complete genome sequence of

HU2014 has been deposited in GenBank under the accession number CP097123.

The antifungal activities of HU2014 and its four fractions (F2, F4, F6 and F8) against *R. cerealis* G11 and *R. solani* YL-3 were conducted in the co-culture trial. The inhibition of mycelial growth by HU2014 against *R. solani* YL-3 and *R. cerealis* G11 were 90.3% and 63.9%, respectively. The aerial hyphae of the two phytopathogenic fungi was hollow, and the cell wall of them was damaged for observation under a scanning electron microscope. F6 and F8 fractions had the maximum inhibition rates of 100% to *R. solani* YL-3 and *R. cerealis* G11, followed by F4 and F2 (less than 60.50%). Spectrophotometric analysis was found that F6 and F8 had the same strong characteristic absorption peaks at 319 nm, 333 nm, and 351 nm. The main active components in the filtrated supernatants were determined to enrich in F6 and F8.

Some active metabolites produced by HU2014 have bacteriostatic effect on pathogens. Ultra-performance liquid chromatography-mass spectrometry on the four extracts from the extracellular filtrate (EF) of HU2014 identified 10 chemical constituents in the Natural Products Atlas with high match levels (more than 90%).

At the beginning of the study of the induce resistance of wheat to phytopathogens, the soil in pots for growing Bainong 4199 wheat was treated with different concentrations of extracellular extract (EF) HU2014 without fungi. Protective reactions of plants were noted by changes in the activity of enzymes. The activity of peroxidase (POD) and phenylalanine ammonia lyase (PAL) could be significantly induced by EF treatment at low concentrations, while the activity of polyphenol oxidase (PPO) had no significant difference compared to the control.

A continuation of the induce resistance study of wheat supplemented with *R. cerealis* G11 showed that POD, PAL and β-1,3-glucanase (GLU) activity in wheat leaves with three different treatments were significantly increased compared to the untreated control at different time points. The induce resistance in wheat varieties Aikang 58 and Bainong 307 was mainly activated by diluted EF, and in Zhoumai 22 by *R. cerealis* G11.

Concurrently, six specific gene expression analyses were performed for Aikang 58, Bainong 307, and Zhoumai 22 at each sampling hour post-inoculation with 500-

fold diluted EF of HU2014 using QRT-PCR. The expression of PR1, PR2, PR3, PR5, PAL, and LOX were markedly induced.

The strain HU2014 had an allelopathic effect on wheat and green bristlegrass. Four fractions in three concentrations were investigated. Seed treatment had a negative allelopathic effect on seedling height and root length of the two tested plants, except for F2 at a concentration of 1 mg/ml, which increased wheat seedling height.

Promoting plant-growth has indirect effect on controlling wheat sharp eyespot. The test of promoting plant growth was established in vitro and pots. The results demonstrated that HU2014 can produce indoleacetic acid, siderophores, extracellular enzymes, and solubilized phosphate, and it can promote plant growth. The shoot height increased significantly by 12.9% to 22.4% in comparison to the non-inoculated control in the three cultivars at 28 days post-inoculation. Similarly, HU2014 was able to improve shoot dry weight in comparison to the non-inoculated control. The chlorophyll content increased by 27.3% to 87.1% compared with the non-inoculated control. The root dry weight increased significantly by 45.5% to 83.4% in comparison to the non-inoculated control at 60 days post-inoculation.

The colonization of an exogenous microorganism and the synergistic effect of it with other rhizosphere microbes are important, which affect the antibacterial and promoting growth functions of this microorganism. The effect of HU2014 inoculation on soil and native soil microorganisms was investigated. From the results, we found that HU2014 increased the malondialdehyde content of wheat leave, while decreased catalase activity, peroxidase activity. With HU2014 inoculation, the concentrations of total nitrogen, nitrate nitrogen, total phosphorus, and Olsen-phosphorus in the wheat rhizosphere increased. The diversity of rhizosphere bacteria, but not fungi clearly decreased by HU2014 inoculation. The compositions of bacterial and fungal community differed after HU2014 inoculation with Proteobacteria, Acidobacteriota, and Ascomycota being the dominant phyla in all treatments.

In order to optimize the environmental and nutritional parameters for improving the production of antifungal components by HU2014 through statistical approaches, five factors of fermentation condition, carbon and nitrogen sources, and inorganic salt

were investigated. The optimized fermentation conditions and medium components with shake-flask were as follows: incubation time, 10 days; temperature, 25°C; agitation speed, 150 rpm; inoculum size, 5% (v/v); initial pH, 7.0; dextrin 39.59 g/L, yeast extract 8.52 g/L, and KNO3 2.49 g/L.

At last, we investigated the biocontrol ability of HU2014 against WSE in field, the physiological parameters, as well as characteristic traits in promoting growth were assessed. In 2022 and 2023, the most promoting growth indicators after HU2014 inoculation showed increasing, but the amplification of them in field was less than in laboratory conditions.

Key words: *Wheat, winter wheat, Streptomyces, plant protection, biocontrol, phytopathogens, diseases, active secondary metabolites, genotype, plant growth-promoting bacteria, plant development, biometric indicators, pre-sowing treatment of seeds, induced enzyme activity, microbial diversity*

АНОТАЦІЯ

Чжу Хунся. Механізми біоконтролю *Streptomyces* sp. HU2014 проти Rhizoctonia spp., збудників ризоктоніозної гнилі пшениці. – Рукопис дисертації на здобуття наукового ступеня доктора філософії (PhD): спеціальність 202 «Захист і карантин рослин». – Сумський національний аграрний університет, Суми, 2023

Пшениця є важливим джерелом глобального продовольства, і на її врожайність впливають кілька основних факторів, таких як хвороби, шкідники, бур'яни та клімат. Ризоктоніоз пшениці - небезпечна хвороба, яку викликають фітопатогени - *Rhizoctonia cerealis* та *R. solani*. *R. cerealis* має вищій рівень патогенності, ніж *R. solani*, і він є основним збудником ризоктоніозу в Китаї. На сьогодні основним методом боротьби з цією хворобою є використання синтетичних хімічних фунгіцидів. Для розвитку сталого сільського господарства та захисту довкілля можливою альтернативою є біоконтроль, який використовує корисні мікроорганізми для боротьби з патогенами та покращення здоров'я рослин. *Streptomyces* spp. належать до них і досліджуються в галузі захисту рослин. Деякі види з роду *Streptomyces* були ідентифіковані як потенційні агенти біоконтролю проти фітопатогенів. Однак дані про використання *Streptomyces* spp. проти *R. cerealis* відсутні. Було досліджено новий штам *Streptomyces* sp. HU2014, щоб отримати уявлення про детермінанти його корисної дії як потенціального агента біоконтролю. Основними напрямками цього дослідження було оцінити протигрибні механізми HU2014 проти збудників ризоктоніозу та здатність сприяти росту рослин.

У дисертаційній роботі подано теоретичні основи та практичне рішення скринінгового біоконтролю в сільському господарстві.

Попередня робота показала, що штам HU2014 мав сильну протигрибну дію проти фітопатогенів. Для подальшого вивчення цього штаму було проведено секвенування генома та розрахунок середньої нуклеотидної ідентичності (ANI) за допомогою стандартного протоколу Oxford Nanopore Technologies та онлайн-сервісу J Species WS. Розмір генома HU2014 становив 8 170 612 bp, а значення

ANIb і ANIm становили 93,30% і 94,01%, відповідно. Результат показав, що HU2014 є новим видом *Streptomyces*, найбільш близьким до *Streptomyces albireticuli*. Повна послідовність геному HU2014 була депонована в GenBank під номером доступу CP097123.

Протигрибну активність HU2014 та його чотирьох фракцій (F2, F4, F6 та F8) проти *R. cerealis* G11 та *R. solani* YL-3 провели за дослідження їх спільного культивування. Штам HU2014 інгібував ріст міцелію *R. solani* YL-3 і *R. cerealis* G11 на 90,3% і 63,9%, відповідно. Надземні гіфи двох фітопатогенних грибів були порожнистими, а клітинна стінка у них була пошкоджена за спостереження під скануючим електронним мікроскопом. Фракції F6 і F8 мали максимальні показники інгібування - 100% для *R. solani* YL-3 і *R. cerealis* G11, за якими йшли F4 і F2 (менше 60,50%). Спектрофотометричний аналіз виявив, що F6 і F8 мали однакові сильні характерні піки поглинання при 319 нм, 333 нм і 351 нм. Було визначено, що основні активні компоненти у відфільтрованих супернатантах містили F6 і F8.

Деякі активні метаболіти, які продукує HU2014, мають бактеріостатичну дію на патогени. Ультраефективна рідинна хромато-мас-спектрометрія чотирьох екстрактів із позаклітинного фільтрату (EF) HU2014 виявила 10 хімічних компонентів в Атласі натуральних продуктів із високим рівнем відповідності (понад 90%).

На початку вивчення індукованої стійкості пшениці до фітопатогенів ґрунт у горщиках за вирощування пшениці сорту Bainong 4199 обробили різними концентраціями позаклітинного екстракту (EF) HU2014 без грибів. Захисні реакції рослин відмітили за змінами активності ферментів. Активність пероксидази (POD) і фенілаланін-аммоній-ліази (PAL) за обробки EF у низьких концентраціях може бути істотно індукована, тоді як активність поліфенолоксидази (PPO) не мала істотної різниці в порівнянні з контролем.

Продовження вивчення індукованої стійкості пшениці з додатковим внесенням *R. cerealis* G11 показало, що активність POD, PAL та β-1,3-глюканази (GLU) у листі пшениці з трьома різними обробками значно зросла порівняно з

необробленим контролем у різні моменти часу. Індукована стійкість у сортів пшениці Aikang 58 і Bainong 307 була в основному активована розведеним EF, а у сорту Zhoumai 22 - R. cerealis G11.

Одночасно було проведено шість специфічних аналізів експресії генів для Aikang 58, Bainong 307 і Zhoumai 22 через кожну годину відбору проб після інокуляції 500-кратним розведенням EF HU2014 за допомогою QRT-PCR. Експресія *PR1*, *PR2*, *PR3, PR5, PAL* і *LOX* була помітно індукована.

Штам HU2014 мав алелопатичний вплив на пшеницю та мишій зелений. Було досліджено чотири фракції у трьох концентраціях. Обробка насіння мала негативний алелопатичний вплив на висоту проростків і довжину коренів двох досліджуваних рослин, за винятком F2 у концентрації 1 мг/мл, яка збільшила висоту проростків пшениці.

Стимулювання росту рослин має непрямий вплив на боротьбу з ризоктоніозом пшениці. Дослідження стимуляції росту рослин було проведено in vitro та в горщиках. Результати показали, що HU2014 може продукувати індолоцтову кислоту, сидерофори, позаклітинні ферменти та солюбілізований фосфат, а також може сприяти росту рослин. Висота пагонів значно зросла на 12,9% - 22,4% порівняно з контролем у трьох сортів через 28 днів після інокуляції. HU2014 зміг покращити суху вагу пагонів. Вміст хлорофілу збільшився на 27,3% - 87,1% порівняно з необробленим контролем. Суха вага коренів значно зросла на 45,5% - 83,4% порівняно з контролем через 60 днів після інокуляції.

Важливе значення має колонізація екзогенного мікроорганізму та його синергічний ефект з іншими мікробами ризосфери, що впливає на його антибактеріальну та стимулюючу функції. Дослідили вплив інокуляції HU2014 на ґрунт та його мікроорганізми. Було встановлено, що HU2014 збільшив вміст малонового діальдегіду в листках пшениці, а також знизив активність каталази та пероксидази. За інокуляції HU2014 концентрації загального азоту, нітратного азоту, загального фосфору та Olsen P у ризосфері пшениці зросли. Різноманітність ризосферних бактерій, але не грибів, явно зменшилась після інокуляції HU2014. Склад бактеріального та грибного співтовариства змінився з

домінуванням Proteobacteria, Acidobacteriota та Ascomycota в усіх обробках.

З метою оптимізації екзогенних та поживних параметрів середовища для покращення виробництва протигрибних компонентів з HU2014 за допомогою статистичних підходів було досліджено п’ять факторів умов ферментації, джерел вуглецю та азоту та неорганічної солі. Оптимізовані умови ферментації та компоненти середовища зі струшуванням колби були такими: час інкубації 10 днів; температура 25°C; швидкість перемішування 150 об/хв; об’єм інокулята, 5%; початковий pH 7,0; декстрин 39,59 г/л, дріжджовий екстракт 8,52 г/л і KNO3 2,49 г/л.

Нарешті, було досліджено здатність HU2014 до біоконтролю ризоктоніозу в польових умовах, де оцінили фізіологічні параметри, а також характерні ознаки сприяння росту. У 2022 та 2023 роках найефективніші ростові індикатори після інокуляції HU2014 показали зростання, але їх підвищення в полі було меншим, ніж у лабораторних умовах.

Ключові слова: *пшениця, пшениця озима, Streptomyces, захист рослин, біоконтроль, фітопатогени, хвороби, активні вторинні метаболіти, генотип, ростостимулюючі бактерії, розвиток рослин, біометричні показники, передпосівна обробка насіння, індукована активність ферментів, мікробне різноманіття*

LIST OF PUBLISHED WORKS ON THE TOPIC OF THE DISSERTATION

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CONTENT

ABBREVIATIONS

WSE： wheat sharp eyespot

PGPM: plant growth promoting microorganisms

BCA: biological control agents

ISR: systemic disease resistance

SAR: systemic acquired resistance

RSM: response surface methodology

AK58: aikang 58

BN307: bainong 307

ZM22: zhoumai 22

HIST: Henan Institute of Science and Technology

GPY: glucose, soybean peptone, and yeast extract

POD: peroxidase

PPO: polyphenol oxidase

PAL: phenylalanine ammonia lyase

GLU1: β-1, 3-Glucanase

MDA: malondialdehyde

SEM: scanning electron microscope

PCR: polymerase chain reaction

QRT-PCR: quantitative real-time PCR

ANI: average nucleotide identity

UPLC-MS: ultra-performance liquid chromatography-mass spectrometry

EF: extracellular filtrate

F3: the pots treated with the 1000-fold diluted EF

PF3: the pots infected *R. cerealis* G11 24 hours post F3 treatment

CKP: the pots infected *R. cerealis* G11 singly

CK: without other treatment

PDA: potato Dextrose Agar

NBRIP: National Botanical Research Institute's Phosphate

ICP-OES: inductive coupled plasma emission spectrometer

IAA: indoleacetic acid

dpi: days post-inoculation

F2: fraction of the filtered supernatant of HU2014 culture using 20% ethanol extracted

F4: fraction of the filtered supernatant of HU2014 culture using 40% ethanol extracted

F6: fraction of the filtered supernatant of HU2014 culture using 60% ethanol extracted

F8: fraction of the filtered supernatant of HU2014 culture using 80% ethanol extracted

RI: response index

S: HU2014 inoculation with the concentration of 4 g/L soil

SOC: soil organic carbon

TN: soil total nitrogen

NO3-N: nitrate nitrogen

NH4+-N: ammonium nitrogen

TP: total phosphorus

Olsen-P: olsen phosphorus

ISP: international streptomyces project

GLU2: glucose

RAF: raffinose

STA: starch

MAL: maltose

GLY: glycerol

DEX: dextrin

PE: potato extract

BE: beef extract

YE: yeast extract

SP: soybean peptone

ME: malt extract

OE: oat extract

FCCD: face-centered central composite design

ANOVA: analysis of variance

RT: retention time

LSD: least significant difference

PR1: pathogenesis-related protein 1

PR2: beta-1,3-glucanase

PR3: chitinase

PR5: thaumatin-like protein

LOX: lipoxygenase

SD: standard deviation

P: the soil with R. cerealis G11 infection

SP: the soil with HU2014 and *R. cerealis* G11 treatments

NMDS: Non-metric multidimensional scaling

PCA: principal component analysis

RDA: redundancy analysis

INTRODUCTION

Bacteria, actinomycetes, algae and fungi are ubiquitous microorganisms in soil. They can promote the nutrient absorption of the host and improve the host's resistance to abiotic stress. As an important host of microorganisms, plants have learned how to use microorganisms to protect themselves from diseases in the long-term coexistence relationship. Wheat sharp eyespot (WSE) is a soil-borne disease that affects almost all temperate wheat-growing regions worldwide. In China, it is common in several main provinces for wheat production, such as Jiangsu, Anhui, Henan, Shandong, Shaanxi and Hubei, with the wheat losses from 10% to 40% totally.

This disease is caused primarily by the soil-borne fungus *Rhizoctonia cerealis* Van der Hoeven and *R. solani* Kuhn. *R. cerealis* anastomosis group CAG-1 and *R. solani* anastomosis group AG- 5 are the main causes of cereal root disease in China.

The current main control against WSE is still chemical fertilizers and pesticides with the advantages of economy and high-efficiency. However, these methods have negative consequences, including environmental pollution and the resistance of virulent strains to chemical treatments. Biocontrol bacteria are considered as a potential method in agriculture production, which plays an important role in ecological balance. *Streptomyces* spp. produce active metabolites that inhibit phytopathogen growth, such as *Rhizoctonia* spp., *Botrytis cinerea* and *Fusarium culmorum*, and promoting plant growth.

Actuality of theme. Wheat sharp eyespot (WSE) is a serious disease caused by the phytopathogens *R. cerealis* and *R. solani*. The pathogenicity of *R. cerealis* was significantly higher than that of *R. solani* and it is a main pathogen in WSE in China. Currently, the principal method of controlling this disease is by the use of synthetic chemical fungicides. In order to develop sustainable agriculture and protect the ecological environment, biocontrol approach is a possible alternative, which uses beneficial microorganisms to control pathogens and improve plant health. Some species in the genus *Streptomyces* have been identified as potential biocontrol agents against phytopathogens. However, reports on using *Streptomyces* spp. against *R. cerealis* are lacking. Therefore, the study of biocontrol mechanisms of *Streptomyces* sp.

HU2014 against *Rhizoctonia* sp. is relevant (Law et al., 2017).

Connection of work with scientific programs, plans, themes. The research was carried out in accordance with the thematic plans of research works of the department of plant protection of the Sumy National Agrarian University and within the framework of the topics "Optimization of fermentation conditions of *Streptomyces* sp. HU2014 and study on antibacterial active substances" (the Key Science and Technology Program of Henan Province, China. Grant Number 162102210106), "Study on the active components and antibacterial lead compounds from the extracts of Carpesium abrotanoides L. (Asteraceae) against phytopathogenic fungi" (the Key Science and Technology Program of Henan Province, China. Grant Number 212102110148), and "Study and application of a beneficial *Streptomyces* strain in disease prevention and growth promotion of wheat" (the Key Science and Technology Program of Henan Province, China. Grant number 232102111015).

The purpose and objectives of the study. To get insight into the determinants of the beneficial activity of *Streptomyces* sp. HU2014 and explore its potential as a biocontrol agent, the main objectives of this study were to assess its antifungal activities and ability to promote plant growth.

The goal was to solve the following tasks:

1. To identify HU2014 strain with its whole genome sequencing.
2. To study the antifungal mechanism of HU2014 against WSE pathogen.
3. To separate the active compounds produced by HU2014.
4. To determine plant-growth promotion of HU2014 in wheat.
5. To investigate the allelopathy of HU2014 on wheat.
6. To explore the effects of HU2014 inoculation on soil factors and rhizosphere microbial structure.
7. To screen the fermentation conditions of HU2014.
8. To verify the biocontrol effect of HU2014 in field.

Object of study. Investigation the potential of HU2014 as a biocontrol agent with its antifungal activities and ability to promote plant growth.

Subject of study. Rhizoctonia cerealis Van der Hoeven and *R. solani*,

Streptomyces sp. HU2014, active secondary metabolites produced by HU2014, wheat.

Research methods. General scientific methods: analysis, induction, deduction, synthesis; field methods - phenological observations of the collection of winter wheat varieties and biometric parameters of plant growth and development, in particular measuring, height, weight and control effectivity analysis; statistical methods - to generalize and determine the reliability of certain experimental results (variation, correlation, dispersion, cluster, factual).

The scientific novelty of the obtained results is the isolation of a new strain of *Streptomyces* sp. HU2014, which will help to effectively regulate soil pathogens of wheat rhizoctoniosis.

On the basis of analytical and experimental research, the work for the first time:

- a new strain of *Streptomyces* sp. HU2014 was isolated and identified;
- effective antifungal effect of strain HU2014 against pathogens of wheat rhizoctoniosis has been proven;
- the mechanisms of induced resistance of wheat by treating *Streptomyces* sp. plants were studied. HU2014
- proved that *Streptomyces* sp. HU2014 promotes the growth of wheat by stimulating growth processes, improving soil nutrition of plants by changing the microbial structure of the rhizosphere;
- optimal cultivation conditions of HU2014 were determined.

The improvement of biological protection of wheat by using a new strain of *Streptomyces* sp. HU2014, which will expand the range of existing bioagents.

The practical significance of the results. Research results show that a new strain HU2014 has been identified, which has proven to be effective in protecting wheat against Rhizoctonia spp. and can be used for the production of new biofungicides. Also, the determination of ten biologically active metabolites in the extracellular filtrate of strain HU2014 will be useful for the production of biofungicides. The studied strain of *Streptomyces* sp. HU2014 showed a phytotoxic effect on seed germination by the green mouse, so it is promising in the fight against segetal vegetation. The stimulating effect of the new strain on the growth and development of wheat plants due to the

improvement of soil nutrition and plant metabolism has been proven. The obtained results are included in the training programs of bachelors in the specialty 202 "Protection and quarantine of plants" at the Sumy National Agrarian University.

The personal contribution of the applicant is to plan and conduct research, summarize scientific data of references (literature) on the topic of the dissertation, analysis of experimental data, format conclusions and recommendations for selection, and write of scientific papers. Scientific articles have been published both independently and in co – authorship as well.

Approbation of dissertation results. The results of the research were published and discussed at “International science and practice conference of the Faculty of Plant Protection of the KhNAU named after V.V. Dokuchaeva” (Kharkiv, 2019), “International scientific and practical conference of plant protection of KhNAU named after V.V. Dokuchaeva, dedicated to the 130th anniversary of the birth of academician VASGNIL, corresponding member of NASU, doctor of biological sciences, professor and first dean of the faculty T.D. Strahov” (Khnaiv, 2020), “International Scientific and Practical Conference dedicated to the 100th anniversary of the birth of doctor of biological sciences, professor B.M. Lytvynov” (Kharkiv, 2021), The 3rd International scientific and practical conference (Kharkiv, 2021), The All-Ukrainian scientific and practical conference (Zhytomyr, 2021), International Scientific and Practical Conference dedicated to the 93rd anniversary of the birth of the Doctor of Agricultural Sciences, Professor Mykola Goncharov (Sumy, 2022).

Publications. Based on the results of the research, six articles were published in professional journals and seven papers were exhibited in conferences as the first author.

The structure and scope of the dissertation. The dissertation structure contains an annotation, a list of abbreviations, introduction, six chapters, conclusions, a list of references, appendixes. In this dissertation, it includes twenty one tables and twenty six figures.

CHAPTER 1
PATHOGENESIS AND CONTROL PROGRESS OF WSE (LITERATURE REVIEW)

Wheat (*Triticum aestivum* L.) is an important table crop with the widest distribution, the highest planting area, yield and trade volume in the world (Balkovič et al., 2014; Wei et al., 2016). With the development of wheat production and the expansion of planting area, the harm of wheat diseases is becoming more and more prominent. WSE is not only a soil-borne disease, but also a worldwide disease, which is almost all over the temperate wheat planting areas in the world (Lemańczyk & Kwaśna, 2013). As early as 1934, WSE was reported abroad. Since the middle and late 1970s, with the replacement of wheat varieties and the promotion of high-yield cultivation measures, the disease has generally occurred and has become one of the main diseases in winter wheat areas in China (Hamada M. S. et al., 2011). Sharp eyespot was mainly caused by the soil-borne fungus *Rhizoctonia cerealis* van der Hoeven (Van der Hoeven & Bollen, 1980) and *Rhizoctonia solani* Kuhn (Cubeta & Vilgalys, 1997). The pathogenicity of R. cerealis is significantly higher than that of *R. solani* in WSE disease. This disease mainly affects the stem of wheat in seedling and booting stage, causing water-loss around the infection point and further reducing the yield and quality.

1.1. Pathological study on WSE

1.1.1. Symptoms and pathogen of WSE

WSE has different symptoms in different growth stages of wheat. From seed germination to seedling emergence, once wheat is infected by sharp eyespot, the young bud sheath turns brown and rots; After emergence, light brown spots appear in the leaf sheath in the soil, gradually expand, and then penetrate into the plexus in the leaf sheath, causing yellow and dead seedlings; Green returning stage later, disease spots on the base leaf sheath of stem begin to develop vertically and horizontally, and flower rod-shaped disease spots like brown moire is formed. With the invasion of pathogens, the disease spots gradually expand; Subsequently, the pathogen invade the stem with gray

brown in the middle and brown or oval around, resulting in water loss, decay and necrosis of the stem wall; Finally, the diseased plants appear withered white ears due to the shortage of nutrients and water.

The main pathogen causing WSE is *Ceratobasidium cornigerum (*Bourd.) Rogers which belong to basidiomycetes. *Rhizoctonia cerealis* van der Hoeven called *R. cerealis* and *R. solani* Kühn called *R. solani* are the genus *Rhizoctonia* (Sneh et al., 1991). *Rhizoctonia* (*Ceratobasidium* spp.) is a kind of fungus widely existing in nature (Vilgalys & Cubeta, 1994; Rashed et al., 2021) and can be easily isolated from infected plants and soil. Many plant diseases are caused by this kind of fungus with its very wide range of host. They mainly cause plant seedling collapse and sheath blight of cereal crops (Rush & Lee, 1983; Nicholson & Parry, 1996; Nerey et al., 2010; Hanson & McGrath, 2011; Sun et al., 2015; Shi et al., 2021). Therefore, this kind of fungus has attracted the interest of many plant pathologists and fungal taxonomists (Vilgalys & Cubeta, 1994; Ohkura et al., 2009; Li Y. Q. et al., 2011; Rashed et al., 2021). Early studies suggested that the pathogen causing WSE was *R. solani* (Pitt, 1964; Clarkson & Cook, 1983). Since Parmeter put forward the theory of distinguishing *Rhizoctonia* according to mycelial fusion in 1969, great progress has been made in the identification of WSE. The main pathogens of WSE are *R. cerealis* and *R. solani* (Hamada Mohamed Sobhy et al., 2011)，both strains have their own hyphal fusion groups. Many Chinese researchers believe that *R. cerealis* CAG-1 anastomosis group and *R. solani* anastomosis group AG- 5 are the main cause of cereal root disease (Chen et al., 1986; Xia & Li, 1989; Pan & Wu, 1992). The pathogenicity of *R. cerealis* was significantly higher than that of *R. solani* and it is a main pathogen in WSE in China (Hamada Mohamed Sobhy et al., 2011; Li Z. et al., 2011; Rong et al., 2016; Lu et al., 2020; Liu C. Y. et al., 2021). Domestic and foreign scholars have done a lot of research on the pathogenicity of different anastomosis groups and different strains of the same anastomosis group (Ogoshi, 1987; Naito & Kanematsu, 1994; Sharma-Poudyal et al., 2015; Melzer et al., 2016; Blanco et al., 2018; Kucharska et al., 2018; Zhang J. M. et al., 2020).

1.1.2. Pathogenic mechanism of WSE

Rhizoctonia infect initially the root of the plant and gradually leaf sheath or leave, which comply with the mode of penetrating claw infection. On the lower surface of the two infected structures, penetrating claws are formed at right angles to the leaf sheath, passing through the stratum corneum and cell wall, and entering the intercellular space and cells. Marshall & Rush (1980) found that the mycelia of this kind of pathogen first grew along the sheath vein, began to branch after a few hours, and then formed two infection structures on the surface of leaf sheath. Liu and Xiao (1999) believed that the pathogen could produce infected areas before penetrating the host. The mycelia at the base of the infected areas invade the host directly or through stomata. After the mycelia invade the host epidermis, they rapidly expand in a network, directly penetrate the adjacent cell wall and expand vertically and horizontally to other cells. This leads to the plasma membranes close to the mycelia separated and broken, chloroplasts deformed or nearly disappeared, thylakoids destroyed, and mitochondrial disintegration and other tissue lesions. It was reported that this kind of pathogen has evolved combinations of plant cell-wall-degrading enzymes to deconstruct host plant cell walls (Dou et al., 2021).

1.1.3. Pathogenesis and damage of WSE

WSE is a saprophytic soil-borne disease, which overwinters and summering in the soil with the mycelium on the sclerotia or diseased residue (Lemańczyk & Kwaśna, 2013). It can infect wheat shortly after wheat seeds sowing. The field occurrence process of WSE can be divided into five stages. That is, the onset period before winter, the rest period of overwintering, the recovery period of the disease (horizontal expansion period), the peak period of the disease (severity growth period), and the stable period of the disease (dry and white ear occurrence period). It is generally indicated that the disease forms two peaks in winter and jointing stage, and the incidence before winter is closely related to the dry and white ear rate in the later stage. Due to the influence of ecological factors, there are some differences in the length and peak value of each stage. The harm of sharp eyespot to wheat plants is not only caused

by rotten teeth, dead seedlings, dead tillers and withered white ears of wheat seeds, but also hindered the water and nutrient transport in the infected plant, resulting in plant lodging and yield reduction (Clarkson & Cook, 1983; Lemańczyk & Kwaśna, 2013).

The occurrence of WSE is affected by many environmental conditions. Temperature, water and fertilizer conditions, soil types and cultivation measures are the main factors. In the warm winter season, the growth and development of pathogenic bacteria in the soil will accelerate. With the increase of temperature in spring, bacteria in the soil accumulate, which can quickly and massively infect wheat seedlings. The amount of rain has a great influence on the incidence of WSE. When the soil moisture reaches humidity, the growth and development of the pathogen of WSE will accelerate and infect the plant more easily. The disease will be aggravated in the early sowing of field production. Excessive sowing amount of wheat leads to the increase of wheat population density. There are many weeds in the field, which will reduce the ventilation and light transmittance of the field and be conducive to the growth of pathogens.

The yield loss caused by sharp eyespot was generally 10-20%, and more than 70% in severe cases. The severity of yield loss was closely related to the onset time and location of wheat plant. The range of yield loss caused by different disease grades was 5-40%, and the difference between disease grades was significant. The biggest yield loss caused by sharp eyespot was at wheat booting stage. The decrease of 1000 grain weight is the primary factor of yield loss caused by sharp eyespot, followed by the decrease of grain number per panicle.

1.2. Control status of WSE

1.2.1. Current situation of WSE control

Cereals represent the main carbohydrate food source in the world. For millennia, wheat has played a major role in the development of healthy societies and has supported economic and social stability, which accounts for near 40% of the cereal supply worldwide (Charmet, 2011). Henan Province is a major wheat planting province in China, with a planting area of 5.69 million hm2 and an annual wheat output of more than 38 million tons. However, in many cases, productivity has been reduced by the

spread of soil diseases, soil degradation, and adverse environmental conditions (Lobell, 2010; Liu et al., 2011; Mavrodi et al., 2012; Barnett et al., 2017). Wheat productivity needs to be rapidly increased in order to cater to the demand of the burgeoning human populations expected during the coming decades (Fischer & Edmeades, 2010). With the deepening of China's supply structural reform, the "reduction and efficiency increase" of chemical fertilizers and pesticides is an important strategic deployment for the quality of agricultural products and the sustainable development of agriculture. The technology that combines ecological regulation, biological prevention and control with precision application will guarantee the green and high-quality development of agriculture in the future.

As a soil borne disease affecting all temperate wheat planting areas in the world, the incidence of WSE in Henan Province has shown an increasing trend year by year with the change of planting patterns. The average area affected by WSE has reached 2.8399 million hm2, which is the largest disease during wheat production in this province (Lemańczyk & Kwaśna, 2013; Zhang et al., 2022). In the field of wheat planting, the control of WSE mainly depends on chemical fungicides. At present, the fungicides used to control WSE in China are mainly triazole fungicides, jinggangmycin, thiafuramide and methyl ricofop (Li M. L. et al., 2020; Xiao X. et al., 2020; Yang et al., 2020). However, previous studies have shown that the resistance of the pathogen causing WSE to triadimefon and jinggangmycin has become prominent, as well as this kind of pathogen has cross resistance with frequently-used control agents. The risk of control failure due to drug resistance cannot be ignored (Xu et al., 2019; Yang et al., 2020).

In recent years, biological control has been paid more and more attention by plant protectionists. A variety of microbial agents (including pesticide, fungicide and herbicide) have been used in agricultural production. *Trichoderma harzianum* and fewer *Trichoderma viride* strains can control leaf rust (Omara et al., 2019). Gram-negative bacteria, such as *Pseudomonas* spp., *Agrobacterium radiobacter, Bacillus* spp. and *Erwinia* spp., *B. subtilis* shows good biocontrol effect on WSE (Alimi et al., 2012). Crop rotation can avoid the accumulation of pathogens in soil and have shown yield

advantages (Khakbazan et al., 2010; Taveira et al., 2020; Zhao et al., 2020). However, due to the limited plots available for rotation, the long survival time of pathogens in the soil, and the short rotation interval, it is difficult to obtain the ideal control effect. Therefore, crop rotation measures can only reduce the occurrence of the disease to a certain extent. Seed coating agent can keep stable effect and released continuously, which is safer for seeds and seedlings, and save chemical pesticides. However, the application of this agent breaks the ecological balance of soil microorganisms. Another side, due to the lack of wheat immune varieties resistant to WSE and the molecular mechanisms underlying wheat defense against this disease largely unknown, the research progress of conventional breeding is at a slow pace (Leng et al., 2011; Guo et al., 2021). Natural resistance of wheat plant to WSE is inadequate and new strategies are urgent desired to control this serious soil-borne disease (Ji et al., 2019).

1.2.2. Advances in biological control of WSE

Many microorganisms in nature can inhibit plant pathogens (Sellamani et al., 2016; Bunbury-Blanchette & Walker, 2019; Marimuthu et al., 2020). Developing and excavating beneficial microorganisms and their metabolites to control plant diseases, especially plant rhizosphere microorganisms with the properties of disease prevention and growth promotion, is the focus of research in recent years (Burketova et al., 2015; Omara et al., 2019; Zheng et al., 2019; Marimuthu et al., 2020). The research on biological control against WSE using rhizosphere bacteria started lately at home and abroad (Peng et al., 2014; Xu et al., 2020). For example, the culture broth of B. subtilis EBS05 can more effectively control WSE (Wen et al., 2011). Plant growth-promoting rhizobacterial strain S58 has good control effect on WSE (Gu et al., 2020). B. cereus 0-9, a Gram-positive endospore-forming bacterium isolated from healthy wheat roots, has biological control capacity against several soil-borne plant diseases of wheat such as WSE and take-all (Zhang J. M. et al., 2020).

1.3. Research progress of plant growth promoting microorganism (PGPM) and biological control agents (BCA)

1.3.1. Concept of PGPM and BCA

Plants are teeming with microorganisms, including those colonize internal tissues and adhere to external surfaces. The microbiome is relative to plant health and serves as a reservoir of additional genes for plants (Berendsen et al., 2012; Rout, 2014). The concept of plant rhizosphere was first proposed by German microbiologist Lorenz Hiltner in 1904 to describe the special relationship between legume roots and bacteria. In 1978, Kleopper first put forward the concept of plant growth promoting rhizobacteria, which is a group of bacteria colonizing the plant rhizosphere, being closely related to the plant roots, and promoting plant growth and increase crop yield. In fact, the rhizosphere is undoubtedly the most complex microhabitat, comprised of an integrated network of plant roots, soil, and a diverse consortium of bacteria, fungi, eukaryotes, and archaea (Hakim et al., 2021). Some researchers expand the concept of rhizosphere growth promoting bacteria. A considerable number of soil and rhizospheric microorganisms collectively known as plant growth promoting microorganisms (PGPM) have demonstrated ability to colonize plant roots and to provide benefits to their hosts (Groppa et al., 2012; Bartelme et al., 2018). Specifically, PGPM can change the condition of ineffective mineral elements, and secrete plant hormones to promote plant growth (Lugtenberg & Kamilova, 2009; Compant et al., 2010; Soumare et al., 2021). BCA are mainly used to resist harmful microorganisms and produce antibiotics against plant infection, thus reducing the invasion of plant pathogens (Bae et al., 2016; Niu et al., 2020). However, the functions of these two types of microorganisms are not independent. Many microorganisms have both growth promoting and antibacterial functions.

1.3.2. Research progress of PGPM and BCA

The biological control system of plant soil-borne diseases involves four factors: plants, microorganisms, pathogens and soil environment. In natural environments, plants are exposed to diverse microbiota and they interact in complex ways (Rodriguez

et al., 2019). The beneficial rhizosphere microorganisms can be divided into two groups according to their action mechanisms: PGPM (Bartelme et al., 2018) and biological control agents (BCA) (Wright & Bennett, 2018). They are very important for the normal growth of plants.

Studies have reported that microorganisms in plant rhizosphere possess their unique characteristics in species and function. Plants provide nutrients for root microbes through photosynthesis. Microorganisms can improve acquisition of nutrients, resilience against pathogens, and improve resistance against abiotic stress conditions such as heat, drought, and salinity (Rodriguez et al., 2019; Suarez-Moreno et al., 2019; Raymaekers et al., 2020). The core microbial communities in plant rhizosphere are mainly A*rthrobacter, Bacillus, Bradyrhizobium, Flavobacterium, Pseudomonas, Sphingomonas, Sporidium, Stenotrophomonas, Streptomyces* spp., mutant genus and followed by fungi at a small proportion rate (Jan et al., 2011; O'Brien, 2017; Newitt et al., 2019; Araujo et al., 2020). *B. subtilis* in rhizosphere can induce and improve the drought resistance of plant seedlings (Barnawal et al., 2013; Wang et al., 2019). *Burkholderia ambiaria* isolated from barley rhizosphere can use fusaric acid as the sole carbon source and energy source, and control plant Fusarium Wilt and root rot caused by *Fusarium* species (Simonetti et al., 2018). The growth of wheat seedlings under salt stress was significantly promoted by *Enterobacter* and *Bacillus* (Liu et al., 2015; Upadhyay & Singh, 2015; Sarkar et al., 2018). *Streptomyces*, belong to actinomycetes, has the function of the promoting growth and disease prevention and been widely studied and applied all over the world (Xue et al., 2016; Colombo et al., 2019; Zheng et al., 2019; Strub et al., 2021).

In agricultural production, plant disease biocontrol refers to the antagonism between beneficial microorganisms and phytopathogens (Mendes et al., 2011). Beneficial microorganisms can also trigger the immune mechanism of plants to enhance plant disease resistance (Bacete et al., 2018). They fight against pathogenic microorganisms as follows: competition, antibiotic, and parasitism (Berendsen et al., 2012).

1.3.3. Allelopathy and allelochemicals produced by PGPM and BCA

Allelopathy is a common biological phenomenon by which one organism produces biochemicals that influence the growth, survival, development, and reproduction of other organisms. Organisms include animals, plants and microorganisms. These biochemicals are known as allelochemicals and have beneficial or detrimental effects on target organisms (Cheng & Cheng, 2015). Therefore, allelopathy is accepted as a technology to implement for weed control and biological control of other useful traits (Ozcatalbas & Brumfield, 2010). Allelochemicals are released into the soil rhizosphere by a variety of mechanisms, including metabolites, decomposition of residues, and exudation. What's more, these chemicals play a role of plant defense, nutrient chelation, and regulation of soil biota (Aslam et al., 2017). The allelochemicals found so far are mostly secondary metabolites of plants or microorganisms. Among them, the most common are low molecular weight organic acids, phenols and terpenoids. Volatile organic compounds have the allelopathic effects on plant physiological and biochemical processes including growth, content of reactive oxygen species, enzyme activity, and photosynthesis except plant-to-plant communication as a signaling substance (Xie et al., 2021). Many allelochemicals of plants had been reported, such as Sorgoleone separated from Sorghum (Hussain et al., 2021), veratric acid, maltol, and (-)-loliolide from crabgrass (Zhou et al., 2013) and cinnamic acid and vanillin in eggplants root exudates (Chen et al., 2011). However, there are few reports on the allelopathy of microorganisms which also play an important role in the biological chain or ecosystem. 2,2'-methylenebis[6-(1,1-dimethylethyl)-4-methyl-Phenol and 1,2,3,4-Butanetetrol identified from *Bacillus amyloliquefaciens* inhibited the seeds germination of *C. equisetifolia* (Chen et al., 2021).

1.4. Research progress and biocontrol application of *Streptomyces* spp.

1.4.1. Introduction of *Streptomyces* spp.

Streptomyces constitute a major clade of the phylum *Actinobacteria*. These Gram-positive, filamentous prokaryotes are ubiquitous in soils and marine sediments,

and are commonly found in the rhizosphere or inside plant roots (Hassan et al., 2015; Rey & Dumas, 2017; Xu et al., 2017; Jakubiec-Krzesniak et al., 2018). They have well-developed branching hyphae, which have no septa and differentiate into nutrient hyphae, aerial hyphae, and spore hyphae. Nutrient hyphae, also known as basal hyphae, are light colored and thin, capable of absorbing nutrients and excreting metabolic waste; Aerial hyphae are branching hyphae with darker colors and thicker diameters; Aerial hyphae mature and differentiate into spore filaments, which then form conidia. The morphology and color of spore filaments and spores vary from species to species, and are one of the main identification traits for species differentiation. The members of the genus *Streptomyces*, which was first proposed by Waksman and Hinrici (1943), are well known as sources of bioactive molecules. They produce more than half of the known antibiotics derived from microbial sources, amongst which 75% are produced by *Streptomyces* strains (Goodfellow & Fiedler, 2010). Indeed, the most gifted actinomycetes have the capacity to produce around 30-50 secondary metabolites (Katz & Baltz, 2016). The genus *Streptomyces* remains a major source of novel pharmaceutically relevant compounds with antibacterial, antifungal, anti-inflammatory and antitumor activities (Barka et al., 2016)). Most (over 500 species) of *Streptomyces* are non-pathogenic polluting or colonizing bacteria. However, the exception is *Streptomyces somali*, which can cause foot swelling and occasionally cause invasive infections. Other strains rarely cause diseases. *Streptomyces griseus* (also known as *Streptomyces circularis*) is the most commonly isolated strain from human specimens, but it is believed to be an occasional pathogen that causes infections; More well-known, it is the original source of streptomycin. Isolated strains are usually only identified at the genus level (if species are to be identified, molecular biology detection methods are needed).

1.4.2. Bioactive components produced by *Streptomyces*

Secondary metabolites synthesized by *Streptomyces* mainly include antibiotics, decomposing enzymes and other components. From the structure of all compounds, they are mainly polyketones, alkaloids, peptides, flavonoids and phenols. Polyketones

are a class of polymeric organic compounds composed of alkenyl and carbonyl segments. Alkaloids are a kind of nitrogen-containing organic compounds, most of which have complex cyclic structure and a variety of biological activities. Peptides with high efficiency, low toxicity, strong target selectivity and strong modifiability have become the focus of pharmacists and natural product scientists in recent years. Antibiotics are widely used in medicine. For example, erythromycin, spectinomycin, actinomycin, streptomycin, and kanamycin (Kakinuma et al., 1976; Kim et al., 2008; Cheng et al., 2015). Oxytetracycline is commonly used as an antibiotic and feed additive (Wang & Yates, 2008). In agriculture, oligomycin A, polyoxin, validamycin and ningnanmycin (made in China) are or have been playing their anti- disease role (Sun et al., 2009; Qian et al., 2011; Li X. Y. et al., 2017; Xiao et al., 2021). Some newly discovered compounds in *Streptomyces* with active activity had been reported in these years (Igarashi et al., 2011; Wu et al., 2013; Jiang et al., 2015; Hashizume et al., 2017; Lu et al., 2017; Rathod et al., 2018). These new substances are lead compound materials for development of new drugs. In addition to producing antibiotic metabolites, *Streptomyces* can also secrete a variety of enzymes, which can protect plants from pathogens and accelerate the utilization of some organic substances. The hydrolases secreted by many *Streptomyces* strains can usually destroy the cell wall of pathogenic fungi and cause the leakage of cell contents. Enzymes are produced from second metabolites of *Streptomyces*, such as oxidase, lipase, amylase, cellulase, chitinase, urease and protease. These enzymes mostly belong to oxidoreductases (Tuncer et al., 2009; Fodil et al., 2011; Ham et al., 2021) and hydrolases (Hwang et al., 2013; Dubey et al., 2014; Wu et al., 2014; dos Santos et al., 2017; Abo-Zaid et al., 2021), except urease which maintains pH environment. therefore, these enzymes are applied in food, textile, pharmaceutical industry and agriculture production.

Beyond that, researchers still found other components. Two new compounds, tryptamine and tryptoline were isolated from the metabolites of *S. eurocidicus* JXJ-0089 strain, which had strong algicidal activity on *Microcystis* sp. FACHB-905 (Zhang et al., 2016). 6-prenylindole, founded from *Streptomyces* sp. TP-A0595 had suppressive effect on infection by *Alternaria brassicicola* by inhibiting the formation

of infection hyphae (Sasaki et al., 2002). Kim et al. found protocatechualdehyde in a *S. lincolnensis* M-20 extract using GC-MS (Kim et al., 2008). An antioxidative bioactive compound was reported, 3-Isobutylhexahydropyrrolo [1,2-a] pyrazine-1,4-dione from *S. mangrovisoli* sp. nov. (Jog et al., 2014).

1.4.3. Bcontrol mechanism of *Streptomyces* spp.

Streptomyces spp. are the most attractive microorganisms for the active secondary metabolites. For example, S. 12-09-4 and 12-09-11 showed strong protective effects (> 70%) on wheat powdery mildew (Wang X. et al., 2018). The *Streptosporangium becharense* strain SG1 exhibited remarkable protective effect by all seed bacterization treatments (Boukaya et al., 2018). In field trials, strains, EN23, and EN27 were effective in suppressing fungal root diseases of wheat when added as spore coatings to wheat seed (Franco et al., 2016). *S. mutabilis* strain, named IA1, reduced both disease occurrence (64.7%) and decrease severity (79.6%) caused by *Fusarium culmorum* (Toumatia et al., 2016). In vitro and growth chamber studies were conducted on several *Streptomyces* species as biocontrol agents against WSE (Tian et al., 2004; Adesina et al., 2007; Wan et al., 2008; Patel et al., 2018; Wu et al., 2019). Concurrently, some *Streptomyces* species can promote plant-growth which enhance the plant resistance (Aldesuquy H.S. et al., 1998; Jog et al., 2012; Li H. Y. et al., 2020; Li Y. L. et al., 2020). *Streptomyces* has been used in biological control of plant diseases. The biocontrol mechanisms of them mainly include antagonism, niche competition, induced systemic disease resistance and effective rhizosphere colonization.

Antagonism mainly refers to the production of antibacterial substances due to the assimilation of microorganisms, inhibiting the growth of harmful pathogens or directly killing pathogens. In the biological control of plant diseases, the same biocontrol strain can produce a variety of antibiotics, and different biocontrol bacteria can also produce the same antibiotics (Parry et al., 2011). Many microorganisms in nature can produce active substances, such as *Bacillus* (Chatterjee et al., 1992), *Pseudomonas* (Gamard et al., 1997; Touioui et al., 2018), and actinomycetes (Lam, 2006; Niyomvong et al., 2012). Among the natural producers of bioactive substances,

as a genus of actinomycetes, *Streptomyces* produces more than 80% of the total active substances in actinomycetes (Barka et al., 2016; Yun et al., 2018). The research of the metabolites produced by *Streptomyces* reached its peak in the 1970s, on the contrary, the discovery rate of new substances decreased continuously in the following decades and the repeated discovery rate of known compounds increased.

The targets of antagonistic mechanism mainly involve the following aspects: i) Acting on the cell wall of pathogen. The MHCE0811 produced the maximum chitinase which probably degraded the fungal cell wall and limited growth of test fungal pathogens (Jog et al., 2014). ii) acting on the cell membrane of pathogen. Pimaricin selectively binds to ergosterol in fungal cell membranes, causing changes in cell membrane permeability, leakage of intracellular substances and cell death. (Kitajima et al., 1976). iii) Acting on the energy metabolism system. *Streptomyces* sp. strain SM8, isolated from *Haliclona simulans*, possessed antifungal and antibacterial activities and inhibits the calcineurin pathway in yeast (Almeida et al., 2018). iv) Acting on protein synthesis system. For example, streptomycin acted on the whole process of protein synthesis.

Induced resistance. Plant rhizosphere growth promoting bacteria can induce plant systemic disease resistance (ISR) (Farag et al., 2013; Sadeghi et al., 2017; Abbasi S. et al., 2019). Another pathway is systemic acquired resistance (SAR) which need pathogen or chemical trigger mediated (Kloeppe et al., 1999; Pieterse et al., 2002; Milikisiyants et al., 2017). ISR, mediated by jasmonic acid and ethylene signaling pathways, is as an important mechanism by which selected plant growth-promoting bacteria and fungi in the rhizosphere cooperate with plants for enhancing defense against a broad range of pathogens (Pieterse et al., 2014). SAR is induced by the accumulation of salicylic acid and pathogenesis-related protein. Many reports have shown that biocontrol bacteria can induce ISR or SAR production of plants to improve their resistance to pathogens through enhancing the activity of defense enzymes and the expression of resistance-related genes. For example, the enzymatic activities of polyphenol oxidase (PPO), phenylalanine ammonia-lyase (PAL), -1,3-glucanase (GLU), and chitinase were significantly enhanced in the rice treated with

antifungalmycin N2 plus *R. solani* (Zhang S.-w. et al., 2020). *Paenibacillus kribbensis* PS04 could induce the expression of defense related genes (*OsPR1, OsPR2, OsPR3, OsPR4, OsPR5, OsPR10* and *OsPR16)* in the rice (Cheng et al., 2020). *S. rubrogriseus* HDZ-9-47 enhanced the activity of PPO, POD, PAL and superoxide dismutase in tomato roots (Jin et al., 2016). *S. roche* D74 and *S. partum* Act12 (Liu et al., 2018) and *S. rochei* strain ZZ-9 (Xie et al., 2019) strongly enhanced the defense activity in wheat leaves. Shahabivand et. al. (2016) reported that antioxidant activity and gene expression associated with cadmium toxicity in wheat affected by mycorrhizal fungus. Some studies investigated antimicrobials at very low concentrations have high inhibiting effect or eliciting activities (Winding et al., 2004; Boukaew et al., 2017; Hennessy & Stougaard, 2017).

Effective rhizosphere colonization. The effective colonization of rhizosphere bacteria in plant roots is the key factor for their role in biological control and growth promotion (Liu et al., 2014; Zboralski et al., 2022). Therefore, understanding the root colonization dynamics of specific rhizosphere microorganisms is the basis for the effective use of beneficial microorganisms to control diseases and promote plant growth. Studies have shown that biocontrol bacterial strains control plant diseases through accurately and successfully colonizing the ecological niche (Zhang Y. et al., 2019). However, the microbial operational taxonomic units were distinct in the greenhouse and field of biocontrol experiments (Araujo et al., 2020). It is critical to understand the microbial networks formed in rhizosphere soils to clarify the impact and effects of potential biocontrol strains (Poudel et al., 2016; Araujo et al., 2019). Overall, the selection of biocontrol agents becomes complex owing to their diversity and interactions with hosts (Moto et al., 2017).

1.5. Effect of exogenous microorganism on soil properties and microbial diversity

Beneficial microbe had an enormous potential to promote plant growth. In the future precision agriculture, these microbe applicated may be a promising way to help plants coping with biotic and abiotic stress and ensure crops yield. *Actinobacteria, Bacteroidetes, Firmicutes,* and *Proteobacteria* mainly consist of the microbial

communities in the plant rhizosphere (Fierer et al., 2009; Schlaeppi et al., 2014; Edwards et al., 2015). Meanwhile, plants release more than 40% carbon into the soil around the rhizosphere, which shapes the microbial communities (Bais et al., 2006; Ulbrich et al., 2022). We have known that microorganisms interact each other by several main patterns, as those were noted previous. Thus, direct cooperative or competitive interactions among the community can influence microbial composition and their effect on plants (Rodriguez et al., 2019). Similarly, the effect of microbial agents in practical application is essentially the result of biological balance in nature. Various factors such as ecological environment, farming system, crop growth status and plant rhizosphere microbial species may affect the biocontrol effect of microbial agents (Moto et al., 2017; Vurukonda et al., 2018; Araujo et al., 2020). In the process of symbiosis, it is possible to identify positive and negative co-occurrence correlations between microbial community members, which may reflect synergistic and antagonistic functional relationships (Faust & Raes, 2012). Biocontrol agents consisted of microorganism with the function of increasing crop yield, are researched and developed in the current agriculture. At present, there are two kinds of streptomyces biocontrol agents Mycostop on the market ® And Activate ® (The effective ingredients are *Streptomyces* strain *S. griseoviridis* K61 and *S. lydicus* WYEC 108 respectively). In the field test, their effects (bacteriostasis and growth promotion) are inconsistent with those in the laboratory (Newitt et al., 2019). Therefore, it is necessary to further understand the factors affecting the competitiveness of strains and their long-term establishment in crop rhizosphere microbiota. A detailed understanding of how these factors affect the success of biological control and how to mitigate them is a priority for developing a sustainable and effective biological control strategy.

1.6. Optimization of fermentation conditions and medium composition

1.6.1. Importance of fermentation process

BCA is a group of microorganisms producing a wide variety of bioactive substances that have the potential to inhibit plant pathogens (Wright & Bennett, 2018). It presents the advantages of safety, environment-friendliness, and sustainable

development. Currently, the most widely used method to control phytopathogenic fungal diseases is the routine application of chemical pesticides and fertilizers; however, these chemicals have been proven to be harmful for agro-ecosystems. Thus, BCAs are increasingly becoming a preferred alternative to traditional control measures. But the use of microbial fermentation to produce various useful metabolites involves a wide variety of cultivated components and complex interactions among them. Therefore, the optimization of microbial cultivation is very important.

1.6.2. Microbial fermentation application

Actinomycetes, as an important microorganism, can produce different kinds of bioactive secondary metabolites, which have significant application potential (Coombs & Franco, 2003; Qin et al., 2009; Song et al., 2012; Hei et al., 2021). Many reports have shown that *Streptomyces*, a genus belonging to Actinomycetes, could suppress a range of phytopathogenic organisms, including *Magnaporthe oryzae* (Patel et al., 2018), *Fusarium* spp. (Tian et al., 2004; Jung et al., 2013), *Rhizoctonia* spp. (Wu et al., 2019), *Gaumannomyces graminis* (Coombs et al., 2004), and *Pythium* spp. (Clermont et al., 2010), both in vitro and in vivo (Coombs & Franco, 2003; Wan et al., 2008; Wang et al., 2013; Viaene et al., 2016), and the yield of bioactive metabolites from *Streptomyces* spp. is crucial for related research and application. Furthermore, it is also necessary to determine the various factors that regulate the production of secondary metabolites with antifungal properties. Strain fermentation, also known as shake-flask fermentation, is significantly influenced by medium components and culture conditions (Sun et al., 2011; Zhao S. L. et al., 2012; Kamoun et al., 2022). Factorial design and response surface methodology (RSM) are powerful statistical tools, which can overcome the shortcomings of single-factor and orthogonal experimental design methods. While factorial design explores the role of each factor in the production of metabolites, RSM comprising mathematical and statistical approaches is used to assemble empirical models based on selected factors (Thompson, 1982).

1.6.3. Common optimization design methods

The usual methods for optimizing fermentation medium are single factor method

and orthogonal experimental design method. The single factor method is only used to discuss the influence of one factor. Due to the frequent interaction between factors, this method may not always achieve the best optimization conditions. Orthogonal experimental design focuses on how to scientifically and reasonably arrange experiments, considering several factors at the same time to find the optimal combination of factor levels (Jiang et al., 2020). However, it cannot find a clear functional expression between the factors and response values in the given entire region, that is, a regression equation, and thus cannot find the optimal combination of factors and response values in the entire region. Therefore, people expect to find a regression analysis method with fewer experiments, shorter cycles, high accuracy of the obtained regression equation, and the ability to study the interaction of several factors. The response surface analysis method largely meets these requirements.

Conclusions to chapter 1

The analysis of the literature shows that it is necessary to develop biological fungicides to cope with wheat diseases. Wheat is one of the most important food crops in China and even in the world. The increase of total wheat production is of practical significance to the rapid growth of China's national economy and social stability. Plant diseases, especially soil-borne diseases, are one of the most important factors affecting wheat yield reduction. Breeding and popularizing new resistant wheat varieties is the most economical and ecological effective measure to control WSE. However, due to the lack of wheat immune varieties, wheat varieties planted in a large area, and their core parents not resistant to WSE, the progress of conventional breeding is slow. The control of WSE mainly depends on chemical pesticides for a long time. However, the consequence of chemical control is the increase of crop drug resistance, destroying soil ecosystem, and impacting on human health. In recent years, with the sustainable development trend of economic crops, it is urgent to find a safe and effective way of disease control.

Biological control has the properties of economic efficiency, wide sources and environmental safety. In recent decades, researchers have carried out a lot of studies

on the biological control against different plant diseases, in which beneficial microorganisms and their metabolites are one of main contents. As an important aspect of biological control, the mechanism of plant rhizosphere growth promoting bacteria (for example, PGPM and BCA) involves antagonism, competition, inducing disease resistance and regulating the level of plant hormones. *Streptomyces* have been widely studied all over the world as biocontrol bacteria for plant diseases, especially soil-borne diseases. They have the properties of disease prevention and/or growth promotion. Equally important, *Streptomyces* has allelopathy on plants, and some positive effects also confirmed its growth promoting mechanism in some certain. Beneficial microorganisms are not effective if it is independent from plant and microbial system. Hence, the interaction, such as target strain, native microorganisms and soil, become one of the research hotspots. The application of *Streptomyces* can reflect its antifungal and growth promoting functions by affecting soil factors and the structure of plant rhizosphere microorganisms. Assuming that the treatment of *Streptomyces* can increase the content of soil microbial nutrients and the abundance of beneficial microorganisms for plant growth, such results are satisfactory.

The above functions of *Streptomyces* can be well applied in agricultural production on the premise that its fermentation capacity can meet the application needs. The optimization of fermentation conditions should only focus on the carbon and nitrogen sources and inorganic salts of the culture medium, as well as the associated temperature, pH value, rotational speed, and inoculation amount during the cultivation process. When selecting fermentation conditions, single factor method and orthogonal experimental method are generally used. The combination of these two methods can effectively optimize the combination of more reasonable fermentation conditions.

CHAPTER 2
MATERIALS AND METHODS OF RESEARCH

2.1. Experiment 1. Study on antifungal mechanism of Streptomyces sp. HU2014 against *R. cerealis* G11 and *R. solani* YL-3

2.1.1. Materials

Three wheat cultivar, Bainong 4199 (BN4199), Aikang 58 (AK58), Bainong 307 (BN307) and Zhoumai 22 (ZM22) were afforded by Henan Engineering Research Center of Crop Genome Editing (HERCCGE) and the Breeding Center of Henan Institute of Science and Technology (HIST) in China.

BN 4199

A semi winter medium-late variety with good lodging resistance. Whole growth period 226.5 days, and plant height about 71.5 cm. The average number of grains per spike 32, and the average weight of 1000 grains 44.1g. High susceptibility to wheat leaf rust, wheat scab, wheat powdery mildew and medium resistance to WSE. Yield per mu 547-580 kg.

Aikang 58

A semi winter medium variety with strong frost resistance, high lodging resistance and disease resistance. Plant eight about 70 cm. Number of grains per spike 38-40, and weight of 1000 grains 42-45g. High resistance to powdery mildew, stripe rust, leaf blight, medium resistance to sharp eyespot. Yield per mu 500-650 kg.

Bainong 207

A semi winter medium-late variety with cold resistance and good lodging resistance. Whole growth period 231 days, and plant height about 76 cm. High susceptibility to wheat leaf rust, wheat scab, wheat powdery mildew and WSE, and medium resistance to wheat stripe rust. Yield per mu 550-650 kg.

Zhoumai 22

A semi winter medium variety with good cold resistance, strong drought resistance and strong lodging resistance. Plant height about 80 cm. Number of grains per spike 35-37, and weight of 1000 grains 44-46g. High resistance to stripe rust, leaf

rust, moderate susceptibility to powdery mildew, sharp eyespot, high susceptibility to scab and stem rust. Yield per mu 500-550 kg.

A *Streptomyces* sp., named *Streptomyces* sp. HU2014 (HU2014) (preserved in the China Center for Type Culture Collection; Preservation No. M2015207), *R. cerealis* G11 and *R. solani* YL-3 was kindly provided by Dr. Hu Linfeng of HIST, cultured on potato dextrose agar (PDA) for 3-6 days, and HU2014 was cultured on PDA for 12 days.

2.1.2. Main reagents

●Ethanol (Kaitong Chemical Reagent Co., Ltd, Tianjin, China. Lot#:20220318);

●Acetonitrile (Kaitong Chemical Reagent Co., Ltd, Tianjin, China. Lot#:20040315);

●Formic acid (Kaitong Chemical Reagent Co., Ltd, Tianjin, China. Lot#:20200515);

●Glutaraldehyde (Macklin Biochemical Co., Ltd, Shanghai, China. Lot#:C13143368);

●Phosphate Buffer Solution (Beijing Solarbio Science & Technology Co., Ltd, China. Lot#:20210922);

●Tert butanol (Macklin Biochemical Co., Ltd, Shanghai, China. Lot#:C12842742);

●Nadic methyl anhydride (Structure probe, Inc. USA)

●POD kit (Beijing Solarbio Science & Technology Co., Ltd, China);

●PPO kit (Beijing Solarbio Science & Technology Co., Ltd, China);

●PAL kit (Beijing Solarbio Science & Technology Co., Ltd, China);

●GLU kit (Beijing Solarbio Science & Technology Co., Ltd, China);

●Trizol Reagent (Thermo Fisher Scientific, Waltham, USA);

●PrimeScript™ II 1ST Strand cDNA Synthesis Kit (TaKaRa, Dalian, China);

●PrimeScript™ RT reagent kit (TaKaRa, Dalian, China);

●PDA medium (g/L): potato, 200; dextrose, 12; and agar, 12; pH 7.0–7.2;

●GPY medium (g/L): glucose, 20; soybean peptone, 5; and yeast extract, 5; pH

7.0–7.2.

2.1.3. Experiment equipment

●Scanning electron microscope (SEM) (Quanta 200, FEI, Hillsboro, USA);

●Electrospray ionization ion trap mass spectrometer (AB Sciex Triple TOF® 4600, SCIEX, Boston, USA)

●Thermo Scientific Microplate Reader (Varioskan Flash, Waltham, USA); Cryogenic centrifuge (Eppendorf 5424, Hamburg, Germany);

●Autoclave (Sanshen YM75FGN, Shanghai, China);

●Constant temperature shaker (Miulab ES-60C, Hangzhou, China);

●QRT-PCR instrument (Quant Studio 6 Flex, ABI, USA);

●PCR instrument (Thermo Fisher Scientific 2720, Waltham, USA);

●Constant temperature incubator (Zhicheng ZXJP-R1230, Shanghai, China).

2.1.4. Experimental design

Mechanism of Bioactive Metabolites from HU2014 against *R. cerealis* G11

Genome sequencing and average nucleotide identity (ANI) calculation

Genomic DNA of HU2014 was extracted using the Bacterial Genomic DNA Extraction Kit (Takara Biomedical Technology Co. Ltd, Beijing, China) in accordance with the manufacturer's instructions. Polymerase chain reaction (PCR) was used to amplify the 16S rRNA gene with the conserved primer pair PrimeF: 5′-GAGCGGATAACAATTTCACACAGG-3' and PrimeR: 5'-CGCCAGGGTTTTCCCAGTCACGAC-3' (Reilly et al. 2002; Weisburg et al. 1991). The genomic sequencing was performed using the Oxford Nanopore Technologies standard protocol. Prior to sequencing, a genomic DNA library was constructed using the Ligation Sequencing Kit (SQK-LSK109) provided by the manufacturer (Oxford Nanopore Technology, Oxford, UK). The resulting library was quantified using a Qubit DNA HS Assay Kit and a Qubit fluorometer (Thermo Fisher Scientific, Bedford, MA, USA). The whole genome was sequenced using the PromethION/GridION platform at BioMarker (Beijing, China). The ANI values among 10 genome sequences, including HU2014 and the closest type strains, were calculated using the J Species WS Online

Service (Richter et al., 2016).

Suppression of mycelial growth of YL-3 and G11

The pathogens *R. solani* YL-3 and *R. cerealis* G11 were cultured on PDA for 4 days and HU2014 was cultured on PDA for 15 days, respectively. Antifungal activity was assessed using the dual culture method modified as described previously (Royse & Ries, 1978; Zivkovic et al., 2010). Briefly, 4-mm fungal plugs were placed on PDA, and HU2014 was co-inoculated by streaking opposite to the fungal plugs. Each treatment was replicated three times, and a single fungal plug served as a control. The plates were incubated at 25°C for approximately 4 days. The fungal colony diameters were measured, and the inhibition rate was calculated as [1-R1/R2] × 100, where R1 represents the radial colony growth on the dual-culture plate and R2 represents the radial colony growth on the control plate.

Observation of the hyphae

Fixation

The samples (extracted biomass) were fixed with 2.5% glutaraldehyde for two hours, and then rinsed with phosphate buffer (0.1 M, Ph=7.4) for ten minutes, a total of three times. Subsequently, the samples were fixed using 1% osmium tetroxide for one hours and rinsed with phosphate buffer as described above.

Dehydration

The samples treated by last step were gradually dehydrated with different concentration of ethanol (ratios of 30%, 50%, 70%, 90%, and 95% (v: v) with water), and the interval time between two concentration was twenty minutes. In the process, the samples were not exposed to air. Finally, the samples were dehydrated with tert butanol for ten minutes with a total of three times.

Lyophilization

The samples after last step were lyophilized by (Christ *ALPHA 1-4 LSC, Osterode, Germany*) using the following settings: 72 h, -10°C, 0.5 mbar (primary drying), and 24 h, 20°C, 0.01 mbar (final drying) (Grossmann et al., 2018).

Gold sputtering

Sample was coated with gold and the SEM were used to photograph the micromorphology of aerial mycelia (Quanta 200, FEI, Hillsboro, USA).

Enrichment of Bioactive Metabolites and Bioassay

In the present study, *Streptomyces* sp. HU2014 was cultured in GPY medium to examine its antifungal components. The filtered supernatant of *Streptomyces* sp. HU2014 culture for 12 days was subjected to gradient elution using 20%, 40%, 60%, and 80% ethanol from CHP-20P resin, evaporated, and then lyophilized (Christ ALPHA 1-4 LSC, Osterode, Germany). The fractions (hereafter denoted as F2, F4, F6, and F8) were diluted with 70% methanol to a concentration of 0.1 mg/mL (Vellingiri et al., 2021), and their absorbances were determined as previously mentioned. 70% methanol was as a control. To trace the distribution of antifungal components in the fractions, antifungal activities assay against *R. solani* YL-3 and *R. cerealis* G11 were performed. Briefly, the 0.5 mg/mL solution of each fraction dissolved in 2% dimethylsulfoxide was mixed with melted PDA medium at a ratio of 1:9 (v/v) and poured into a petri dish. The control comprised PDA medium with the equal volume of 2% dimethylsulfoxide. Then, *R. solani* YL-3 discs were placed at the center of the petri dish and incubated at 25°C for 3-5 days. Subsequently, the fungal colony diameter was measured using the growth rate method, and the mycelial growth inhibition rate was calculated as follows: Mycelial growth inhibition rate = (1 − Colony diameter of treatment / Colony diameter of control) × 100% (Zhang et al., 2009; Fan et al., 2020). The experiment was performed in triplicate.

Identification of bioactive metabolites using UPLC-MS

UPLC-MS was used to examine the potential components of the four fractions (Gruz et al., 2008). Sample was dissolved in acetonitrile/water, and 5 μL was injected into a C18 column (2.1× 100 mm, 1.8 μm; Acquity UPLC HSS T3, Waters Corp., Milford, USA) at 35°C. The mobile phase consisted of water with 0.1% formic acid and acetonitrile with 0.1% formic acid. The following gradient program was applied: 10% B, 0–2 min; 10% –20% B, 2–6 min; 20%–30% B, 6–13 min; 30% B, 13–19 min; 30%–10% B, 19–20 min. The column effluent was delivered to an electrospray ionization ion trap mass spectrometer (AB Sciex Triple TOF® 4600, SCIEX, Boston,

USA). Peaks were identified by comparison with the mass spectra data from the Natural Products Atlas (NP Atlas, https://www.npatlas.org/).

Induce of HU2014 on Defense Enzyme and Resistance-related Genes Expression in Wheat

The pre-test for the change of enzyme activity in wheat treated by HU2014

HU2014 was pre-cultured on PDA medium at 4℃ until required. The mycelia discs were transferred to sterile GPY broth in 250 mL flasks, incubated at 25℃ with shaking at 150 rpm for 12 days. The fermentation broth was centrifuged at 12000 rpm for 15 minutes to separate the supernatants. The supernatants were filtrated through 0.22 μm candle filters, and then the extracellular filtrate (EF) was stored at 4℃ until use.

The EF of HU2014 was considered as original concentration, which was further diluted by adding sterile water to concentration of 1×10^{-1}, 1×10^{-2}, 1×10^{-3}, 1×10^{-4} and 1×10^{-5}. An aliquot was applied by soil drench of 50 mL with 3 replications. BN4199 seeds were disinfected in 30 % H_2O_2 for 2 min and washed thoroughly with sterile distilled water. The seeds were germinated in a plastic tray (30 cm × 20 cm × 3 cm) contained wet gauze for 12 hours. Subsequently, the germinated seeds were transferred to sow in each pot (12 cm × 12 cm × 9 cm), placed into growth chamber. Each experiment used a randomized design, sterile water as untreated control. Ten days after soil drench treatment, leave tissues were collected and weighted, 0.1 g per aliquot for one enzyme activity assay. After weighted, the tissues were immediately submerged in liquid nitrogen. Material was ground in mortar with a pestle under liquid nitrogen, transferred into centrifugal tube. The enzyme activity was determined by POD colorimetry (Doerge et al., 1997), PPO colorimetry (Tang & Newton, 2004) and PAL colorimetry (Aydaş et al., 2013). All detailed steps referred to the instruction of kits (Beijing Solarbio Science & Technology Co., Ltd, in China).

Enzymes activity in wheat treated by HU2014 and R. cerealis G11

Preparation of diseased wheat grains: In order to increase the survival rate of HU2014 in the soil, we adopted the method of inoculating diseased wheat grains (Zhou

et al., 2020). Firstly, wheat seeds are placed in a round bottom flask and sterilized at high temperature, and then inoculated with *R. cerealis* G11 colony and cultured for 28 days at 25°C.

According to the previous test, The EF was diluted 1000-fold with sterile water (F3) (Zhu et al., 2020). This experiment was divided into three groups: AK58, BN307 and ZM22; Four same treatments for each group were: (i) the pots treated with 100 mL of F3; (ii) the pots infected *R. cerealis* G11 24 hours post inoculation (PF3); (iii) the pots infected *R. cerealis* G11 singly (CKP) and (iv) the pots without other treatment (CK). Three wheat seeds were pre-treated as the above and germinated. Subsequently, the germinated seeds were transferred to sow in each pot (12 cm × 12 cm × 9 cm), placed into growth chamber. Each experiment used a randomized design, sterile water as untreated control. 28 days later, 100 mg leave tissues were collected and immediately submerged in liquid nitrogen at 1 day (D), 2 D, 3 D, 4 D, 5 D and 6 D after treatment. POD and PAL activity was determined as described above, and GLU activity was referred to GLU colorimetry (Mohammadi & Karr, 2002).

The expression of resistance-related genes

As previously described, this experiment was carried out with pregerminated seeds from the wheat cultivars AK58, BN307, and ZM22. After 6 d plant growth in the growth chamber, 100 mL of 500-fold diluted EF was irrigated into each pot filled with 1 kg non-sterile soil. A randomized design was used in each experiment, with sterile-water treatment as a control. A total of 100 mg leaves were harvested from both treatment and control plants at 0, 2, 6, 12, 24, 48, 72, and 96 h, placed in a 2 mL tube, immediately frozen in liquid nitrogen, and stored at −80°C before further analysis.

Total RNA was isolated from both cultivars using Trizol™ Reagent (Thermo Fisher Scientific, Waltham, USA). Briefly, 100 mg sample was added 1 mL of Trizol™ Reagent used for lysis, centrifuged for 10 min at 12,000 rpm at 4°C after incubating for 10 min. The supernatant was added 0.2 mL chloroform, centrifuged for 15 min at 12,000 rpm at 4°C after stewing 10 min, and the newly supernatant was added with 0.4 mL isopropanol as described as chloroform. After discard the third supernatant, resuspend the pellet in 1 mL of 75% ethanol, vortex the sample for 15 s, then centrifuge

for 2 min at 12,000 rpm at 4°C. Discarding the supernatant with a micro-pipettor, resuspending the pellet in 30 μL of RNase-free water, 0.1 mM ethylenediamine tetraacetic acid by pipetting up and down, and then stored the RNA at -80°C.

First-strand cDNA synthesis was performed using PrimeScript™ RT reagent kit with gDNA Eraser (TaKaRa, Dalian, China). Briefly, the 10 μL reaction system of erasing genomic DNA included 1μg RNA, 1μL gDNA Eraser, 2μL 5 × gDNA Eraser buffer, and 7 μL RNase free dH_2O. After incubating it at 42°C for 2min, the 20 μL reverse transcription system, consisting of 10 μL reaction solution of the previous step, 1 μL PrimeScript™ RT enzyme mix I, 1 μL RT primer mix, 1 μL 5 × PrimeScript buffer 2, and 4 μL RNase free dH_2O, incubated at 37°C for 15 min, 85°C for 2 s.

Quantitative real-time PCR (QRT-PCR) reactions were carried out according to the manufacturer's instructions (Takara Biotechnology, Dalian, China), and amplified for 40 cycles with each cycle consisting of 30 s at 95°C, 5 s at 95°C, and 30 s at 60°C on a real-time PCR detection system (ABI QuantStudio 6 Flex, Waltham, USA). The primer sequences for QRT-PCR are shown in Table 2.1.

Table 2.1

Gene-specific primers used in this study for quantitative reverse transcription polymerase chain reaction (qRT-PCR)

No.	Gene name	Accession No.	Direction	Sequence (5′ to 3′))
1	*Actin*	AB181991	F	GCTATGAGATGCCTGATGGTC
			R	GAACCTCCACTGAGAACAACA
2	*Ta* pathogenesis-related 1 (*PR1*)	AF384143	F	AACCTCGGCGTCTTCATCA
			R	TTTACTCGCTCGGTCCCTCT
3	*Ta* beta-1,3-glucanase (*PR2*)	JF718349	F	TGCCGTTGCTCTCTTCAT
			R	ATGCCCTTGGACCTGTAGA
4	*Ta* chitinase I (*PR3*)	AB029934	F	AGAGATAAGCAAGGCCACGTC
			R	GGTTGCTCACCAGGTCCTTC
5	*Ta* thaumatin-like protein (*PR5*)	AF442967	F	ACAGCTACGCCAAGGACGAC

			R	CGCGTCCTAATCTAAGGGCAG
6	phenylalanine ammonia-lyase (*PAL*)	AY005474	F	CCAATGTTCTGTCCGTCCT
			R	GCTGCTTCAATCTGTCCAG
7	Lipoxygenase (*LOX*)	HQ913602	F	AGGCAACTACATCTACGCTTC
			R	GCCATCAACACCAGAGTCA

As an internal control, the wheat *Actin* gene was used. Each gene's expression was replicated three times. Gene expression was quantified using the comparative $2^{-\Delta\Delta Ct}$ method (Schmittgen & Livak, 2008).

Statistical analysis

Statistically significant differences ($P < 0.05$) were evaluated by an analysis of variance (ANOVA) followed by the Duncan's test using SPSS version 16.0 (SPSS Inc. Chicago, IL, United States). Simple linear regression analysis was carried out in SPSS 16.0.

2.2. Experiment 2. Determination of plant-growth promotion

2.2.1. Materials

Soil samples were collected from the field where commonly plant wheat in Xinxiang, China (Benton Harbor: N 113.9351°, E 35.3829°), air dried at room temperature, thoroughly sieved to remove roots and other plant tissue, and stored until use. Three wheat cultivars as described previously. Green bristlegrass seeds were purchased on line (https://www.lvbad.com/)

2.2.2. Main reagents

●Calcium phosphate tribasic (Tianjin Kaitong Chemical Reagent Co., Ltd, China. Lot#:20120408);

●Salkowski reagent: $FeCl_3 \cdot 6\ H_2O$ 0.027 g，5 ml 70 %的 $HCLO_4$, 5 ml H_2O;

● Chrome azurol S detection medium (Qingdao Hope Bio-Technology co., Ltd, China. Lot #: HB 9132)

● Tryptic Soy Broth medium (g/L): Tryptone 17, Soytone 3, NaCl 5, KH_2PO_4 2.5, Glucose 2.5, pH 7.1–7.5;

● National Botanical Research Institute's Phosphate (NBRIP) medium (g/L):

Glucose 10, $Ca_3(PO_4)_2$ 5, $MgCl_2$ 5, $MgSO_4 \cdot 7H_2O$ 0.25, KCl 0.2, $(NH_4)_2SO_4$ 0.1, pH 7.0–7.2;

● CMC medium (g/L): CMC-Na 10, $MgSO_4$ 1, KNO_3 1, K_2HPO_4 0.3, Yeast extract 2, NaCl 0.5, Agar 15, pH 7.0–7.4;

●Colloidal chitin medium (g/L): Na_2HPO_4 6; KH_2PO_4 3; NH_4Cl 1; NaCl 0.5; Yeast extract 0.05; Agar 15, Colloidal chitin 1% (w/v).

●Skimmed milk medium (g/L): Skimmed milk 50-100 mL, Yeast extract 3, Agar 20, pH 7.0–7.2;

●MDA kit (Beijing Solarbio Science & Technology Co., Ltd, China).

2.2.3. Experimental equipment

●Thermo scientific microplate reader (Varioskan Flash, Waltham, USA);

● Inductive coupled plasma emission spectrometer (ICP-OES, Optima 2100DV, PerkinElmer, Norwalk, USA)

2.2.4. Experiment design

Growth promoting properties of HU2014

Indoleacetic acid synthesis

The colorimetric Salkowski's assay was used to determine the quantitative and qualitative levels of indoleacetic acid (IAA) (Tang & Bonner, 1948). HU2014 was cultured in 150 mL of Tryptic Soy Broth supplemented with 60 mmol/L tryptophan for 8 days. At room temperature, 1 mL of culture supernatant was mixed with 2 mL of Salkowski reagent for 30 min (Bonaldi et al., 2015). Color development indicated IAA production, and the intensity of the color was measured spectrophotometrically at 530 nm.

Phosphate solubilization

For phosphate solubilization by HU2014, qualitative and quantitative analysis were conducted. In the qualitative analyses test, HU2014 was incubated on the NBRIP medium at 28°C for 6 days (Xiao et al., 2009), and the phosphate solubilization was determined based on the presence of an orange zone. In the quantitative analysis test, HU2014 was incubated in 150 mL of NBRIP liquid medium at 28°C for 5 days with

$Ca_3(PO4)_2$ as the sole phosphate source. Subsequently, the solubilized phosphate in the supernatant of the fermentation broth was measured using an Inductive Coupled Plasma Emission Spectrometer (ICP-OES, Optima 2100DV, PerkinElmer, Norwalk, USA) (Bandura et al., 2002).

Siderophore production

Siderophore production was measured on Chrome azurol S agar (Schywn & Nielands, 1987; Hu & Xu, 2011). Five plugs (4 mm in diameter) of HU2014 were inoculated on the medium and incubated at 30°C for one week. The presence of a color change around the colonies indicated the presence of siderophore production. The test was repeated twice with three replicates.

Chitinase production

The qualitative assay of chitinase production was carried out according to Saima et al. (Saima et al., 2013). HU2014 was streaked on a colloidal chitin medium (chitin as a single carbon source) and inoculated for 20 days at 30°C. A clear halo forming around colonies indicated positive activity. The experiment was repeated twice with three replicates.

Cellulase and protease production

The cellulolytic activity was determined by inoculating HU2014 plugs (4 mm in diameter) on carboxymethylcellulose media plates with CMC as the sole carbon source. After the plugs were incubated at 30°C for 7 days, the strains were assayed for their ability to degrade CMC by flooding plates with a 0.5% Congo red solution, immersed for 10 min, and then washed with 1 M NaCl. The haloes surrounding the colonies revealed cellulase production (El-Sersy et al., 2010). Protease production was measured in skimmed milk medium, and positive activity was determined by the formation of an opaque halo around each colony (Palaniyandi et al., 2013).

Plant growth promotion experiments in pots

The pre-test for growth promotion was carried out using wheat cultivar BN307 with pre-germinated seeds. The culture of HU2014 in GPY liquid medium at 25°C for 6 days, with the strain in logarithmic growth, was centrifuged at 12,000 rpm. After discarding the supernatant, the pellets were rinsed in sterile water and filtrated three

times using a 200-mesh sterile fabric filter. Five concentrations of HU2014 (wet weight) were prepared by homogeneously mixing with non-sterile soil: 1 g/kg, 5 g/kg, 10 g/kg, 15 g/kg, and 20 g/kg soil. The germinated seeds were sown in each pot (12 cm × 12 cm × 9 cm) in a growth chamber at 25°C. Each treatment was replicated six times, and pots without the HU2014 treatment served as a control. After 6 days of growth, the seedlings were thinned to 20 plants. Plant shoot length was recorded at 7 days post-inoculation (dpi), 20 dpi, and 28 dpi. A second investigation was carried out using wheat cultivars AK58, BN307, and ZM22, as described above. For these assays, 18 pots (20 plants per pot) of each wheat cultivar were equally divided into three groups: two groups with the strain concentration 4 g/kg soil and 8 g/kg soil per pot, respectively, and one group with the same volumes of non-sterile soil as a control. No fertilizer was applied during the process. Shoot fresh weight, dry weight, shoot length, chlorophyll content, and root dry weight were recorded at 28 dpi, and 60 dpi. The dry weight of each plant was determined after drying the plant material at 60°C until a constant weight was obtained.

Effect of HU2014 inoculation on soil properties and microbial diversity

Soil sampling

Rhizosphere soil of ZM22 without HU2014 inoculation and HU2014 inoculation with the concentration of 4 g/L soil in the above test were physically brushed away from the root surface using a sterile soft bristle paintbrush after 40 days of plant growth. The soil samples were divided into two groups. One group was passed through a 2-mm sieve after air drying for physicochemical analyses. The other group was immediately stored at -80°C to extract genomic DNA.

Soil physiochemical analysis

The basic soil characteristics were assessed in accordance with the standard acceptable methods. Total phosphorus (TP) and Olsen-phosphorus (Olsen-P) in soil were measured using molybdenum antimony blue colorimetry method (Fang et al., 2017). Soil total nitrogen (TN), nitrate nitrogen (NO_3^--N), ammonium nitrogen (NH_4^+-N), total phosphorus (TP), and olsen phosphorus (Olsen-P) were all measured using

assay kits manufactured by Sinobestbio Technology co., Ltd., (Shanghai, China) according to the manufacturers' instructions.

DNA extraction, HiSeq sequencing and bioinformatics analysis

Total bacterial DNA was extracted from the 0.5g of soil samples by using a FastDNA® Spin Kit (MP Biomedicals, Santa Ana, CA) according to the manufacturer's instructions. DNA concentrations were quantified by a QuantiFluor dsDNA analysis kit (Promega, WI, USA) and were conducted by a Quantum Level 3.0 fluorometer (Thermo Fisher Scientific Inc., Waltham, USA). The fungal ITS region was amplified using ITS1FI2 primers (5′-GTGARTCATCGAATCTTTG-3′) and ITS2 (5′-TCCTCC GCTTATTGATATGC-3′) (Karlsson et al., 2014). The universal primers used in V3-V4 region of the bacterial 16S rRNA gene are 341F: (5′-CCTACGGGNGGCWGCAG-3′) and 805R: (5′- GACTACHVGGGTATCTAATCC-3′) (Logue et al., 2016). PCR amplification and conditions were followed the method as described previously (Yang et al., 2022). High-throughput sequencing analyses of bacterial and fungal rRNA genes were submitted to the NovaSeq PE250 platform (LC-BIO, Hangzhou, China). The accession numbers of bacterial and fungal DNA sequences in the NCBI Sequence Read Archive are PRJNA871079 and PRJNA870704 respectively.

Allelopathic effect of four fractions from HU2014 on wheat and green bristlegrass

Pre-germinating

Wheat: Washed the seeds with distilled water, spread them on the tray, covered with gauze to keep the seeds moist, changed the water 2 - 3 times a day, and loosen the air. After about 24 hours, the exposed seeds were selected for use.

Green bristlegrass: kept the seeds in distilled water at 55℃ for 10 min, the seeds were wrapped with gauze for germination, loosen once every 5 hours for air exchange and added water in the process, and the exposed seeds were selected for use when most seeds germinated.

Allelopathy assessment

In the previous work, we had separated four fractions (F2, F4, F6, and F8) from the extracellular fermentation of *Streptomyces* sp. HU2014 (Zhu et al., 2023). F2 and

F4 were dissolved with sterile water and F6 and F8 were dissolved in ethanol solution (0.4% v/v), which reached to 1 mg/mL, 5 mg/mL and 10 mg/mL concentration, respectively. Sterile water and 0.4% ethanol solution were as controls. 10 mL per treatment was added on the filter paper which fully covered the inside petri dish, then 5 per-germinated seeds were cultured in plate for 5 days at (25 ± 1) ℃. Every treatment was in triplicates. The shoot length, root length and fresh weight were measured.

The response index (RI) calculated using the method as Williamson reported (Williamson & Richardson, 1988). When T<C; RI = 1-C/T; when T ≥ C, RI = C/T-1. (C is the control value; T is the experimental value; RI is the allelopathic effect without unit;) It is a widely accepted international standard in recent years to use RI value to express the intensity of allelopathy between organisms. RI > 0 indicates that the medicament can promote the growth of plants, RI < 0 indicates that the medicament can inhibit the growth of plants.

Statistical analysis

Statistically significant differences ($P < 0.05$) were evaluated by an analysis of variance (ANOVA) followed by the Duncan's test using SPSS version 16.0 (SPSS Inc. Chicago, IL, United States). Simple linear regression analysis was carried out in SPSS 16.0. All data shown are the average value of independent replicates ± standard deviations (SDs). The microbial amplicon sequences were analyzed using Quantitative Insights Into Microbial Ecology 2 (Rai et al., 2021). The indices of alpha diversity and principal component analysis (PCA) were determined using the R package *phyloseq* (McMurdie & Holmes, 2013). The distances of Bray-Curtis coefficients visualized using non-metric multidimensional scaling (NMDS) and a redundancy analysis (RDA) were conducted using the R package Vegan (Oksanen et al., 2016).

2.3. Experiment 3. Study on fermentation processing of HU2014

2.3.1. Organism

R. solani YL-3 and HU2014 stored on PDA at 4°C until further use.

2.3.2. Basal culture media

●PDA medium (g/L): potato, 200; dextrose, 12; and agar, 12; pH 7.0–7.2.

●PD medium (g/L): potato, 200 and dextrose, 12; pH 7.0–7.2.

●GPY medium (g/L): glucose, 20; soybean peptone, 5; and yeast extract, 5; pH 7.0–7.2.

●Gause's No. 1 medium (g/L): soluble starch, 20; NaCl, 0.5; KNO_3, 1; $K_2HPO_4 \cdot 3H_2O$, 0.5; $MgSO_4 \cdot 7H_2O$, 0.5; and $FeSO_4 \cdot 7H_2O$, 0.01; pH 7.4–7.6.

●Czapek's medium (g/L): $NaNO_3$, 3; $K_2HPO_4 \cdot 3H_2O$, 1; KCl, 0.5; $FeSO_4 \cdot 7H_2O$, 0.01; sucrose, 30; and $MgSO_4 \cdot 7H_2O$, 0.5; pH 7.0–7.2.

2.3.3. Experiment equipment

●Spectrophotometer (Thermo Varioskan Flash, Waltham, USA)

●Lyophilizer (Christ ALPHA 1-4 LSC, Osterode, Germany)

2.3.4. Experimental design

Spectrophotometric Analysis of Cell-free Filtrates of HU2014 culture

HU2014 spores were inoculated into GPY medium and incubated as described earlier, and the fermentation broth was collected at 2-day intervals from days 2 to 20. Subsequently, the broth was centrifuged to separate the mycelia and supernatant. Then, the supernatant was filtered through a 0.22-μm candle filter. The collected supernatants were diluted 10-folds with sterile water, and their absorbance values were determined at 200–500 nm using a spectrophotometer (Thermo Varioskan Flash, Waltham, USA), multiplied with dilution factor, and denoted as Abs_{HU2014}.

Screening of Basal Culture Media

To increase the active metabolites production in HU2014, it is essential to optimize the culture conditions. The spores were scraped off the surface of a HU2014 colony using an inoculation needle and suspended in sterile water at a density of 1.5×10^5 spores/mL as determined using a haemocytometer. Briefly, 1.5×10^5 spores/mL HU2014 (5%) were inoculated into different media and incubated at 150 rpm for 10 days at 25 °C, such as PD, GPY, Gause's No. 1, Czapek's, and ISP (International streptomyces project) 1, 2, 3, 4, 5, 6, 7 (Shirling & Gottlieb, 1966). The mother solution of the heat sensitive components was sterilized with a bacteriological filter before use. HU2014 was cultured in PDA medium at 25°C for 12 days. Subsequently, the broth

was centrifuged using a high speed refrigerated centrifuge (Beckman Avanti J-25, California, USA) at 12,000 rpm for 10 min to separate the mycelia and supernatant. Then, the supernatant was filtered through a 0.22-μm candle filter. The resulting strain pellets were lyophilized (Christ ALPHA1-4 LSC, Osterode, Germany) and then individually weighted (Sartorius BSA 223, Shanghai, China). The bioassay was conducted as described above. The control was PDA medium without the cell-free filtrate and the experiments were performed in triplicate. The basal medium was predominantly selected based on the antifungal activity of the fermentation metabolites from *Streptomyces* spp. (Song et al., 2012; Al-Dhabi et al., 2020).

Single-factor Experiments for Optimization of Fermentation Conditions

The fermentation conditions were optimized by one-factor at a time approach. To determine the effect of incubation time, temperature, agitation speed, inoculum size, and pH on the antifungal activity of the bioactive metabolites, univariate analysis was performed using HU2014 (1.5×10^5 spores/mL) cultivated in GPY medium under the following conditions: fermentation time (at 3-day intervals over 28 days), temperature (10, 20, 25, 30, 35 and 40°C), agitation rate (60, 90, 120, 150 and 180 rpm), inoculum size (0.5%, 1%, 5%, 10% and 15%), and initial pH (5.0, 6.0, 7.0, 8.0, 9.0 and 10.0). The bioassay and biomass measurements were conducted as indicated previously.

Single-factor Experiments for Optimization of Medium Components

Medium components play a crucial role in microbial fermentation. To screen the best medium for achieving higher bioactive metabolites production in *Streptomyces* sp. HU2014, various carbon sources (including glucose (GLU), mannose (MAN), arabinose (ARA), galactose (GAL), starch (STA), maltose (MAL), glycerol (GLY), and dextrin (DEX)), nitrogen sources (including potato extract (PE), beef extract (BE), yeast extract (YE), soybean peptone (SP), malt extract (ME), and oat extract (OE)), and inorganic salt content (KH_2PO_4, $MgSO_4 \cdot 7H_2O$, NaCl, $CaCl_2$, $FeSO_4$, and KNO_3) were examined using OFT approach (Tortora et al., 2015). First, by employing GLU as the sole carbon source and 10 g/L nitrogen source (PE, BE, YE, SP, ME, and OE, respectively), the *R. solani* YL-3 mycelial growth inhibition rate was evaluated as described earlier and the optimal nitrogen source was selected. Subsequently, the effect

of optimal nitrogen source concentration (1.0, 2.5, 5.0, 10.0, 15.0, 20.0, 25.0, and 30.0 g/L) on mycelial growth inhibition rate was studied. Similarly, by using SP and YE as the nitrogen sources and 20 g/L carbon source (GLU, RAF, STA, MAL, GLY, and DEX, respectively), the *R. solani* YL-3 mycelial growth inhibition rate was evaluated as described earlier and the optimal carbon source was selected. Then, the effect of optimal carbon source concentration (15.0, 20.0, 25.0, 30.0, 35.0, 40.0, and 45.0 g/L) on the mycelial growth inhibition rate was assayed. Finally, by adding 1 g/L inorganic salt to the GPY medium, and the optimal inorganic salt was selected based on the *R. solani* YL-3 mycelial growth inhibition rate as described earlier. Subsequently, the effect of inorganic salt concentration (0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 g/L KNO_3) on mycelial growth inhibition rate was evaluated. As no significant differences in *R. solani* YL-3 mycelial growth inhibition rates were observed among the 10-fold diluted filtered supernatants of HU2014 incubated with the above-mentioned combination of medium components, 50-fold diluted filtered supernatant was employed from this experiment.

Optimization of the Main Factors by Placket-Burman (PB) Design

In this study, to screen the main factors in fermentation process. A total of eight parameters such as DEX, YE, KNO_3, pH, inoculum size, temperature, agitation speed, and incubation time were provided in Table 2.2 and Table 2.3.

Table 2.2

Experimental variables at two levels used for the antifungal effect of the EF from HU2014 broth using PB design

Parameters	Code	Levels	
		-1	+1
Dextrin (g/L)	A	30	40
Yeast extract (g/L)	B	5	10
KNO_3 (g/L)	C	1.5	2.5
pH	G	6.8	7.2
Inoculum size (%)	H	10	20
Temperature (°C)	D	20	30
Agitation speed (rpm)	E	120	180
Incubation time (days)	F	8	12

Note: A total of eight parameters such as DEX, YE, KNO_3, pH, inoculum size, temperature, agitation speed, and incubation time were screened at two levels, namely, maximum (+) and minimum (-).

Table 2.3

PB design for the optimization of parameters influencing antifungal activity of the EF from HU2014 broth

Run	A	B	C	D	E	F	G	H	Inhibition rate (%)
1	1	1	-1	-1	-1	-1	-1	-1	70.26
2	1	1	1	-1	-1	1	1	1	75.64
3	1	1	-1	1	1	1	-1	1	88.61
4	1	1	-1	-1	-1	1	-1	-1	75.37
5	1	1	-1	1	1	1	1	-1	88.35
6	1	1	1	1	-1	-1	1	-1	90.21
7	1	1	1	1	1	-1	-1	-1	93.78
8	1	1	-1	-1	1	-1	1	1	74.90
9	1	1	-1	1	-1	-1	1	1	83.66
10	1	1	1	-1	1	-1	-1	1	84.53
11	1	1	1	1	-1	1	-1	1	92.33
12	1	1	1	-1	1	1	1	-1	83.61

Note: A represents dextrin; B represents yeast extract; C represents KNO_3; D represents pH; E represents inoculum size; F represents temperature; G represents agitation speed; H represents incubation time.

Optimization of the Selected Factors Using Face-centered Central Composite Design

Each experiment point was tested according to the principle of randomness, and the data of inhibition rate were obtained and statistically analyzed. Each of the three input factors was varied at three levels, as shown in Table 2.4. A total of 20 experiments were conducted using RSM-based face-centered central composite design (FCCD) matrix (Table 2.5).

Table 2.4

Input factors with their ranges and levels

Input parameters	Minimum value (g/L)	Maximum value (g/L)	Level
Dex	30	40	3

YE	5	10	3
KNO_3	1.5	2.5	3

Note: DEX represents dextrin; YE represents yeast extract.

Table 2.5

RSM-based FCCD experimental design matrix for three factors

Run	C.S: Dextrin (g/L)	N.S: yeast extract (g/L)	S: KNO_3 (g/L)	IR (%)
R1	40.00	10.00	1.50	89.56
R2	30.00	10.00	1.50	81.31
R3	30.00	10.00	2.50	86.53
R4	35.00	10.00	2.00	90.17
R5	40.00	10.00	2.50	92.96
R6	30.00	5.00	2.50	74.88
R7	35.00	7.50	2.50	96.36
R8	35.00	7.50	2.00	88.83
R9	35.00	7.50	2.00	87.14
R10	40.00	5.00	2.50	82.52
R11	30.00	5.00	1.50	72.33
R12	35.00	7.50	2.00	89.20
R13	35.00	7.50	2.00	93.08
R14	35.00	7.50	1.50	94.54
R15	40.00	7.50	2.00	96.24
R16	40.00	5.00	1.50	76.70
R17	35.00	7.50	2.00	90.41
R18	35.00	7.50	2.00	94.66
R19	35.00	5.00	2.00	80.22
R20	30.00	7.50	2.00	87.01

Note: C.S represents carbon sources; N.S represents nitrogen sources; I.S represents Inorganic salt; IR represents inhibition rate.

Statistical analysis

A value of $P < 0.05$ was considered significant in this study. Simple linear

regression analysis was performed in SPSS 16.0. All the data shown are the average value of three biological replicates ± SD. Minitab software (version: 20.3.0, Minitab LLC, Pennsylvania, USA) was used for Placket-Burman design experiments. Expert design (version:12.0.3.0 Stat-Ease, Inc., Minneapolis, USA) was used to generate the experimental designs and construct 3D surface plots and 2D contour plots to illustrate the relationship between response and effect of the investigated parameters and their experimental levels used in the present study.

2.4. Experiment 4. Field application of HU2014 in wheat plants

2.4.1. Conditions

The experimental field is located in Xinxiang City, Henan Province, China. Xinxiang is a part of the alluvial plain of the Yellow River and around the edge of the Taihang Mountains. The parent material of soil is river alluvium, and the whole texture is heavy. The arable layer is loamy clay, and the soil or silty clay is below it. The reaction of the soil solution is close to slight alkaline (pH =7.9-8.3). The content of organic matter is 1.21%, the total nitrogen is 0.078%, the available phosphorus is 7 ppm and the available potassium is 215 ppm. Xinxiang is characterized by a temperate-subtropical climate and belongs to the zone of humidity and monsoon. The soil has strong cohesiveness, good water and fertilizer conservation performance and high content of available nutrients.

The weather conditions of the research period were close to the long - term average with some tendency to warming and aridization.

The average long - term data on the dynamics of monthly temperatures and precipitation during the growing season are presented in Figure 2.1.

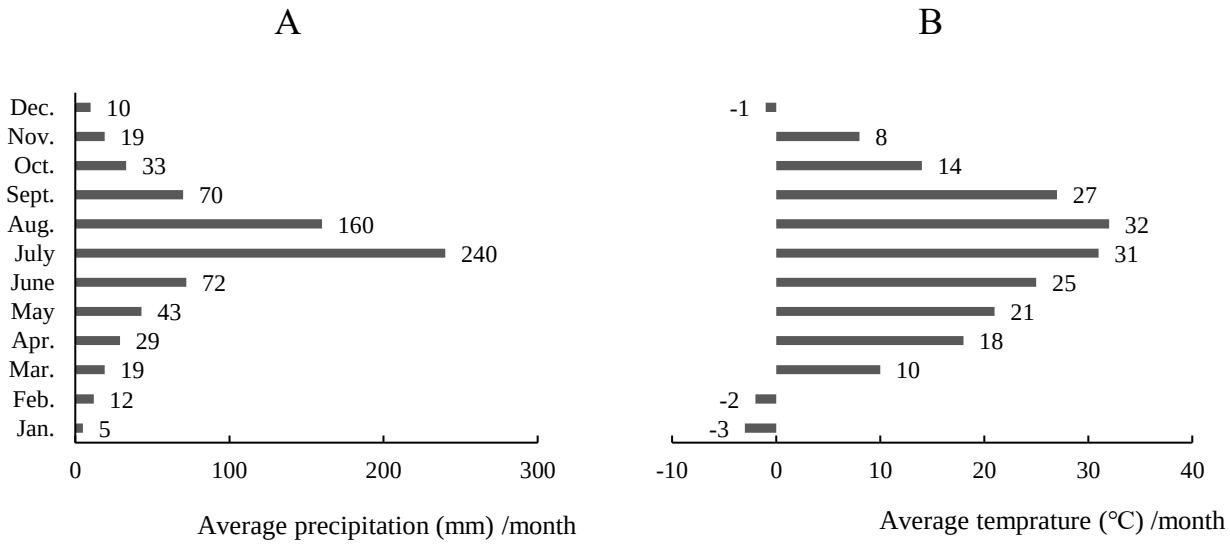


Figure 2.1. Long - term average monthly temperatures and precipitation in the study area (the experimental field of Henan Institute of Science and Technology).

From Figure 2.1, The winter wheat sowing in China generally starts in the cold dew of the 24 solar terms, that is to say, in the middle and late October of each year. The harvesting time is in late May. The annual average temperature is 14.4℃, the annual precipitation is 739.4 mm and the frost free period is about 219 days. The total indicators of the active temperatures sum (>=10℃) during the agronomic year of the region were 4727ºC. Steady heavy rainfal was recorded from 14.07.2021 to 26.07.2021.we concluded that the average temperatures of October was 14ºC and the average precipitation was 33 mm. This month was characterized by a lower humidity level. Average emperatures in the winter months ranged from - 1 in December to - 3 in January. The last month is considered to be the coldest, which is favourable to the winter crops. March is the tillering stage of wheat, there was a transition of average temperatures through the mark of + 10ºC. The aveage precipitation was 19 mm, which showed that proper irrigation was necessary in this stage. The last month of spring (May) was characterized by the higher temperatures and precipitation. This temperature dynamics provides the accumulation possibility of the sum of active temperatures. deviations in the dynamics of temperatures (especially precipitation) cause fluctuations in yields.

In general, the complex of hydrothermal conditions, the years of research were

favorable for the growth, development and formation of a high level of productivity of winter wheat.

2.4.2. Materials

R. cerealis G11 colony were inoculated on the sterilized grain and cultured for 28 D at 25℃ (Zhou et al., 2020).

HU2014 and ZM22 were supplied as described above.

2.4.5. Main reagents

●GPY medium (g/L): glucose, 20; soybean peptone, 5; and yeast extract, 5; pH 7.0–7.2.

●Ethanol and acetone (Kaitong Chemical Reagent Co., Ltd, Tianjin, China).

●MDA kit (Beijing Solarbio Science & Technology Co., Ltd, China)

●Root activity kit (Beijing Solarbio Science & Technology Co., Ltd, China)

2.4.6. Methods of research

Preparation of Microbial Soil

The 1×10^5 spores/mL of HU2014 was cultured (5% inoculation amount) in 10 L fermentation tank filled with GPY medium at 25°C for 6 days, with the strain in logarithmic growth. Then the fermentation broth was centrifuged at 12,000 rpm. After discarding the supernatant, the strain pellets were mixed with a small amount of sterilized soil, covered with plastic for 3 days.

Preparation of Wheat Seeds Infected by *R. cerealis* G11

After cleaning 2L of wheat seeds with distilled water, the seeds were put into a 250ml conical flask (200 mL per flask), in which an amount of water was left. These seeds were sterilized at 120℃ for 15 minutes, infected by *R. cerealis* G11, and then were incubated at 25℃ for 28 days. During this period, the seeds were shaking at regular intervals to make the strains e grow evenly.

Field Experiment

Trails by randomized block design

The field experiment was conducted in the trial zone of HIST, which was wasteland transformation with medium - low soil fertility. The plot area is 56 m^2. There

are 7 lines in each cell with the line spacing 0.25 m and the length 1.6 m. Two treatments in 2021 were as follows: the soil without treatment (CK) and the soil with HU2014 inoculation (S). Four treatments in 2022 were as follows: the soil without treatment (CK), the soil with HU2014 inoculation (S), the soil with *R. cerealis* G11 infection (P) and the soil with HU2014 *R. cerealis* G11 co-inoculation (SP). Each treatment had three duplicates. In the cell with HU2014 inoculation, the strain mixed with soil at the ratio of 5 g:1000 g. The main tillage, harrowing and cultivation were done according to the scheme. 100 germinated seeds of ZM22 were sowed in the lines per cell, respectively. The treatments with *R. cerealis* G11 infection were conducted: 100 mL/line of the infected seeds were followed after the germinated seeds sowing.

20 plants were sampled from each treatment to investigate the shoot height, number of tillers, chlorophyll content, malondialdehyde (MDA) and root activity at the wheat tiller stage. At the wheat maturation stage, spike length, grains per spike and 1000 grain weight were carefully evaluated.

Determination of chlorophyll content

Acetone extraction method was used for extracting chlorophyll of wheat leave and spectrophotometer method was used for determining the chlorophyll content (Miao et al., 1998). The extraction solution was prepared by mixing acetone and ethanol at a volume ratio of 1:1 and the surface of wheat leaves was cleaned and cut into pieces. 0.2 g leaves were filled into a 25 mL volumetric flask with 15 mL of solution, kept in dark place at room temperature for 24 h, and then the extraction solution was added to 25 mL. The absorbance value was measured at 646 nm and 663 nm, calculated the concentration of chlorophyll a and b according to the formulas below:

Chlorophyll a: $Ca = 12.21 \times A663 - 2.81 \times A646$;

Chlorophyll b: $Cb = 20.13 \times A646 - 5.03 \times A663$;

Where Ca and Cb represents the mass concentration of chlorophyll a and b, respectively.

Determination of MDA and root activity

0.1 g tissues of wheat were immediately submerged in liquid nitrogen. Material

was ground in mortar with a pestle under liquid nitrogen, transferred into centrifugal tube. MDA and root activity were determined according to the instruction of kits (Beijing Solarbio Science & Technology Co., Ltd, in China).

Statistical analysis

A value of $P < 0.05$ was considered significant in this study. Simple linear regression analysis was performed in SPSS 16.0. All the data shown are the average value of three biological replicates ± SD.

Conclusions to Chapter 2

The investigation of this section shows that the sequence of HU2014 was identified using the Oxford Nanopore Technologies standard protocol at BioMarker, Beijing, China. Based on the genome sequence, the ANI analysis was used the J Species WS Online Service according to Richter et al. (2016). The antifungal activities of this strain against *R. cerealis* G11 and *R. solani* YL-3 in vitro and the antifungal efficiency test on WSE in pots were investigated. UPLC-MS on the four extracts from the extracellular filtrate of HU2014 was used to identify the chemical constituents in the Natural Products Atlas with high match levels (more than 90%).

What's more, we began with POD, PPO, and PAL enzymes in different concentration of M and EF of the strain with the application of soil drench treatment. The enzyme activities were determined by visible spectrophotometry. Subsequently, F3 was used to assess its effect on WSE caused by *R. cerealis* G11. The activities of defense-related enzymes in the leaves of three wheat cultivars (BN 58, BN 307 and ZM 22), POD, PAL, and GLU, were investigated 1^{th} -6^{th} day post treatments. Furthermore, we used quantitative reverse transcription polymerase chain reaction to perform transcriptional profiling of six disease-resistant genes (*PR1, PR2, PR3, PR5, PAL,* and *LOX*) for three wheat cultivars at 2, 6, 12, 24, 48, 72, and 96 h post inoculation, with a fermentation of HU2014.

Next, we aimed at assessing the effect of this strain inoculation on wheat growth and rhizosphere microbial communities. The soil physiochemical properties and the response of rhizosphere microbial communities using high-throughput sequencing of

the 16S rRNA gene and the internal transcribed space region were determined.

Also, we assessed the allelopathy of HU2014 metabolites on wheat and green bristlegrass using the response index (RI) (RI > 0 indicates that the medicament can promote the growth of plants, RI < 0 indicates that the medicament can inhibit the growth of plants).

Another way, we screened the fermentation and medium conditions for this strain's culture using single-factor method, Placket-Burman (PB) Design and RSM-based face-centered central composite design (FCCD) (version:12.0.3.0 Stat-Ease, Inc., Minneapolis, USA).

Finally, we applied this strain in field to verify the effect of HU2014 on wheat growth and coping with wheat resistance to WSE.

CHAPTER 3

STUDY ON ANTIFUNGAL MECHANISM OF HU2014 AGAINST *R. CEREALIS* G11 AND *R. SOLANI* YL-3

3.1. Genome sequencing and ANI calculation

In the previous tests, we found HU2014 had broad-spectrum antifungal activities. To explore further its action mechanism, we identified this strain from molecular level. The results were shown in Figure 3.1and Table 3.1.

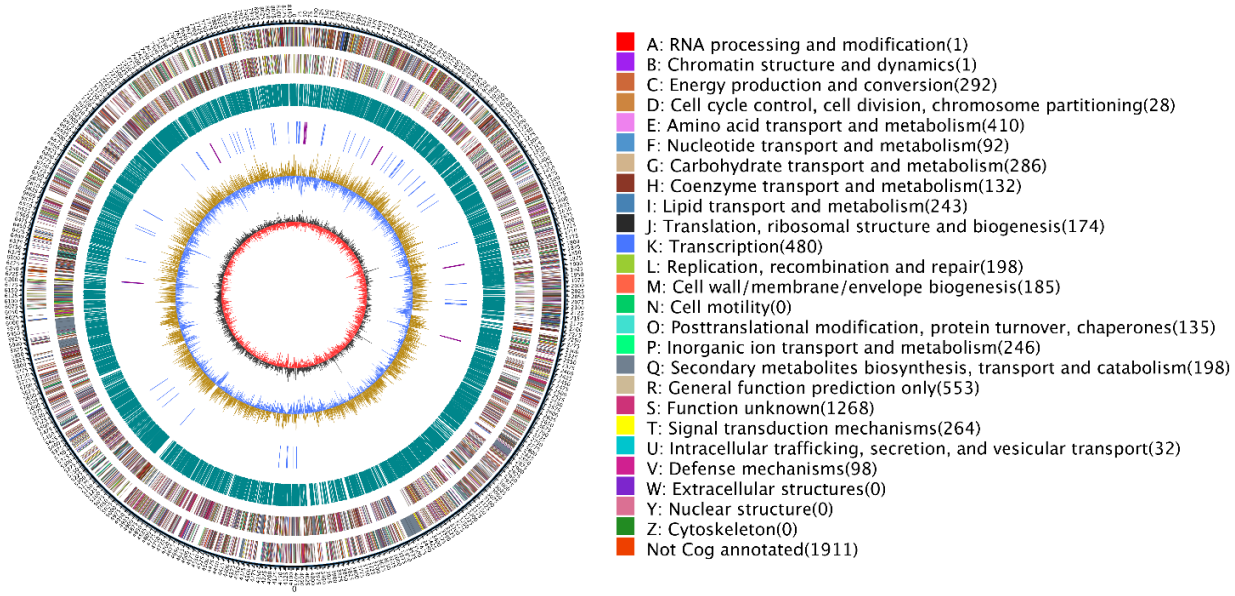


Figure 3.1. Genome sequencing of HU2014 (HERCCGE, HIST, 2020)

As shown in this figure, the size of complete genome sequence of HU2014 was 8,170,612 bp with 72.7% of GC content. Circular genome map for HU2014 using CG View (Figure 3.1). The outermost circle indicates the genome size. The second and third circles indicate CDS on forward and reverse strands colored according to COG category. The fourth circle is the repetitive sequences. The fifth circle shows the tRNA (blue) and rRNA (purple). The sixth circle shows G+C content in light yellow (+) and purple (-). The innermost circle shows the G+C skew in deep grey (G content more than C content) and red (G content less than C content). The scale is shown in the innermost circle. The different colors in the gene outer circle represent the function of the genes in this region.

Table 3.1

ANI analysis between HU2014 and the closest type strains of *Streptomyces* species (HERCCGE, HIST, 2021)

TS	Genome	ANIb and [aligned nucleotides] (%)	ANIm and [aligned nucleotides] (%)
[T]	*Streptomyces albireticuli* NRRL B-1670	93.30 [70.52]	94.01 [81.09]
[T]	*Streptomyces eurocidicus* CECT 3259	91.94 [66.15]	92.94 [75.82]
[T]	*Streptomyces eurocidicus* ATCC 27428	91.92 [66.43]	92.94 [75.70]
[T]	*Streptomyces roseifaciens* MBT76	83.14 [51.41]	87.50 [48.01]
[T]	*Streptomyces hiroshimensis* JCM 4586	82.76 [49.75]	87.39 [45.64]
[T]	*Streptomyces netropsis* CECT 3265	82.34 [49.19]	87.30 [42.98]
[T]	*Streptomyces netropsis* JCM 4063	82.29 [47.85]	87.29 [43.96]
[T]	*Streptomyces griseocarneus* JCM 4580	81.48 [46.65]	86.96 [39.77]
[T]	*Streptomyces abikoensis* JCM 4002	80.92 [45.11]	86.63 [36.73]
[T]	*Streptomyces flaveolus* JCM 4032	76.79 [33.73]	85.31 [18.33]

Note: TS represents type strain; ANIb and ANIm values indicate the pairwise comparisons o given genomic sequences with the genome of HU2014.

From the above chart, after gene sequence alignment, the top 10 species of *Streptomyces* with similar genetic relationships were selected. ANI analyses based on the genome of HU2014 revealed a value below the threshold of 95% needed for species demarcation (Table 3.1). *Streptomyces albireticuli* NRRL B-1670, *Streptomyces eurocidicus* CECT 3259, and *Streptomyces eurocidicus* ATCC 27428 were the closely species to HU2014, with the ANIb and ANIm values more than 91.00% and 92.00%, respectively. Other species were *Streptomyces roseifaciens* MBT76, *Streptomyces hiroshimensis* JCM 4586, *Streptomyces netropsis* CECT 3265, *Streptomyces netropsis* JCM 4063, *Streptomyces griseocarneus* JCM 4580, *Streptomyces abikoensis* JCM 4002, and *Streptomyces flaveolus* JCM 4032, respectively. The ANIb and ANIm values of them all were lower than 90%.

HU2014 was distinct from the type strains of closely related species. Considering these results, we propose that HU2014 is a novel *Streptomyces* species,

named *Streptomyces* sp. HU2014 (preserved in the China Center for Type Culture Collection under preservation number M2015207). The complete genome sequence of HU2014 has been deposited in GenBank under the accession number CP097123. *S. albireticuli* and *S. eurocidicus* were two species with closer genetic relationships to HU2014, and studies had been reported for their active secondary metabolites production and/or plant growth-promoting capacities (Fukushima et al., 2002; Huang et al., 2016; Wang et al., 2018; Zhang et al., 2021).

From the functional annotations of the bacterial genome, we have noticed protein subcellular localization analysis and annotation of antiSMASH metabolic gene clusters (not noted in this text), which will provide a good reference for future molecular level research on the antibacterial mechanism of this bacterium. Gene or gene cluster candidates of HU2014 responding to antifungal activities would be researched further.

3.2. Antagonistic activity of HU2014 against *R. solani* YL-3 and *R. cerealis* G11

To study the antifungal activity of HU2014 against wheat sharp eyespot caused by *R. solani* YL-3 and *R. cerealis* G11, we conducted the co-culture and scanning electron microscope tests, and the results were exhibited as follows (Figure 3.2 and Figure 3.3).

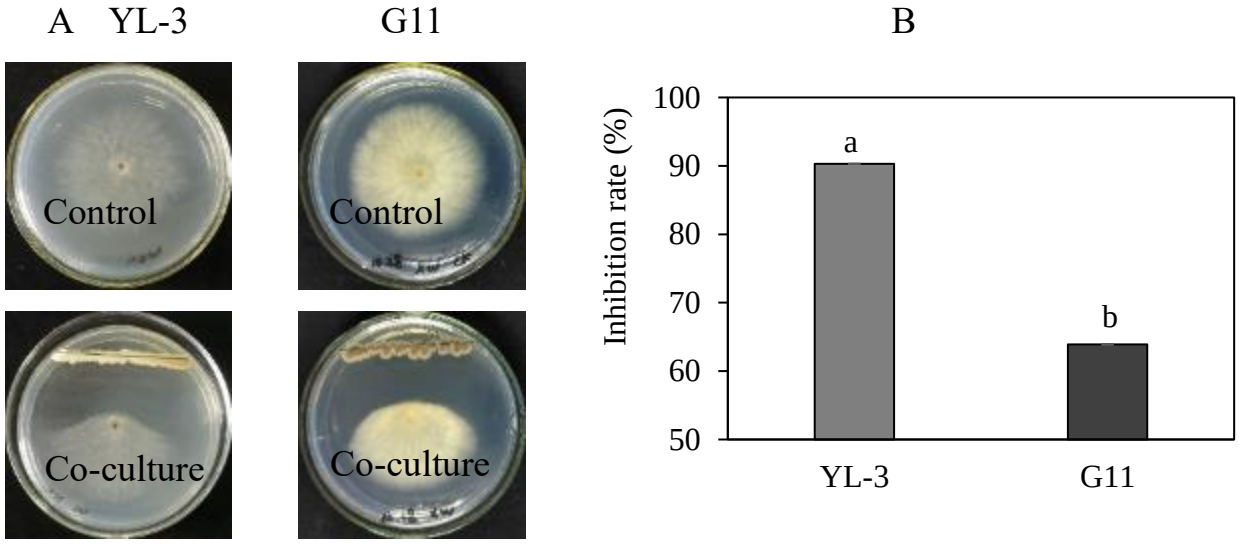


Figure 3.2. The inhibition effects of HU2014 on *R. solani* YL-3 and *R. cerealis* G11 in vitro (HERCCGE, HIST, 2022). (A) Colony diameter of YL-3 and G11. (B) Antagonistic effect of HU2014 against YL-3 and G11. The data are the mean ± SD of

three independent replicates. One way-ANOVA ($p < 0.05$) was used to compare means and bars sharing a letter are not statistically different.

As shown in this figure, we could observe that the mycelia of *R. cerealis* G11 were denser than that of *R. solani* YL-3. The suppressed section of them obviously showed like a fan-shaped and a circular arc, respectively. Based on the results of inhibition rate, the inhibition of mycelial growth by HU2014 against *R. solani* YL-3 and *R. cerealis* G11 were 90.3% and 63.9%, respectively ($p < 0.05$). From Figure 3.2A, we could conclude that the inhibition action induced by HU2014 mainly depended on the active substances produced by this strain.

Streptomyces, as a genus of Actinobacteria, are abundant in soil and have been shown to suppress a range of phytopathogenic organisms, including *Magnaporthe oryaze*, *Fusarium* spp., *R. solani*, *Gaumannomyces graminis* and *Pythium* spp., both in vitro and in vivo (Coombs & Franco, 2003; Coombs et al., 2004; Tian et al., 2004; Wan et al., 2008; Jung et al., 2013; Wang et al., 2013; Viaene et al., 2016; Wu et al., 2019). In this study, HU2014 showed strong antagonism on *R. solani* YL-3 and *R. cerealis* G11, which provided expected values in the field of antifungal reagents development. What's more, reports on using *Streptomyces* spp. against *R. cerealis* are lacking, which is an exciting result.

It is interesting that the activity of this strain against *R. cerealis* was lower than that of *R. solani* in co-culture test, which was supposed the mycelial growth of *R. solani* was faster than that of *R. cerealis.* For in the previous work, we had witnessed that *R. solani* YL-3 covered the petri dish at the third day, but *R. cerealis* G11 at the sixth day. Next, we will investigate the effect of antifungal substance produced by HU2014 on these two fungi in pot experiments to examine their antifungal differences in different environments.

As shown in Figure 3.3, when compared to the control, the aerial hyphae of the two phytopathogenic fungi with HU2014 co-culture was hollow, and the cell wall of them was damaged under SEM. The mycelial morphology showed that the application of HU2014 could be a new method for suppressing *Rhizoctonia* spp.

Many studies about antifungal mechanism of microbe against phytopathogen reported that the cell wall of phytopathogen showed atrophy, splitting and lysis (Cuervo-Parra et al., 2011; Ma et al., 2014; Yang et al., 2019). Fungal cell walls are mainly composed of carbohydrates, such as chitin, deacetylated chitosan, dextran, cellulose, and galactose. The left is composed of proteins and glycoproteins, including enzymes responsible for cell wall growth, specific extracellular enzymes, and structural proteins that crosslink polysaccharides.

YL-3 G11

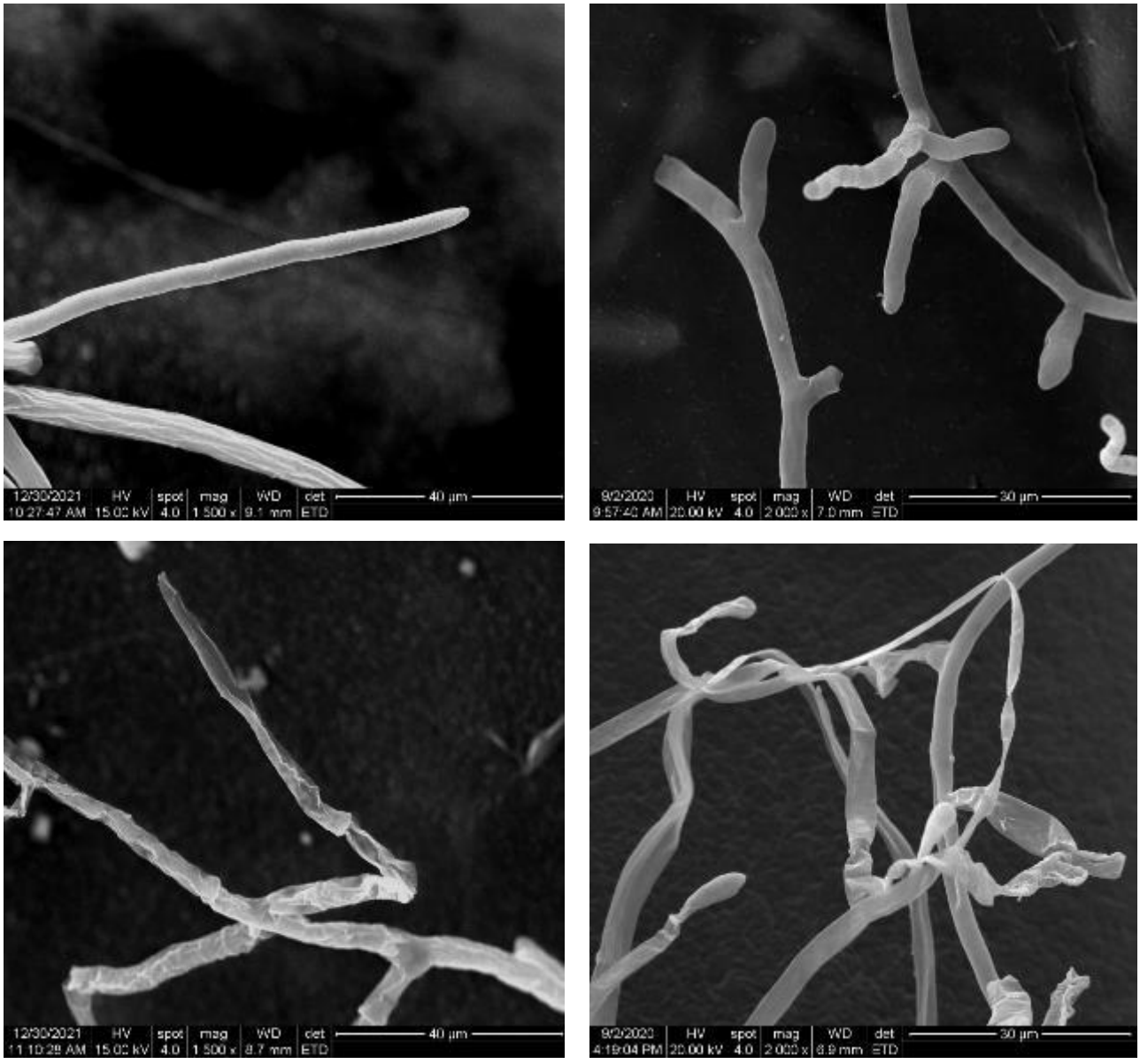
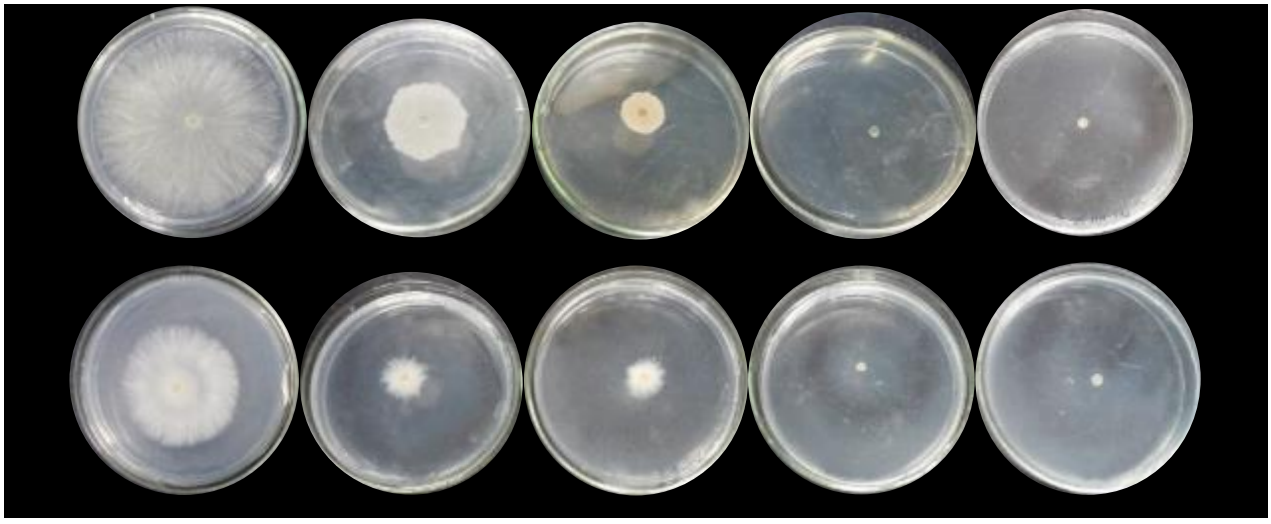


Figure 3.3 Mycelial morphology of *R. solani* YL-3 and *R. cerealis* G11 under a scanning electron microscope (HERCCGE, HIST, 2021).

In addition, there are small molecules such as lipids and inorganic salts. The cell wall is an essential component in fungal homeostasis (Lima et al., 2019). At the same time, fungal cell walls are also targets for attack by other exogenous substances. Macrolactin, a key antifungal substance of *Bacillus amyloliquefaciens* D2WM, could be one of the compounds destroying the cell wall a and decreasing the levels of cell-degrading enzymes (Chen et al., 2019). One study reported that the lipopeptides produced by *Bacillus amyloliquefaciens* C-1 lipopeptides damaged the integrity of *Clostridum difficile* cell wall and cell membrane (Lv et al., 2020). Further research will aim at the target effect of specific antibacterial substances produced by HU2014 on fungal cell walls.

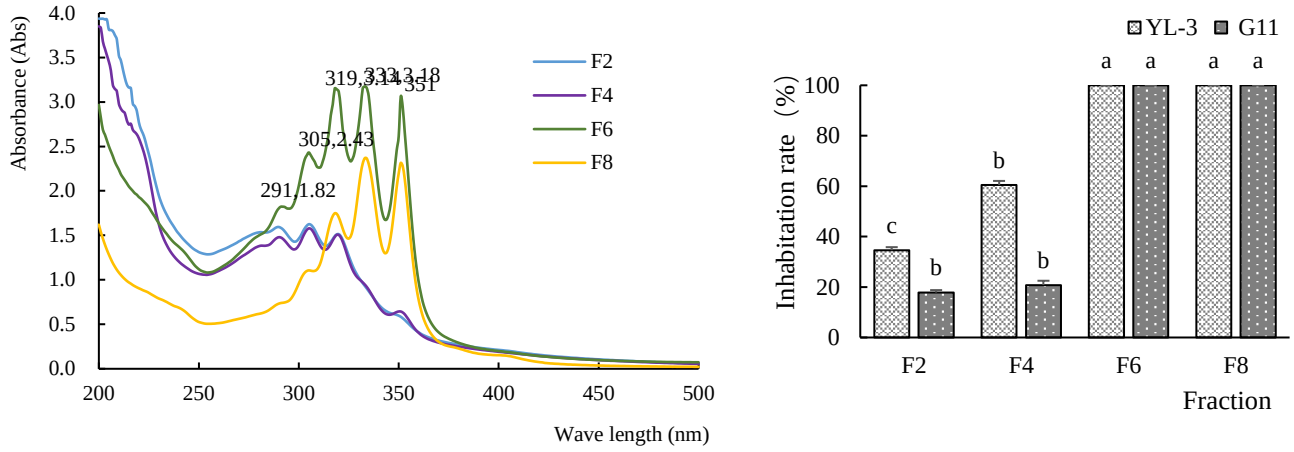
3.3. Enrichment and detection of bioactive metabolites

To study the antifungal mechanism of HU2014, the detection of the active compounds produced by this strain were important. Through these identified active compounds, we can further find the sites and ways in which these compounds act on phytopathogenic fungi, thereby better utilize the strain in agriculture. In the present test, scanning broad spectrum and antifungal activity of four fractions from HU2014 broth were firstly investigated and the results were shown in Figure 3.4 and Figure 3.5. Secondly, the active compounds were identified by UPLC-MS (Table 3.2).



Control F2 F4 F6 F8

Figure 3.4. The antifungal activities of four fractions against *R. solani* YL-3 (first row) and *R.cerealis* G11 (second row) (HERCCGE, HIST, 2022).



A B

Figure 3.5. Scanning broad spectrum of four fractions by spectrophotometer (A) and antifungal activity of four fractions of HU2014 against *R. solani* YL-3 and *R. cerealis* G11 (B) (HERCCGE, HIST, 2022). Values are means ± SD of three independent experiments. Means with the same letter for inhibition rate are not significantly different ($p<0.05$). F2, F4, F6 and F8 represent one of fractions, respectively.

As these figures shown, the result of spectrophotometric analysis was found that F6 and F8 had the same strong characteristic absorption peaks at 319 nm, 333 nm, and 351 nm (Figure 3.5 A). Although F2 and F4 had the absorption peaks at the same points, the strength was lower than F6 and F8. Bioassay revealed that F6 and F8 had the maximum inhibition rates of 100% to *R. solani* YL-3 and *R. cerealis* G11, followed by F4 and F2 (less than 60.50%) (Figure 3.4 and Figure 3.5 B). F6 and F8 had the highest antifungal activities, which were accordance with that of the stronger characteristic absorption peaks. From these results, we could conclude that the main active components in the filtrated supernatants were enriched in F6 and F8.

Streptomyces spp. have been researched much in screening the bioactive substances, which mainly started from their extracts or fractions (Srikesavan & Selvam, 2013; Lee et al., 2015; Naine et al., 2015). Few literatures reported the characteristic absorption spectrum of fraction isolated from *Streptomyces* spp. Spectral scanning of the separated components to determine the characteristic absorption spectra of highly active components is helpful for the detection and tracking of active substances. In this

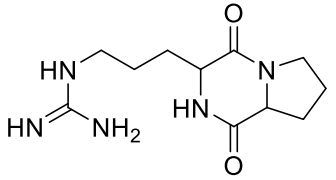
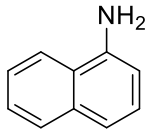
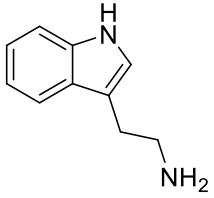
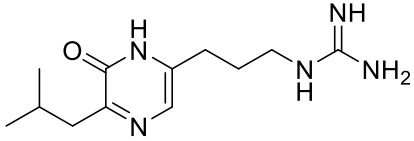
study, we investigated the distribution of the fractions of HU2014 by UV-Vis spectrometer at different nm points. Through this auxiliary analytical method, we could determine the distribution of four fractions combined with biological test. F6 and F8 exhibited high antifungal activities against *R. solani* YL-3 inoculated on the broad bean leaves (Zhu et al., 2023). From another hand, F2 and F4 had the same absorption peaks with F6 and F8 at the same wavelengths, but these don't mean they consist of same compounds. Therefore, the further research is to identify these active components.

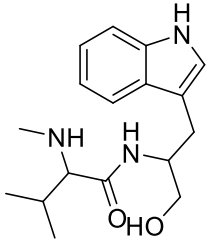
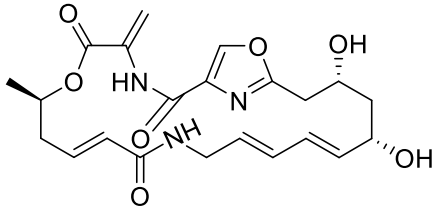
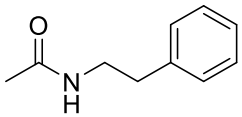
As shown in Table 3.2, UPLC-MS analysis was used for further identification of bioassay-guided extracts from the metabolites of HU2014. In this table, the main constituents were determined as aza-heterocyclic compounds when compared to the NP Atlas with a match degree of more than 90%. Based on their retention time, molecular mass, and chemical structures, these compounds were mainly verpacamide A (1), 1-naphthylamine (2), L-tryptamine (3), arglecin (4), valindolmycin (5), (7R,14E,16E,18S,20R,21E)-18,20-dihydroxy-7-methyl-4-methylene-6,23-dioxa-3,12,25-triazabicyclo[20.2.1]pentacosa-9,14,16,22(25),24-pentaene-2,5,11-trione (6), N-acetylphenethylamine (7), N-acetyltryptamine (8), L-tryptophan (9), and genistein (10) (Table 3.2).

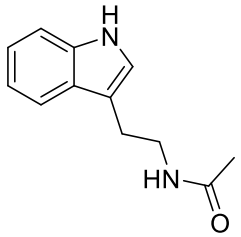
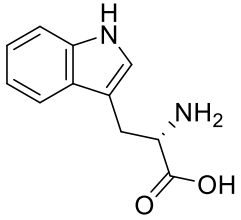
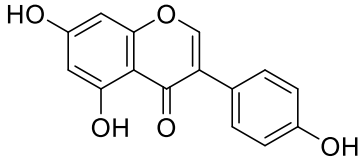
These identified compounds mostly possessed antibacterial, antifungal, antialgae, antitumor and antiangiogenic activities. Some newly discovered compounds with activities from cultures of *Streptomyces* species have been reported in recent years, such as Zunyimycin C isolated from the *Streptomyces* sp. FJS31-2 (Lu et al., 2017), RSP 01 produced by *Streptomyces* sp. RAB12 (Rathod et al., 2018), Xiakemycin A isolated from *Streptomyces* sp. CC8-201 (Jiang et al., 2015) and 3-dechloro-3-bromonapyradiomycin A1 derived from *Streptomyces* sp. strain SCSIO 10428 (Wu et al., 2013). In the further research, we will screen the above compounds and explore their action on *R. cerealis* G11.

Table 3.2

Compounds identified from four crude extracts of HU2014 (Testing Center, HIST, 2022)

Compound name	RT (min)	MF	Activity	Chemistry Structure	References
Verpacamide A	0.97	C11H19N5O2	Biogenetic Precursors		(Vergne et al., 2006) (Forte et al., 2009) (Arima et al., 2010)
1-Naphthylamine	3.53	C10H9N	NF		
Tryptamine	3.64	C10H12N2	Antifungal Excitor		(Greenberg, 1960) (Yan et al., 2017)
Arglecin	4.45	C12H21N5O	Antifungal		(Tatsuta et al., 1971) (Chen et al., 2018)

Compound name	RT (min)	MF	Activity	Chemistry Structure	References
Valindolmycin	5.22	C17H25N3O2	Antibacterial		(Jin & Cui, 1988)
(7R,14E,16E,18S,20R,21E)-18,20-dihydroxy-7-methyl-4-methylene-6,23-Dioxa-3,12,25-triazabicyclo[20.2.1]pentacosa-9,14,16,22(25),24-pentaene-2,5,11-trione（CAS: 2368928-46-3）	5.67	C22H27N3O7	Antimicrobial		(Xie et al., 2014)
N-Acetylphenethylamine	9.14	C10H13NO	NF		(Foster et al., 1988) (Huber et al., 2014)

Compound name	RT (min)	MF	Activity	Chemistry Structure	References
N-Acetyltryptamine	10.25	C12H14N2O	Antialgae Antibacterial		(Yi et al., 2019) (Zhang et al., 2013)
L-tryptophan	14.98	C11H12N2O2	Gastroprotective		(Yan et al., 2017) (Konturek et al., 1997)
Genistein	15.94	C15H10O5	Antitumor Antiangiogenic		(Büchler et al., 2003) (Banerjee et al., 2005)

RT represents retention time; MF represents molecular formula; NF represents not found.

3.4. Defense responses of wheat at the physiological and molecular level by HU2014 induction

Induced resistance in wheat by HU2014 at the physiological level

Inducing plant resistance is one of the main antibacterial mechanisms of microorganisms as biocontrol agents. In this part, our pot experiment was conducted under different concentrations of EF with soil drench treatment to carry the quantitative changes in the plant defense-related enzyme activities of POD, PPO, and PAL in wheat leaves. The changes of enzymes activities in wheat leaves were presented in Table 3.3.

Table 3.3

The effect of the EF on the enzyme activity in wheat leaves (HERCCGE, HIST, 2021)

Concentration	POD activity (U/g)	PPO activity (U/g)	PAL activity (U/g)
Untreated control	3476.61±273.37d	74.51±3.08abc	7.30±0.72c
Original EF	6870.61±219.65b	83.43±1.40ab	8.38±0.66bc
1×10^{-1} dilution	6567.13±135.63b	64.58±6.60c	8.91±1.15b
1×10^{-2} dilution	5619.70±145.51c	87.17±7.55a	9.33±0.62b
1×10^{-3} dilution	9522.38±106.33a	69.3±3.85bc	12.55±1.30a
1×10^{-4} dilution	3340.89±216.28d	86.79±4.15a	5.78±0.37d
1×10^{-5} dilution	2768.14±152.48e	67.23±5.30c	7.99±0.39bc

Note: Data in the table are means ± SD, Different lowercase letters in the same column show values that are significantly different at the $p < 0.05$ level by least significant difference (LSD) test.

As shown in this table, we determined the effect of the EF on three enzyme activities in wheat leaves. The activities of POD and PAL at the concentration of 10^{-3} dilution of the EF significantly increased in comparison with the control, by 173.9% ($p < 0.05$) and 71.9% ($p < 0.05$), respectively. It is shown that the EF can significantly

induce the activities of the above enzymes in wheat at a low concentration. Although the activity of PPO at the concentration of 10^{-2} and 10^{-4} dilution increased when compared to the control, by 17.0% ($p < 0.05$) and 16.5% ($p < 0.05$) respectively, the difference was non-significant.

In plant defense system, POD, PPO and PAL are the major defense enzymes, they are used as physiological indexes to identify plant disease resistance (Peng et al., 2019; Jinal & Amaresan, 2020). Few studies investigated that antimicrobial at very low concentrations have high inhibiting effect or eliciting activities (Winding et al., 2004; Hennessy & Stougaard, 2017; Newitt et al., 2019). In this study, the activities of POD and PAL at the concentration of 10-3 dilution of EF increased significantly to some extent in comparison with control. In other words, EF of *S.* HU2014 can significantly induce the activity of defense enzymes in wheat at low concentrations. From this point of view, the performance of POD and PAL activity is in agreement with the findings of Xie et al .(2019) and Liu et al.(2018), Moreover, the increase of induced enzyme activity was greater in this experiment. Therefore, this experiment showed that the S. HU2014 can significantly improve the resistance of wheat seedlings. The level of enzyme activity with M is lower than that of EF, this result showed that *S*. HU2014 is a soil-borne microorganism and has formed a stable colonization mechanism, its metabolites are stable in shaking flasks incubation. But the mycelia were transferred from lab flask to soil, it is difficult to predict the behavior of bacterial populations in the environment. Their plant-beneficial bioactivities in the environment may differ greatly from laboratory observations (Rey & Dumas, 2017). The enzyme activities measured at the physiological level of plants were easily affected by some factors, which need to be further verified at the molecular level. Further studies should be focused on wheat disease resistance with RT-PCR assay, metabolic pathways and transcriptomics research. According to the pre-test, to determine the quantitative changes in defense-related enzyme activities of POD, PAL and GLU in three wheat cultivar leaves, we conducted the experiments treated with the EE, *R. cerealis* G11, and co-inoculation. The results were shown as follows (Table 3.4, Table 3.5, Table 3.6 and Figure 3.6).

Table 3.4

Effect of the diluted EF on the activity of POD in three wheat cultivars (HERCCGE, HIST, 2021)

Treatment	POD activity (U/g)					
	1 D	2 D	3 D	4 D	5D	6 D
A-CK	8360.15±70.71c	7304.16 ±238.60b	12374.34 ±814.98a	11883.67 ±51.54b	11477.05 ±182.20b	11126.33 ±482.72b
A-F3	12999.79 ±610.32a	11189.37 ±85.37a	10678.83 ±568.60a	11240.78 ±573.73b	11444.77 ±269.94b	8966.98 ±811.79c
A-CKP	10271.94 ±89.16b	11570.46 ±484.22a	12203.22 ±288.58a	13055.00 ±416.58a	12905.97 ±855.31a	13814.62 ±415.24a
A-PF3	10264.55 ±325.23b	10533.13 ±659.22a	9779.47 ±146.55a	13126.62 ±158.60a	15143.10 ±834.56a	11016.78 ±52.63b
B-CK	8061.56±714.22c	8276.44±627.24c	11694.38±190.23b	6719.75±77.70b	7957.56±263.92c	6039.62±133.81d
B-F3	14003.77±597.22a	9346.88±479.41c	12525.84±307.44a	9958.59±446.28a	10078.29±9.33b	10072.17±261.87b
B-CKP	10989.69±517.73b	12780.74±921.75a	11809.31±143.66b	10019.03±752.69a	10553.03±702.00b	8839.99±55.14c
B-PF3	10570.16±353.12b	10536.78±127.52b	10488.78±235.72c	9491.81±192.16a	12819.45±692.56a	10485.72±525.69a
Z-CK	11545.89±498.93c	11825.78±192.88b	8801.11±11.64d	9031.77±0.76c	10699.97±110.36b	10897.94±179.70b
Z-F3	10496.20±364.19c	9924.03±816.25c	14789.72±544.09b	10713.63±307.7b	9610.95±354.27b	12502.48±119.53b
Z-CKP	13219.11±520.18a	13434.57±28.80a	15762.69±34.38a	11288.74±402.78b	15316.26±304.09a	14319.32±343.21a
Z-PF3	12370.11±472.14ab	13249.31±580.09a	13539.72±210.49c	13499.39±22.25a	15225.24±210.04a	8843.86±394.76c

Note: A, AK58; A-CK: control; A-F3: 10^3-fold diluted EF; A-CKP: *R. cerealis* G11 infected; A-PF3: *R. cerealis* G11 infected 24 hours post inoculation F3; B, BN307 and Z, ZM22 (same treatments as A). Data in the table are means ± SD; Different lowercase letters in the same column show values that are significantly different at the $p < 0.05$ level by least significant difference (LSD) test.

Table 3.5

Effect of the diluted EF on the activity of PAL in three wheat cultivars (HERCCGE, HIST, 2021)

Treatment	PAL activity (U/g)					
	1 D	2 D	3 D	4 D	5D	6 D
A-CK	23.14±0.31a	18.41±0.67a	19.77±0.31a	16.93±0.64c	18.75±0.77a	16.80±0.01b
A-F3	24.02±0.56a	19.76±0.42a	19.94±1.41a	22.77±0.26a	20.05±1.47a	16.07±0.10c
A-CKP	19.90±0.59b	19.95±0.86a	17.55±0.69ab	21.66±0.54ab	17.06±0.46b	18.21±0.51a
A-PF3	23.57±0.60a	18.42±0.40a	15.83±0.57b	20.97±0.05b	21.25±0.37a	15.85±0.12c
B-CK	17.29±0.10c	18.64±0.06c	9.72±0.80b	23.45±0.16c	17.74±0.71b	17.32±0.71b
B-F3	25.85±0.25a	23.03±0.95a	20.24±0.90a	27.33±0.33a	21.85±0.73a	19.20±0.72a
B-CKP	22.61±0.10b	19.25±0.39b	19.55±0.33a	25.30±0.24b	21.42±0.23a	17.64±0.23b
B-PF3	22.01±0.38b	21.07±0.64c	18.59±0.28a	20.87±0.04d	18.68±0.41b	17.26±0.42b
Z-CK	19.63±0.21a	16.95±0.49b	13.16±0.48b	24.10±0.01c	19.6±0.75b	18.37±0.67a
Z-F3	18.94±0.15b	19.91±0.64a	19.42±0.12a	27.25±0.06b	21.21±0.76a	17.37±0.76a
Z-CKP	19.96±0.17a	20.23±0.27a	18.89±0.13a	29.14±0.44a	17.42±0.01d	17.99±0.02a
Z-PF3	17.00±0.21c	20.57±0.81a	19.33±0.34a	29.37±0.01a	18.52±0.68c	17.91±0.73a

Note: A, AK58; A-CK: control; A-F3: 10^3-fold diluted EF; A-CKP: *R. cerealis* G11 infected; A-PF3: *R. cerealis* G11 infected 24 hours post inoculation F3; B, BN307 and Z, ZM22 (same treatments as A). Data in the table are means ± SD; Different lowercase letters in the same column show values that are significantly different at the $p < 0.05$ level by least significant difference (LSD) test.

Table 3.6

Effect of the diluted EF on the activity of GLU in three wheat cultivars (HERCCGE, HIST, 2021)

Treatment	GLU activity (U/g)					
	1 D	2 D	3 D	4 D	5D	6 D
A-CK	11.77±0.63ab	10.00±0.09ab	23.81±0.06b	16.48±0.70a	19.91±0.11a	15.65±0.35ab
A-F3	12.32±0.04a	9.05±0.18b	23.41±0.72b	16.60±0.13a	16.11±0.08c	16.07±0.46a
A-CKP	10.30±0.65b	11.35±0.99a	25.38±0.23a	15.78±0.01a	17.53±0.63b	16.37±0.23a
A-PF3	11.20±0.60ab	9.33±0.19b	25.21±0.34a	16.75±0.04a	16.49±0.52ab	14.65±0.47b
B-CK	9.10±0.22b	8.23±0.38a	20.49±0.34c	13.39±0.28d	16.31±0.04c	14.61±0.54b
B-F3	9.45±0.27ab	7.90±0.36ab	25.37±0.32a	17.10±0.01c	17.92±0.01b	15.90±0.32ab
B-CKP	10.20±0.55a	7.74±0.38ab	23.76±0.18b	18.66±0.40a	19.45±0.33a	16.36±0.50a
B-PF3	9.53±0.10ab	7.12±0.32b	24.75±0.50a	17.93±0.07b	17.66±0.46b	16.83±0.54a
Z-CK	5.49±0.15c	7.67±0.55c	23.28±0.30c	16.56±0.37b	20.01±0.35c	16.55±0.33b
Z-F3	11.09±0.08ab	10.56±0.73a	27.11±0.27b	17.21±0.28b	23.46±0.26ab	17.87±0.20a
Z-CKP	10.39±0.50b	9.74±0.12ab	28.09±0.13a	19.02±0.14a	23.14±0.15a	17.54±0.44ab
Z-PF3	11.84±0.33a	8.76±0.01bc	28.45±0.35a	18.90±0.29a	20.64±0.53bc	16.86±0.52ab

Note: A, AK58; A-CK: control; A-F3: 10^3-fold diluted EF; A-CKP: *R. cerealis* G11 infected; A-PF3: *R. cerealis* G11 infected 24 hours post inoculation F3; B, BN307 and Z, ZM22 (same treatments as A). Data in the table are means ± SD; Different lowercase letters in the same column show values that are significantly different at the $p < 0.05$ level by least significant difference (LSD) test.

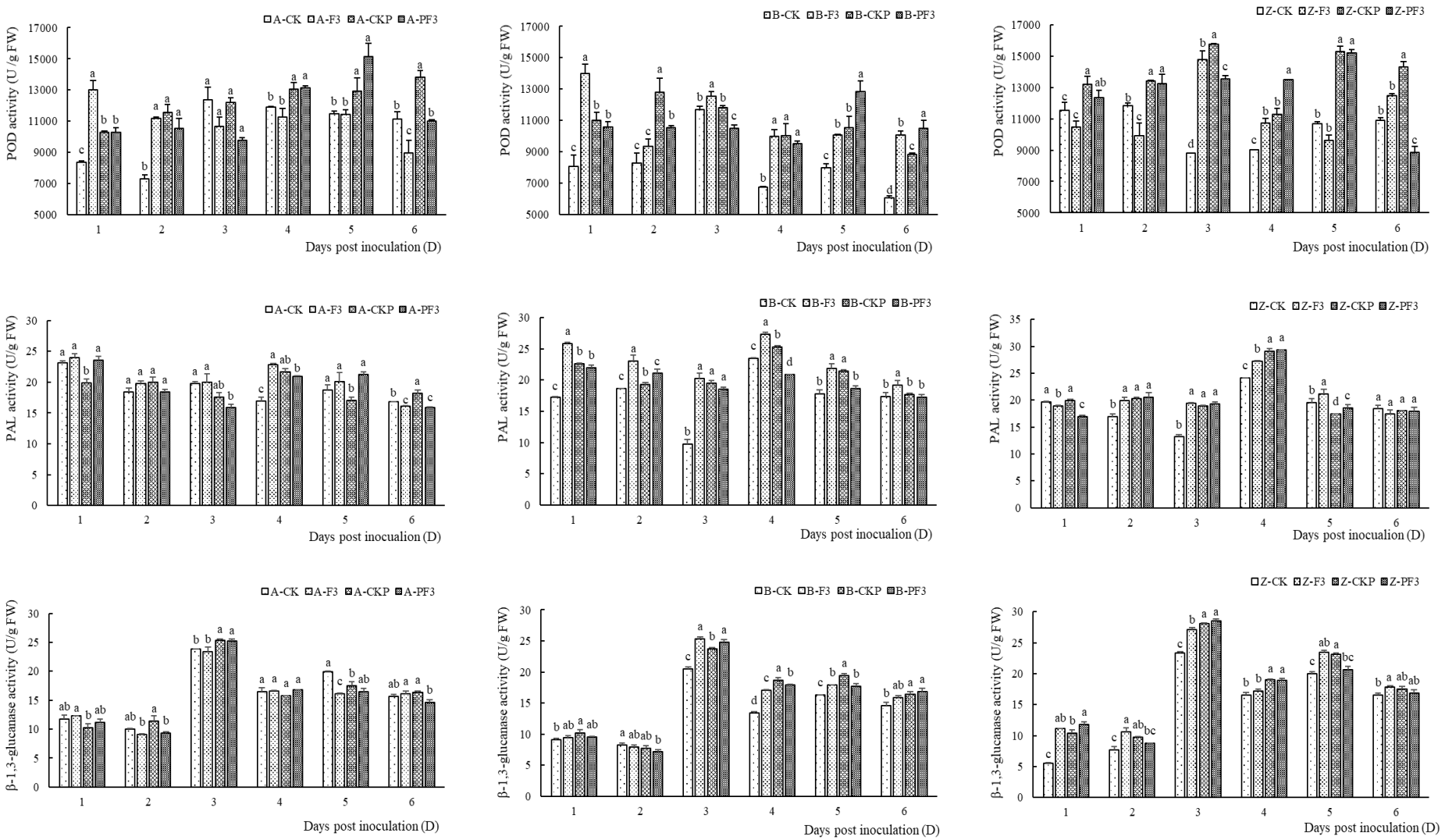


Figure 3.6. Quantitative changes in wheat defense enzymes by the diluted EF of HU2014 (HERCCGE, HIST, 2021). Different lowercase letters in the same column show values that are significantly different at the $p < 0.05$ level by least significant difference

test. A, AK58; A-CK: control; A-F3: 10^3-fold diluted EF; A-CKP: *R. cerealis* G11 infected; A-PF3: *R. cerealis* G11 infected 24 hours post inoculation F3; B, BN307 and Z, ZM22 (same treatments as A). Data in the table are means ± SD; Different lowercase letters in the same column show values that are significantly different at the $p < 0.05$ level by least significant difference (LSD) test.

From the above charts, we found that the activities of POD, PAL and GLU in wheat leaves with three different treatments increased significantly in comparison with untreated control at different time point. The quantitative changes of POD in AK58 and BN307 with F3 treatment had an obvious upward trend and peaked at 1 D, but in ZM22 with CKP treatment had that trend at 1 D and peaked at 3 D. The POD peak value with F3, CKP and PF3 treatments in AK58 was 12999.79 U/g (1.6 fold that of CK) at 1 D, 13814.62 U/g (1.2 fold that of CK) at 6 D and 15143.10 U/g (1.3 fold that of CK) at 5 D respectively; in BN307 was 14003.77 U/g (1.7 fold that of CK) at 1 D, 12780.74 U/g (1.5 fold that of CK) at 2 D and 12819.45 U/g (1.6 fold that of CK) at 5 D respectively and in ZM22 was 14789.72 U/g (1.7 fold that of CK) at 3 D, 15762.69 U/g (1.8 fold that of CK) at 3 D and 15225.24 U/g (1.4 fold that of CK) at 6 D respectively (Table 3.4, Figure 3.6). For PAL, the peak value with F3, CKP and PF3 treatments in AK58 was 22.77 U/g (1.3 fold that of CK), 21.66 U/g (1.3 fold that of CK) and 20.97 U/g (1.2 fold that of CK) at 4 D respectively; in BN307 was 27.33 U/g (1.2 fold that of CK) at 4 D, 25.30 U/g (1.1 fold that of CK) at 4 D and 22.01 U/g (1.3 fold that of CK) at 1 D respectively and in ZM22 was 27.25 U/g (1.1 fold that of CK) , 29.14 U/g (1.2 fold that of CK) and 29.37 U/g (1.2 fold that of CK) at 4 D respectively (Table 3.5, Figure 3.6). As to GLU, the peak value with F3, CKP and PF3 treatments in AK58 was 23.45 U/g (0.1 fold that of CK) 25.38 U (1.1 fold that of CK) and 25.21 U/g (1.1 fold that of CK) at 3 D respectively; in BN307 was 25.37 U/g (1.2 fold that of CK), 23.76 U/g (1.2 fold that of CK) and 24.75 U/g (1.2 fold that of CK) at 3 D respectively and in ZM22 was 27.11 U/g (1.2 fold that of CK), 28.09 U/g (1.2 fold that of CK) and 28.4 5 U/g (1.2 fold that of CK) respectively (Table 3.6, Figure 3.6).

Many studies have reported that rhizosphere microorganisms, such as *Streptomyces,* can induce host plants to produce defense response, form local or systemic acquired resistance and inhibit diseases (Van Loon, 1997; Zhao et al., 2012; Sunpapao et al., 2018; Abbasi et al., 2019). POD, PAL, and GLU selected in this study are the major defense enzymes in plant defense system (Peng et al., 2019; Jinal & Amaresan, 2020). Liu et al. reported that POD activity in wheat leaves significantly increased after soaking seeds using the diluted extracellular filtrate of *Streptomyces*

roche D74 and *S. partum* Act12 cultures (Liu et al., 2018). PAL activity in wheat leaves significantly increased by 58.7% after root irrigation with the fermentation broth of *Streptomyces rochei* ZZ-9 (Xie et al., 2019). In present study, the activities of POD, PAL, and GLU in the leaves of three wheat cultivars with the F3 of *Streptomyces* sp. HU2014, *Rhizoctonia cerealis* G11, and their co-inoculation treatments were changed significantly at different time. The results suggested that the defense system in AK58 and BN307 would be mainly induced by CKP, and that in BN307 mainly induced by F3. The performance of enzyme activity by different microorganism induced would be related to wheat cultivar characteristics. Although the multiple of enzyme activity increased, inducing plant disease resistance may not be the only advantage of this strain.

In another way, these oxidizing enzyme activities changes (POD and PAL) induced by HU2014 and *R. cerealis* G11 were closely related to reactive oxygen species (ROS) accumulation. GLU is a hydrolase and the activity change of it by HU2014 and *R. cerealis* G11 was weaker than POD and PAL. In the later stage, we will focus on the antifungal activity of HU2014 against wheat sharp eyespot in view of the experimental basis from molecular level.

Expression profile of genes induced by EF

Streptomyces can indirectly provide protection to their plant host through the activation of host resistance pathways which include ISR and SAR. To investigate the expression profiles of various disease resistance-related genes of wheat, six specific gene expression analyses were performed for AK58, BN307, and ZM22 at each sampling hour post-inoculation (hpi) with 500-fold diluted EF of HU2014 using QRT-PCR. The results were shown in (Figure 3.7).

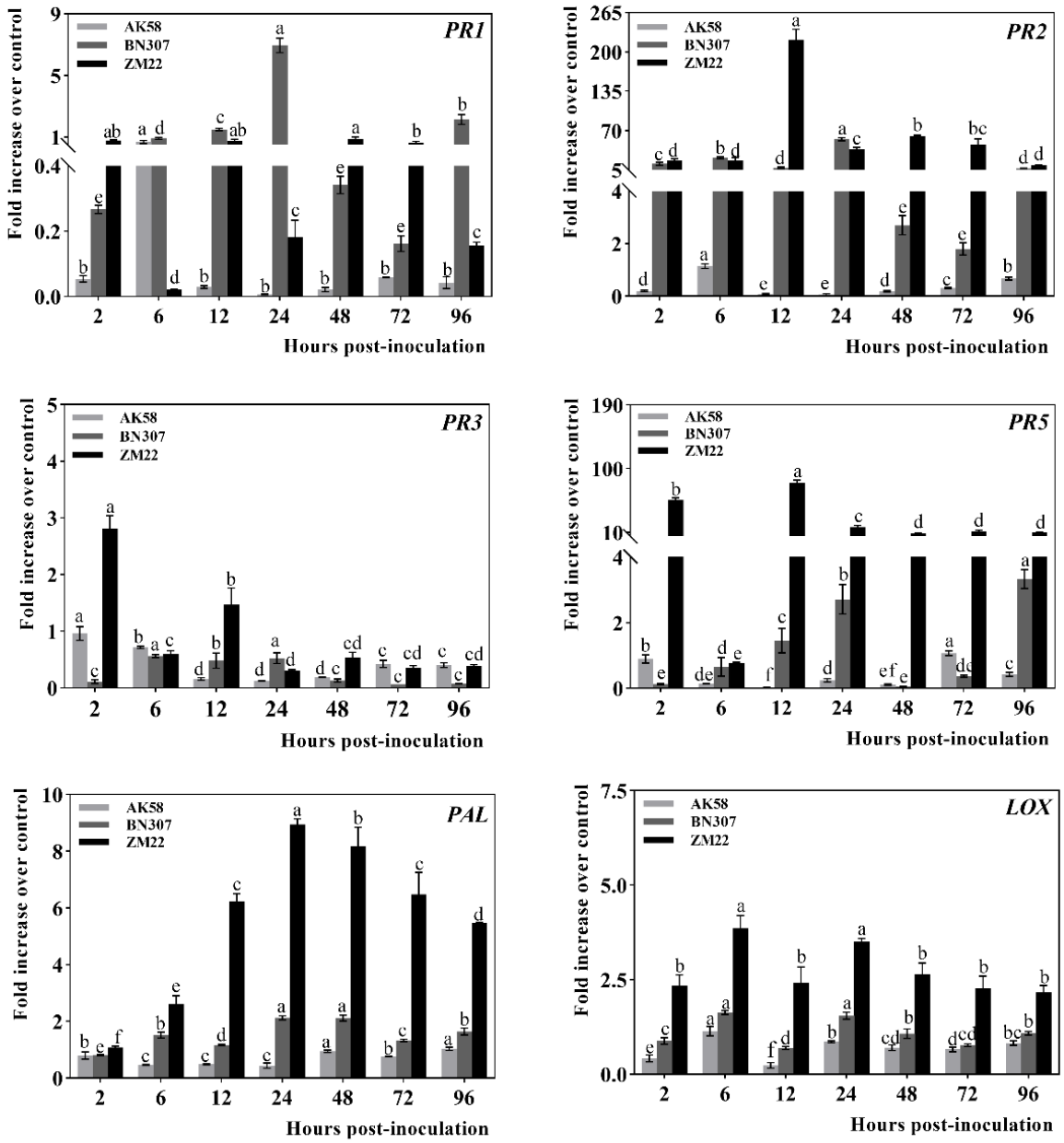


Figure 3.7. QRT-PCR analyses of six differentially expressed genes in three wheat cultivars (HERCCGE, HIST, 2022). Relative gene quantification was calculated by comparative $\Delta\Delta^{CT}$ method. Values are means ± SD of three independent experiments in six biological replicates. Statistical analysis was performed within the cultivar. Means with the same letter for fold change in transcript levels are not significantly different.

As shown in Figure 3.7, we investigated the relative genes expression in the leaves of three wheat varieties treated by HU2014 broth treatment. The accumulation of *PR1* transcripts was high at 6 hpi for AK58, 24 hpi for BN307, and 48 hpi for ZM22

with the fold values of 0.66, 6.95, and 0.82, respectively. The expression of *PR2* was found to have reached the maximum level at 6 hpi for AK58, 12 hpi for BN307, and 24 hpi for ZM22 with the fold values of 1.14, 55.70, and 210.47, respectively. Transcript accumulation of *PR3* gene came to a peak at 2 hpi for AK58, 6 hpi for BN307, and 2 hpi for ZM22 with the fold values of 0.96, 0.56, and 2.81, respectively. *PR5* gene peaked at 72 hpi for AK58, 96 hpi for BN307, and 12 hpi for ZM22 with the fold values of 1.17, 3.33, and 98.67, respectively. *PAL* gene showed the highest fold change occurring at 48 hpi for AK58, 24 hpi for BN307, and 24 hpi for ZM22 with the fold values of .1.02, 2.13, and 8.95, respectively. *LOX* gene expression peaked at 6 hpi for AK58, BN307, and ZM22 with the fold values of 1.14, 1.63, and 3.70, respectively. The data indicated that *PR1* gene was down-regulated in AK58 and ZM22, and *PR3* gene was down-regulated in AK58 and BN307. The highest expression levels of *PR2, PR3, PR5, PAL,* and *LOX* in ZM22 were higher than in AK58 and BN307, and that of *PR1* in BN307 was higher than in AK58 and ZM22.

In addition to direct inhibition via the production of antagonistic compounds, *Streptomyces* spp. can indirectly provide protection to their plant host from pathogens is though the activation of host resistance pathways (Conn et al., 2008; Tarkka & Hampp, 2008; Van Wees et al., 2008; Lugtenberg & Kamilova, 2009) . In oak trees (Quercus robur) inoculated by *Streptomyces* sp. AcH505, the expression of wide variety of genes involved in both ISR and SAR was upregulated, including PR genes and genes contributing to all of the signaling pathways (Kurth et al., 2014). Pathogenesis-related protein 1 (*PR1*), beta-1,3-glucanase (*PR2*), chitinase (*PR3*), and thaumatin-like protein (*PR5*) are defense-related genes. Phenylalanine ammonia lyase (*PAL*) is a rate-limiting enzyme of the phenylpropionic acid synthesis pathway, and lipoxygenase (*LOX*) is a key enzyme of the jasmonic acid pathway. Pathogenesis-related protein 1 (*PR1*) family genes are often used as molecular markers of disease resistance (Gamir et al., 2017; Pecenkova et al., 2017; Castorina et al., 2020). Beta-1,3-glucanase (*PR2*), and chitinase (*PR3*) can degrade beta-1,3-glucans and chitin, which are the main structural components of fungal cell walls, resulting in growth inhibition of fungi (Sharma, 2013; Patil et al., 2015). Thaumatin-like protein (*PR5*) is

widely distributed in higher plants, and its expression can be induced by a variety of biotic and abiotic stresses (Cao et al., 2016; Liu et al., 2021). Phenylalanine ammonia lyase (*PAL*) is a rate-limiting enzyme of the phenylpropionic acid synthesis pathway, and lipoxygenase (*LOX*) is a key enzyme of the jasmonic acid pathway. This study showed that 500-fold diluted EF of HU2014, to varying degrees, triggered four wheat defense-related genes (*PR1, PR2, PR3,* and *PR5*), one salicylic acid pathway gene (*PAL*), and one jasmonic acid pathway gene (*LOX*). The genetic background of wheat cultivars may influence expression patterns after inoculation with the diluted EF of HU2014.

Conclusions to Chaper 3

The results of this section show that HU2014 could produce active constituents and induce defense system of wheat to suppress WSE. In this study, the genomic sequencing of the strain HU2014 and ANI analysis indicate that it is a novel *Streptomyces* species most closely related to *Streptomyces albireticuli*. HU2014 exhibited strong antifungal activity against *R. cerealis* G11 and *R. solani* YL-3. In the co-culture trial, the inhibition of mycelial growth by HU2014 against *R. solani* YL-3 and *R. cerealis* G11 were 90.3% and 63.9%, respectively. The aerial hyphae of the two phytopathogenic fungi was hollow, and the cell wall of them was damaged under SEM.

Bioassay revealed that F6 and F8 fractions had the maximum inhibition rates of 100% to *R. solani* YL-3 and *R. cerealis* G11, followed by F4 and F2 (less than 60.50%). Spectrophotometric analysis was found that F6 and F8 had the same strong characteristic absorption peaks at 319 nm, 333 nm, and 351 nm. The main active components in the filtrated supernatants were determined to enrich in F6 and F8.

UPLC-MS on the four extracts from the EF of HU2014 identified 10 chemical constituents in the Natural Products Atlas with high match levels (more than 90%).

In the pre-test, the result showed that the activities of POD and PAL in wheat drenching with EF of HU2014 could be significantly induced at low concentrations, while the activity of PPO was non-significant difference when compared with that of control. the experiments were conducted.

In the test with the EF treatment and *R. cerealis* G11 infected, the result showed that the activities of POD, PAL and GLU in wheat leaves with three different treatments increased significantly in comparison with untreated control at different time point. The induce resistance in AK58 and BN307 was mainly induced by F3, and that in ZM22 was mainly induced by CKP.

Six specific gene expression analyses were performed for AK58, BN307, and ZM22 at each sampling hour post-inoculation (hpi) with 500-fold diluted EF of HU2014 using QRT-PCR. The expression of *PR1, PR2, PR3, PR5, PAL,* and *LOX* were markedly induced.

CHAPTER 4
DETERMINATION OF PLANT-GROWTH PROMOTION

4.1. Allelopathy of HU2014 metabolites on wheat growth and green bristlegrass

The allelopathy of microorganisms on plants is necessary for a comprehensive understanding of the interaction between microorganisms and plants. In this present study, we assessed the allelopathy of four fractions of HU2014 culture on wheat (Figure 4.1 and Table 4.1) and green bristlegrass (Figure 4.2 and Table 4.2). Green bristlegrass is a weed in wheat planting field, so it was investigated together. The results were shown in the below charts.

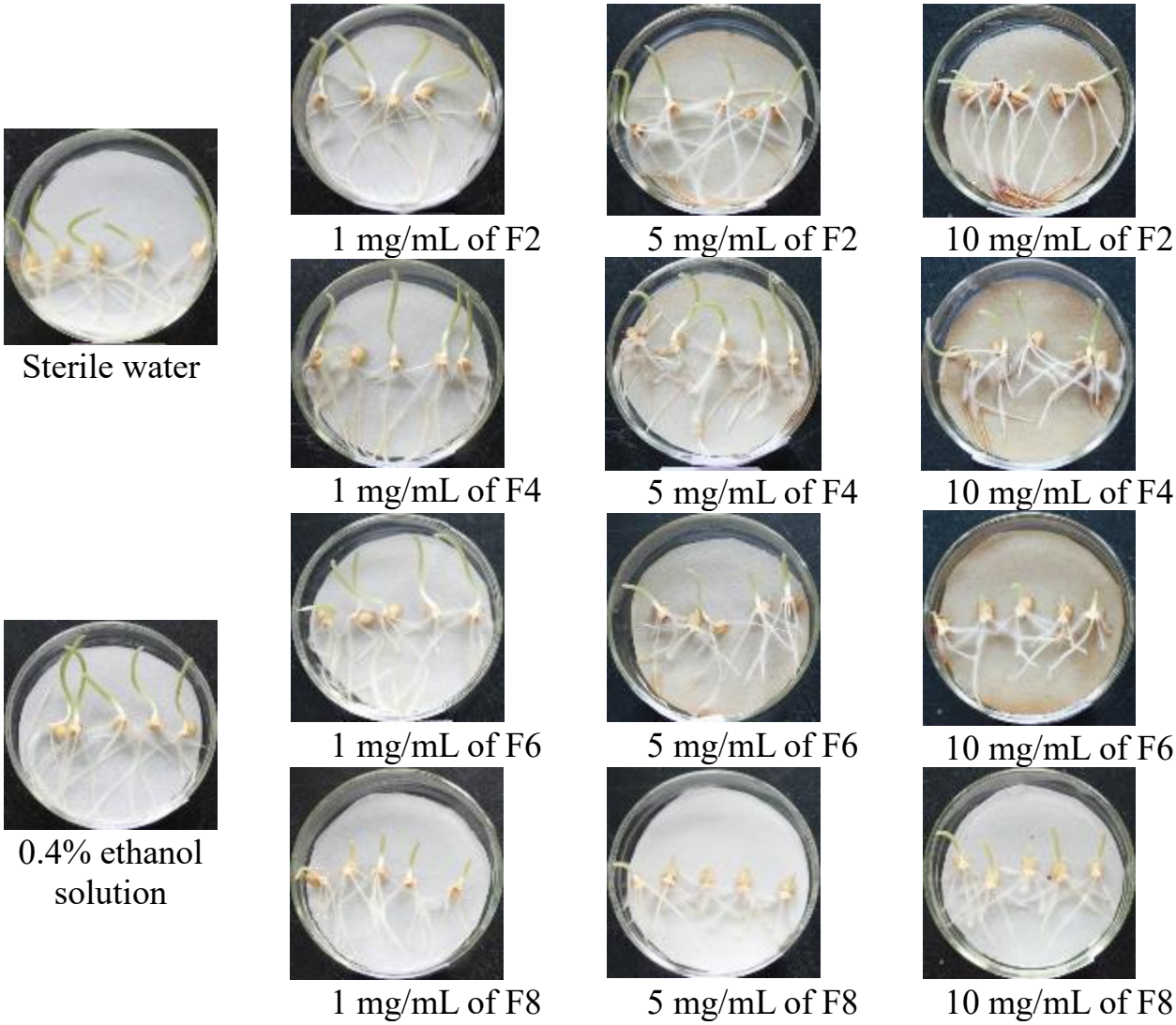


Figure 4.1. The growth state of wheat with four fractions treatment (HERCCGE, HIST, 2022).

Table 4.1

The sensitivity index of wheat shoot height and root length (HERCCGE, HIST, 2022)

Concentration		RI value (Shoot length)	RI value (Root length)
F2	1	0.01±0.45	-0.10±0.99
	5	-0.22±0.72	-0.11±1.09
	10	-0.53±0.32	-0.22±0.55
F4	1	-0.06±0.71	-0.34±0.77
	5	-0.08±0.90	-0.50±0.39
	10	-0.30±0.67	-0.58±0.41
F6	1	-0.02±0.66	-0.34±0.38
	5	-0.45±0.19	-0.54±0.93
	10	-0.76±0.33	-0.62±0.88
F8	1	-0.50±0.67	-0.58±0.97
	5	-0.66±0.58	-0.43±0.59
	10	-0.47±0.97	-0.66±0.43

Note: F2, F4, F6 and F8 represents four ethanol extracts from HU2014 broth respectively. Each treatment was done three times with three biological replicates. 1, 5, and 10 represent 1mg/mL, 5 mg/mL, and 10 mg/mL, respectively.

As shown in the charts, we could obviously observe the wheat shoot and root treated by F4, F6, and F8 shorter than that of the CK. Specifically, the allelopathy of inhibiting the wheat seedling and root growth was more powerful with the increasing concentration of F2 except for 1 mg/mL. F2 at 10 mg/mL had the strongest inhibition on the shoot and root length, the RI value were -0.53 and -0.22, respectively. It is interesting that F2 at 1 mg/mL promoted the shoot length (RI = 0.01). The allelopathy of inhibiting the seedling growth was also stronger with the increasing concentration of F4 and F6. The two fractions at 10 mg/mL had the strongest inhibition on the shoot root length. The RI value for the shoot length were -0.30 and -0.76, and for the root length were -0.58 and -0.62, respectively. F8 at 5 mg/mL had the highest inhibition, whereas F8 at 10 mg/mL had the lowest inhibition on the shoot length (RI were -0.66

and -0.47, respectively). F8 at 10 mg/mL had the highest inhibition, whereas F8 at 5 mg/mL had the lowest inhibition on the root length (RI were -0.66 and -0.43, respectively).

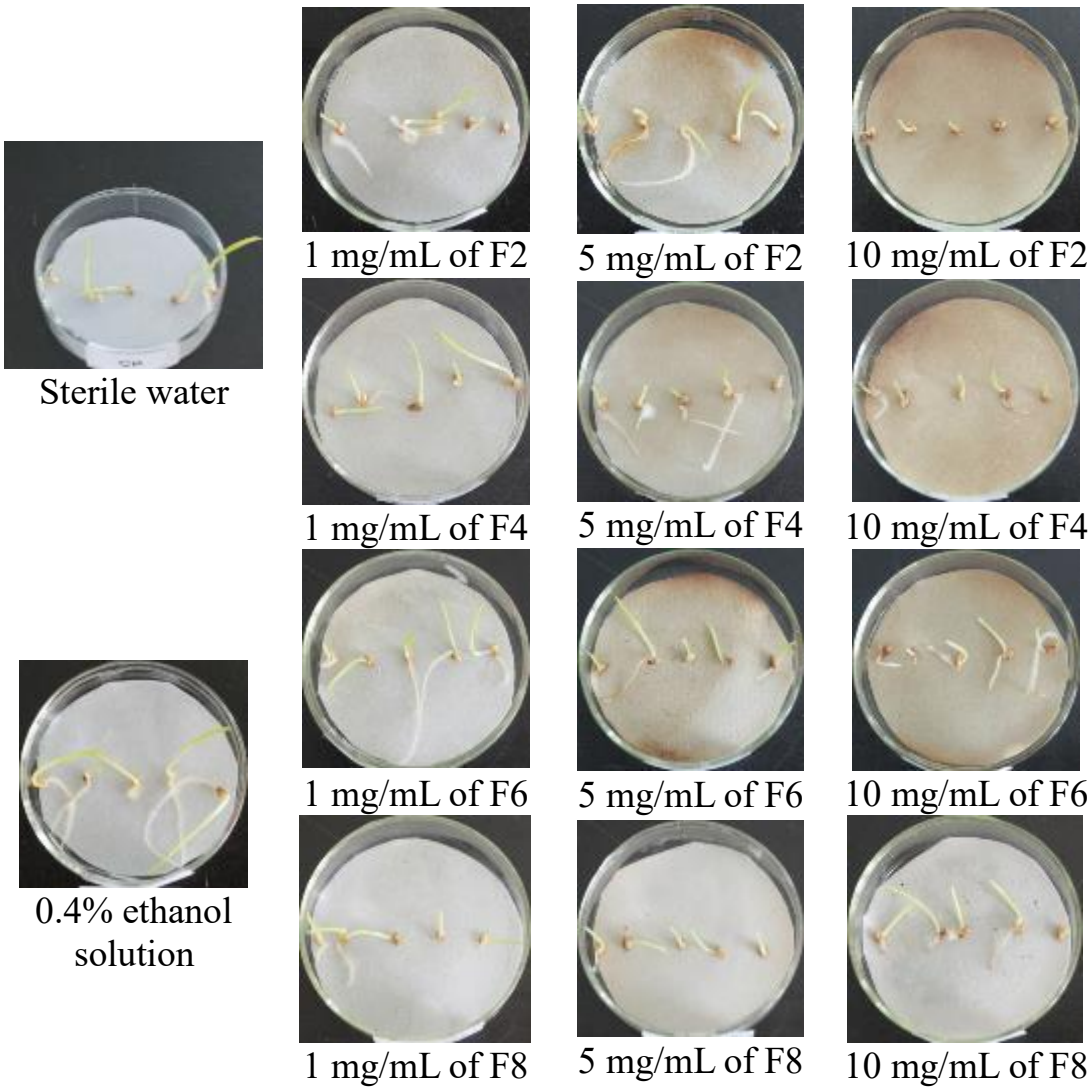


Figure 4.2. The growth state of green bristlegrass with four fractions treatment (HERCCGE, HIST, 2022).

From the above charts, we concluded that four fractions had an inhibitory effect on the green bristlegrass seedling. The allelopathy on its shoot and root length increased with the increasing concentration of F2, with the RI value from -0.13 to -0.73 for shoot length and -0.08 to -1.00 for root length. The allelopathy on shoot length increased with 1mg/mL and 5 mg/mL of F4 (RI = -0.14 and -0.69, respectively), while decreased with 10 mg/mL of F4 (RI = -0.50); That on root length generally increased with the three increasing concentrations of F4 (RI value from -0.30 to -0.85). For F6, the absolute RI value increased with the increasing concentrations for shoot length,

while for root length, the RI value with the concentrations in order were -0.26, -0.80, and -0.45 respectively. It is interesting that the change of RI value was not obvious and the allelopathy on shoot and root length decreased at 10 mg/mL, with the range from -0.37 to -0.47 for shoot length and -0.82 to -0.93 for root length, respectively.

Table 4.2

The sensitivity index of green bristlegrass shoot height and root length (HERCCGE, HIST, 2022)

Concentration mg/mL		RI value (Shoot length)	RI value (Root length)
F2	1	-0.13±0.41	-0.08±0.09
	5	-0.35±0.54	-0.53±0.12
	10	-0.73±0.23	-1.00±0.34
F4	1	-0.14±0.61	-0.32±0.66
	5	-0.69±0.33	-0.30±0.65
	10	-0.50±0.41	-0.85±0.73
F6	1	-0.25±0.83	-0.26±0.07
	5	-0.29±0.76	-0.80±0.45
	10	-0.59±0.54	-0.45±0.32
F8	1	-0.40±0.56	-0.83±0.33
	5	-0.47±0.23	-0.93±0.41
	10	-0.37±0.11	-0.82±0.47

Note: F2, F4, F6 and F8 represents four ethanol extracts from HU2014 broth respectively. Each treatment was done three times with three biological replicates. 1, 5, and 10 represent 1mg/mL, 5 mg/mL, and 10 mg/mL, respectively.

Many studies had shown that the symbiotic mechanism of plants and microorganisms is largely related to allelopathy (Muller et al., 2012). The established role of soil microbes in plant health has been consolidated in studies of allelopathy (Mishra et al., 2013). For example, *Ageratina adenophora* invasion promoted an increase of *Bacillus cereus*, which in turn induced a positive feedback effect on *A. adenophora* (Sun et al., 2021). *Burkholderia* sp. LS-044 is a potential allelochemical-metabolizing bacterium in rice rhizosphere, which got involved in mitigating

autotoxicity produced by bacteriostatic-dose of meropenem (Hameed et al., 2019). Simultaneously, the negative allelopathy of microbes on weeds provides an idea for the research and development of new microbial herbicides (Francisco et al., 2019). Some studies reported that soil microorganisms play the beneficial role in weed management (Mishra et al., 2013; Nichols et al., 2015; Xiao et al., 2020). Xi et al. came to a conclusion that *Streptomyces rochei* D74 combined maize rotation could suppress *Orobanche cumama* seed germination (Xi et al., 2022). In our study, the effect of allelochemicals on wheat and green bristlegrass is related to the concentration of allelochemicals and the part of plants. It should be emphasized that low concentration of F2 promoted the wheat seedling growth, but all fractions of HU2014 at different concentration had inhibition on green bristlegrass. This provides a good experimental basis for developing the function of HU2014 to promote growth and inhibit weeds. The allelochemicals separation would be the next work.

4.2. Assessment of plant growth promotion traits

Promoting plant growth is another way of biological preparations in controlling plant disease. To assess the promoting growth properties of HU2014, first, we conducted the test in vitro and the results were as follows (Figure 4.3 and Table 4.3).

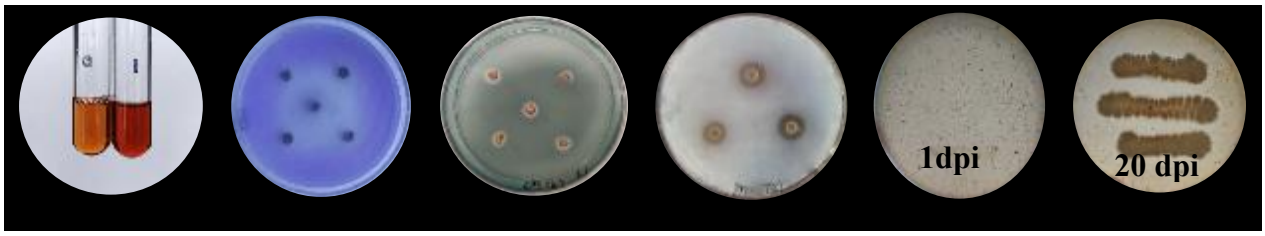


Figure 4.3. Assessment of plant growth promotion traits in vitro (HERCCGE, HIST, 2022). **(A)** IAA qualitative production assay: 0, control and 1, fermentation with the supplement of tryptophan. **(B-F)** Qualitative phosphate solubilization, siderophore qualitative production, protease qualitative production and chitinase qualitative production assay.

Table 4.3

Traits related with direct plant growth promotion by HU2014 (HERCCGE, HIST, 2022)

Quantitative assay		Qualitative assay			
IAA (µg/mL)	Phosphate solubilization (µg/mL)	Siderophore production	Protease production	Chitinase production	Cellulase production
35.0 ± 0.6	199.7 ± 6.2	+	+	+	UD

Note: IAA represents Indole-3-acetic acid; UD represents undetectable activity. Each assay was done two times with three biological replicates, and values represent their means ± standard deviation.

In the charts, the results revealed that HU2014 could solubilize inorganic phosphate and produce IAA, siderophores, protease, and chitinase, but not cellulase. The quantitative assay showed that the production of IAA was 35.0 ± 0.6 µg/mL and that of phosphate solubilization was 199.7 ± 6.2 µg/mL.

It is necessary to determine the properties of microbe in promoting growth in vitro. Many reports had found that most of microbe could produce some active compounds, such as growth hormone, protease, and other volatile substances, and these compounds were beneficial to plant growth (Roca et al., 2013; Zhao et al., 2018; Fan & Smith, 2021). The revelation of these characteristics often originates from experiments in vitro, which is further explored in pot experiments.

Secondly, to verify the effect of HU2014 on wheat growth in pots, then we conducted experiments and the results were presented in Figure 4.4 and Figure 4.5.

As these figures shown, we investigated the effect of HU2014 with concentration gradient treatment on wheat growth in the first pot experiment. At 7 dpi, the shoot height wheat BN307 showed a decreasing trend with the increasing concentrations, and the turning point was that at 5 g/kg of HU2014. While the shoot height treated with 5 g/kg of HU2014 was highest at 20 dpi and 28dpi, followed by 10 g/kg and 15 g/kg when compared to that of wheat in control pots (Figure 4.4). From the results, we could conclude that initially these three concentrations of HU2014 (5 g/kg, 10 g/kg, and 15 g/kg) inhibited wheat growth, however, it showed a trend of promoting wheat growth

with time. In special, 5 g/kg of HU2014 was the most suitable concentration for wheat growth.

A B

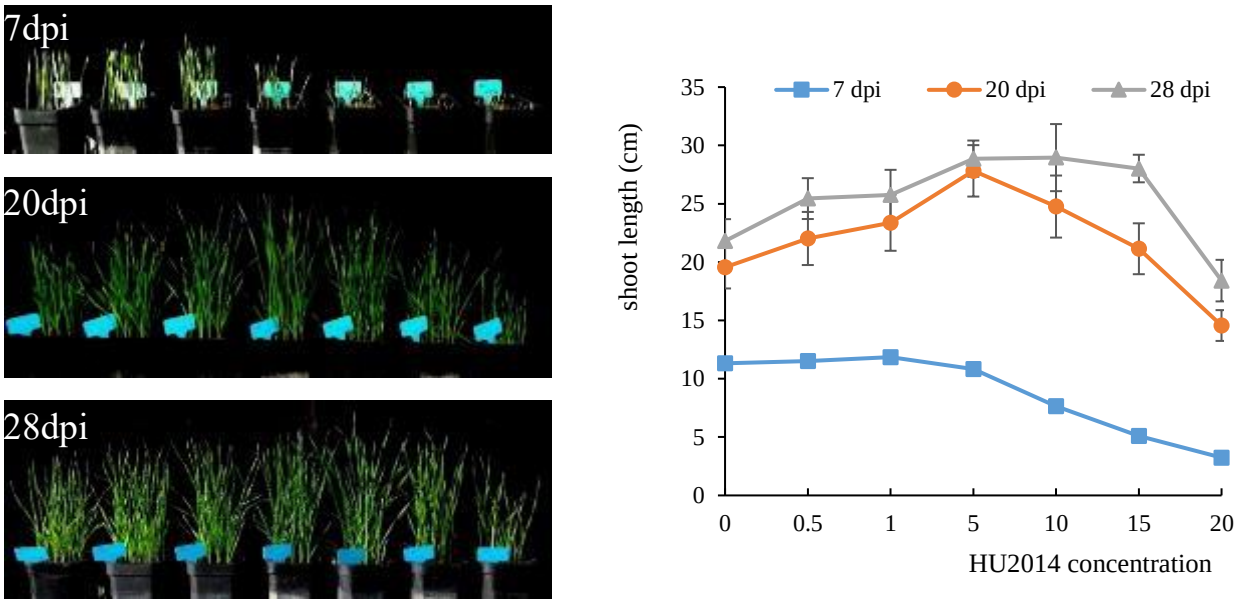


Figure 4.4. Growth promotion experiments of wheat cultivar BN307 treated by six different concentrations of HU2014 (HERCCGE, HIST, 2022). (**A**) Three stages of growth (7 dpi, 20 dpi and 28 dpi from top panel to bottom panel). (**B**) The shoot length in **(A)**. The results are means of twenty values ± SD of three independent replicates.

According to the pre-test, the suitable concentration of HU2014 was applied to the next pot experiments. The growth of three wheat cultivars was investigated after this strain inoculation with soil. Their physiological indicators had significantly changed, and the results are as follows in Figure 4.5.

A

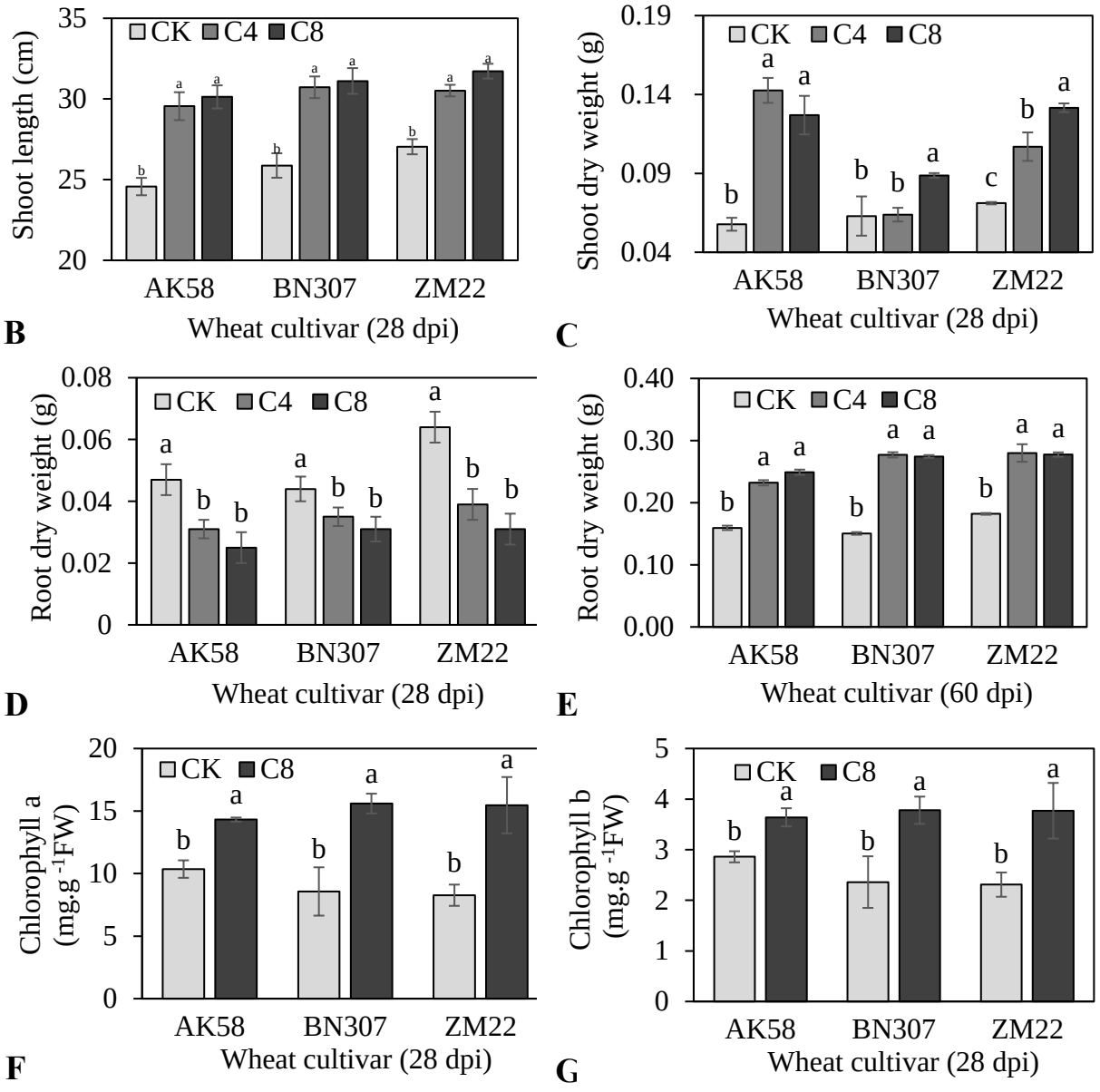


Figure 4.5. Plant-growth promotion experiments with the treatment of HU2014 (HERCCGE, HIST, 2022). **(A)** The growth of three wheat cultivars at 28 dpi. **(B-G)** Analysis of the shoot length and weight, root weight and chlorophyll content in wheat leaves. CK represents the soil without HU2014 inoculation. C4 and C8 represent two concentrations of HU2014, respectively. Statistical analysis was performed within cultivar. The results are means of twenty values ± SD of three independent replicates. One way-ANOVA ($p < 0.05$) was used to compare means and bars sharing a letter are not statistically different.

As Figure 4.5 shown, in the second pot experiment, the biometric properties of wheat cultivars (AK58, BN307, and ZM22) inoculated with two concentrations of HU2014 were examined (Figure 4.5 A-G). The shoot height increased significantly by

12.9% to 22.4% in comparison to the non-inoculated control in the three cultivars at 28 dpi ($p < 0.05$). Similarly, HU2014 was able to improve shoot dry weight in comparison to the non-inoculated control. The chlorophyll content increased by 27.3% to 87.1% compared with the non-inoculated control. The root dry weight increased significantly by 45.5% to 83.4% in comparison to the non-inoculated control at 60 dpi ($p < 0.05$).

Promoting plant-growth has indirect effect on controlling wheat sharp eyespot. Growth promoting properties of some beneficial microbes in vitro had been assessed before further relative research (Zamoum et al., 2015; Monteiro et al., 2017). The growth-promoting properties of HU2014 were tested in pot experiments, demonstrating that the strain could also significantly increase shoot length, shoot fresh and dry weight, root dry weight and chlorophyll content. According to UPLC-MS analysis, N-acetyltryptamine and L-tryptophan were identified, which could be components of a possible IAA synthetic pathway. *Actinomycete* M527 could promote the shoot growth and prevent the development of the disease on cucumber caused by *Fusarium oxysporum f. sp. cucumerinum* (Lu et al., 2016). Aldesuquy et al. reported that the culture filtrates of *Streptomyces* had the abilities to promoting wheat growth (Aldesuquy et al., 1998). Microorganisms have the potential ability to promote crop growth and control weeds in order to make full use of the positive effects of allelochemicals in the agricultural ecosystem. Developing natural pesticides and growth regulators have important theoretical value and practical significance for the effective use of resources, the protection of the ecological environment, and the development of sustainable agriculture. However, the application of these microorganisms in field is different with pot trials (Vurukonda et al., 2018). Next, the research about HU2014 in real farming environment will be continued.

4.3. Effect of HU2014 inoculation on soil properties and microbial community

Soil physicochemical properties in rhizosphere

In long-term coevolution, plants and their relative microbial communities form a complete microbial ecosystem. Rhizosphere bacteria can interact with plants through

various direct and indirect factors. To investigating the effect of HU2014 on the environmental factors in wheat rhizosphere soil, first, we assessed the effect of HU2014 inoculation on soil parameters (Table 4.4).

Table 4.4

The results of showing the effects of HU2014 inoculation on soil properties (HERCCGE, HIST, 2022)

Soil properties	CK	S
TN (g/kg)	1.33 ± 0.02b	1.50 ± 0.05a
TP (g/kg)	1.09 ± 0.05b	1.23 ± 0.10a
SOC (mg/kg)	50.45 ± 0.24a	50.83 ± 0.54a
Olsen-P (mg/kg)	167.11 ± 3.68b	206.42 ± 3.13a
NH4+ (mg/kg)	39.25 ± 2.22a	32.88 ± 1.57b
NO3- (mg/kg)	36.36 ± 3.71b	46.29 ± 3.67a
pH	8.31 ± 0.01a	7.98 ± 0.00b

Note: CK and S represent control soil and inoculating HU2014 soil, respectively. The data shows the means (± SD) of five repetitions of soil samples. One way-ANOVA ($p < 0.05$) was used to compare means and lines sharing a letter are not statistically different.

In this table, the results showed the variations of soil parameters in different treatments. The S treatment had significantly greater TN ($p < 0.05$) than CK treatment. TP and Olsen-P were remarkably increased in the S treatment, when compared with that in the CK treatment ($p < 0.05$). However, SOC in all treatments did not change significantly ($p > 0.05$). $NH4^+$-N content was remarkably decreased in S treatment ($p < 0.05$), when compared with that in the CK treatment. NO_3^--N significantly increased in the S treatment compared to the CK treatment ($p < 0.05$). Soil pH had different variation in all treatments.

The nature of the soil is shaped by the presence of plant roots and the soil microbe (Volpiano et al., 2022). Soil meets the various nutrients required for crop growth and yield, including large amounts of elements such as nitrogen, phosphorus, potassium, calcium, and trace elements. These substances can be directly absorbed and

utilized by crops. Adequate nutrients enhance the soil's water absorption and fertilizer retention capacity. However, crops require a large amount of nitrogen, phosphorus, and potassium, and the supply of soil often cannot meet the needs. Usually, nitrogen, phosphorus, and potassium fertilizers need to be increased. PGPM can produce promoting plant growth factors, enzymes and increase the nutrient elements easily absorbed by plants (Lopes et al., 2021). The combined application of *Pseudomonas* sp. DSMZ 13134 and *Bacillus amyloliquefaciens* FZB42 with ammonium sulphate fertilization greatly enhanced shoot P accumulation (109-235%) as compared with nitrate supply (Mpanga et al., 2019). One study reported that inoculation of Pseudomonas sp. DSMZ 13134 tended to increase shoot N and P concentrations, and shoot N content relative to the control (Nkebiwe et al., 2017). *Bacillus amyloliquefaciens* EZ99 with the addition of sucrose treatment significantly increased total potassium, whereas it decreased available potassium, which enhance the potassium utilization in rhizosphere soil (Tian et al., 2022). In this study, with HU2014 inoculation, soil factors changed, which resulted in the increasing of TN, TP, Olsen-P, and NO_3^-. From these results, we could conclude that HU2014 has the properties of promoting plants growth though enhancing the soil nutrients.

One study showed that multiple PGPM strains had complementary functions, which been proposed as superior, particularly under particular conditions and for protecting beneficial microbial communities in destroyed soil field (Bradácová et al., 2019). These joint microbes can increase the strength of plant roots, promote plant growth, and regulate plant metabolism.

Therefore, the next research will explore the effect of HU2014 with other beneficial microbe inoculation on soil parameters and plants which will provide a certain foundation for the future research and development of biological fertilizer.

Alpha diversity and distribution of bacteria and fungi community structures

To investigating the effects of HU2014 inoculation on the species abundance and community diversities in soil, the alpha diversity and distribution of microbial community were conducted at LC-Bio Technology Co., Ltd (Hangzhou, Zhejiang province, China).

Microbial diversity in soil was investigated by Chao1, Shannon and Sampson indices. First, two alpha indices were shown in Figure 4.6。

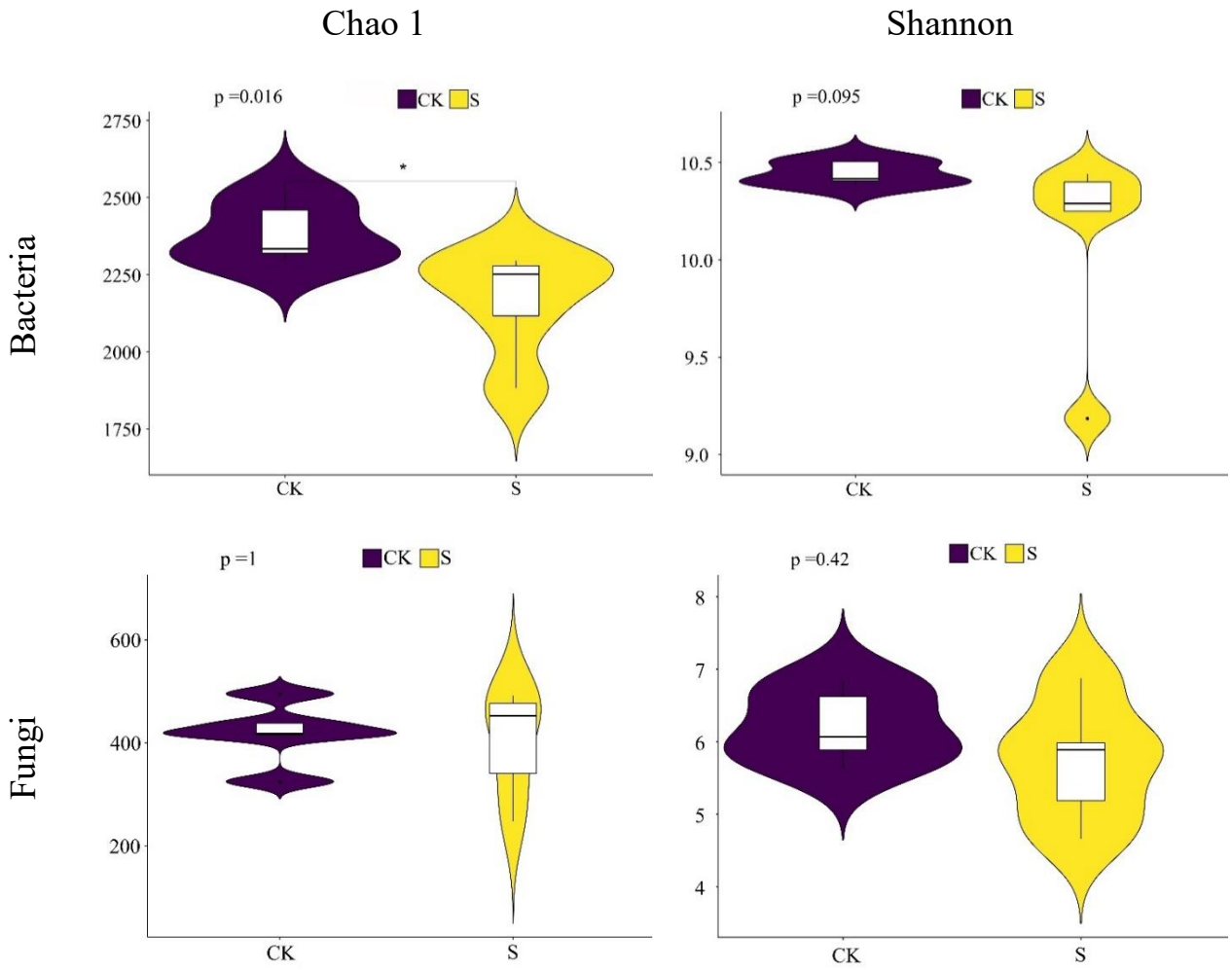


Figure 4.6 Difference in bacterial, fungal alpha indices with the treatments (HERCCGE, HIST, 2022). Values show the mean ± SD of five independent replicates. CK and S represent control soil and inoculating HU2014 soil, respectively. $P < 0.05$ represents significant difference in the treatments.

From this figure, a total of 1,361,631 high-quality microbial sequences of soil samples were identified, of which 673,398 sequences for bacteria were clustered into 7,957 ASVs and 688,233 sequences for fungi were clustered into 1,517 ASVs at the 97% similarity cut-off level. Bacterial and fungal alpha diversity was estimated using Chao 1 and Shannon indices. For bacterial communities, in alpha diversity indices, Chao 1 was significantly affected by HU2014 inoculation, but not Shannon ($p < 0.05$). Which suggested the richness of bacterial composition was changed but not the evenness of them. For fungal communities, HU2014 inoculation were not significantly

correlated with fungal alpha indexes (Chao 1 and Shannon). From the results, we could conclude that the inoculation of exogenous *Streptomyces* HU2014 leads to higher competition with bacterial communities than with that of fungal communities. The more details about the discussion were in the following part.

Second, the distribution of microbial community structures was determined and the results were exhibited as follows (Table 4.5, Table 4.6 and Figure 4.7).

Table 4.5

The effects of HU2014 inoculation on relative abundances of bacterial taxa of top 10 (phylum level) (HERCCGE, HIST, 2022)

Taxa	CK RA (%)	S RA (%)	Total RA (%) and *p* value
Proteobacteria	36.90±0.94	41.45±4.00	39.19 (0.12)
Acidobacteriota	16.60±0.52	11.05±1.79	15.33 (0.01)
Actinobacteriota	6.63±0.67	6.31±0.64	10.23 (0.46)
Gemmatimonadota	6.09±0.69	5.06±0.91	5.89 (0.12)
Bacteroidota	4.95±0.49	6.39±0.71	5.87 (0.02)
Firmicutes	4.35±0.39	6.10±2.67	5.35 (0.12)
Planctomycetota	4.57±0.40	3.76±0.64	4.26 (0.05)
Chloroflexi	3.91±0.29	3.19±0.41	3.55(0.03)
Myxococcota	3.54±0.15	2.97±0.48	3.25 (0.05)
Verrucomicrobiota	2.51±0.23	2.00±0.45	2.26 (0.03)

Note: CK and S represent control soil and inoculating HU2014 soil, respectively. RA represents relative abundance. Values are the means (± SD) of five replicate soil samples.

In the above charts, 10 bacterial phyla were detected throughout the different treatments with a relative abundance > 1%. These main phyla were *Proteobacteria* (39.19%), *Acidobacteriota* (15.33%), *Actinobacteriota* (10.23%), *Gemmatimonadota* (5.89%), *Bacteroidota* (5.87%), *Firmicutes* (5.35%), *Planctomycetota* (4.26%), *Chloroflexi* (3.55%) and *Myxococcota* (3.25%) and *Verrucomicrobiota* (2.26%) in sequence (Table 4.5).

Table 4.6

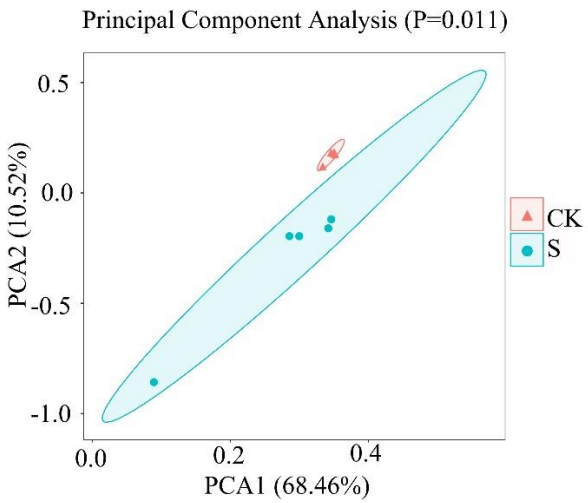
The effects of HU2014 inoculation on relative abundances of fungal taxa of top 6 (phylum level) (HERCCGE, HIST, 2022)

Taxa	CK (RA%)	S RA (%)	Total RA (%) and *p* value
Ascomycota	73.38±8.44	70.17±15.87	71.77 (0.92)
Basidiomycota	6.79±7.81	10.72±14.93	8.75 (0.60)
Fungi_unclassified	3.88±0.72	10.75±11.64	7.31 (0.25)
Zygomycota	7.01±2.51	4.59±2.58	5.80 (0.12)
Chytridiomycota	5.24±8.50	2.12±1.73	3.68 (0.60)
Olpidiomycota	3.25±2.28	1.58±0.93	2.42 (0.17)

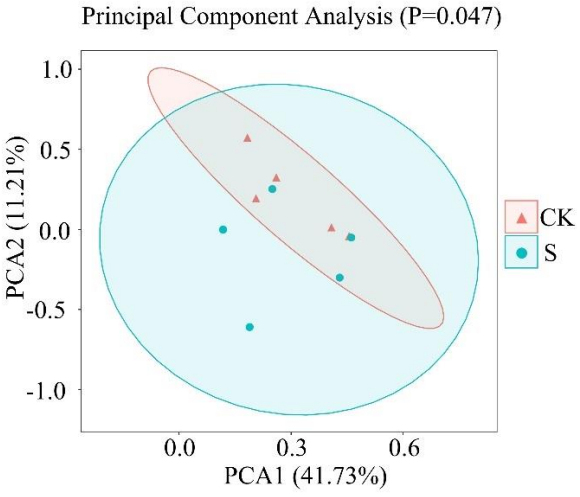
Note: CK and S represent control soil and inoculating HU2014 soil, respectively. RA represents relative abundance. Values are the means (± SD) of five replicate soil samples.

HU2014 inoculation clearly affected the relative abundance of six bacterial phyla. Our study detected nine fungal phyla throughout the different treatments with a relative abundance > 1%. The main taxa were *Ascomycota* (71.77%), *Basidiomycota* (8.75%), Fungi_unclassified (7.31%), *Zygomycota* (5.80%), *Chytridiomycota* (3.68%) and *Olpidiomycota* (2.42%) (Table 4.6).

Bacteria



Fungi



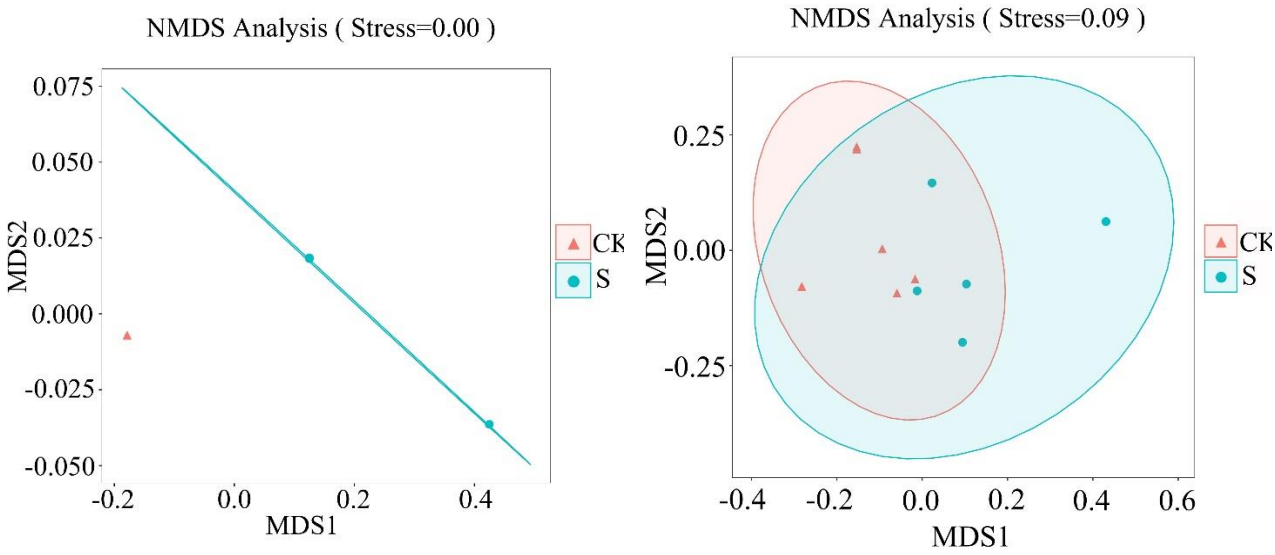


Figure 4.7. Principal component analysis (PCA) of the Bray-Curtis distance for soil microbial communities and Non-metric multidimensional scaling (NMDS) ordination plots based on the Bray-Curtis distance matrix (HERCCGE, HIST, 2022). CK and S represent control soil and inoculating HU2014 soil, respectively.

As the figure shown, based on Bray-Curtis dissimilarity, the PCA analysis of the microbial composition showed marked differences between the S treatment and the CK treatment (PCA=68.46% for bacteria and 41.73% for fungi, respectively). Moreover, NMDS analysis showed that bacteria and fungi communities from the treatments are distinct from each other (Figure 4.7).

Soil microorganisms are mainly composed of bacteria, fungi, and archaea. Among them, bacteria are the most abundant group, followed by fungi (Zhang et al., 2019). Microbial diversity includes species richness and evenness. Richness (Chao I index) refers to the number of species in a community and evenness (Simpson and Shannon indices) refers to the uniform distribution of individuals among different species under the same species abundance. The diversity of soil microorganisms is mainly manifested in the type and quantity of the population. The co-inoculation of rhizobia and arbuscular mycorrhiza fungi could remarkably decrease fungal community diversity and increase bacterial community diversity and the abundance of *Proteobactieria, Actinobacteria, Acidobacteria*, and *Chloroflexi* (Wang et al., 2021). Geo-authentic soil microbe inoculation altered the root of endophytic bacteria diversity in *Atractylodes lancea* and promoted its growth (Wang H. Y. et al., 2022). In this study,

with HU2014 inoculation, bacterial diversity was significantly affected, but fungal diversity was not remarkably different. Concurrently, the enrichment of

Proteobacteria and *Acidobacteriota* were abundant taxa in bacterial communities. The changes of these microbial composition would alter the soil factors, and then plants growth, which was discussed in the following findings.

Relationship between soil properties, microbial communities

We conducted A redundancy analysis (RDA) and correlation analysis to evaluated relationships between soil factors and microbial community structures. The results were shown in Figure 4.8 and Figure 4.9.

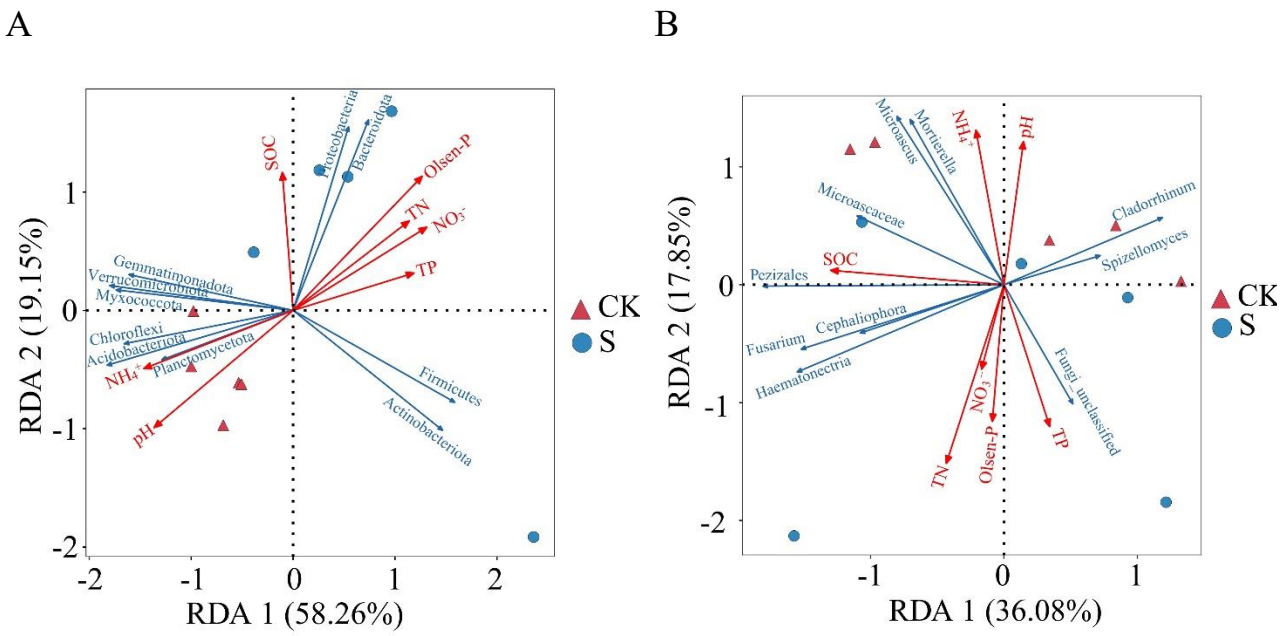


Figure 4.8. RDA of the different bacterial communities (A) or fungal communities (B) and soil properties (red arrows) (HERCCGE, HIST, 2022). SOC represents soil organic-carbon content; TN represents total nitrogen content; NH_4^+ represents NH_4^+-N content; TP represents total phosphorus content; NO_3^- represents NO_3^--N content; Olsen-P represents olsen-phosphorus content.

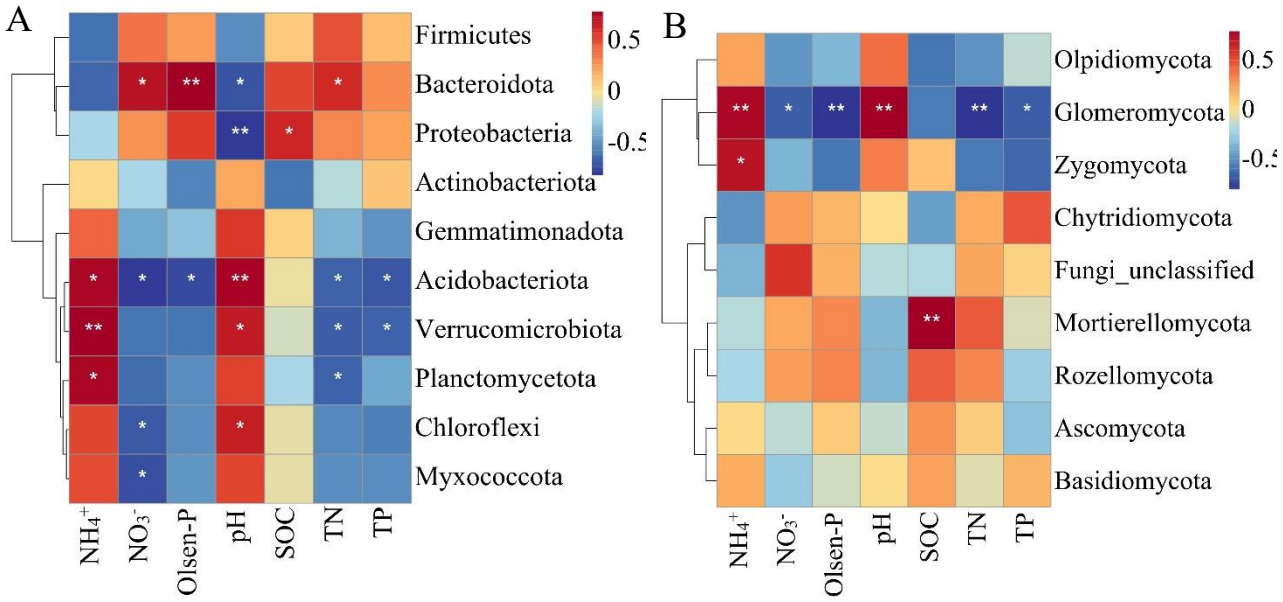


Figure 4.9. Correlation heat map of microbial taxa (A and B, phylum level) with soil parameters (HERCCGE, HIST, 2022). SOC represents soil organic-carbon content; TN represents total nitrogen content; NH_4^+ represents NH_4^+-N content; TP represents total phosphorus content; NO_3^- represents NO_3^--N content; Olsen-P represents olsen-phosphorus content.

From the figures, the first and second RDA axes explained 58.26% of the variance in bacterial communities, while the first and second RDA axes explained 36.08% of the variance in fungal communities. According to the correlation heat map and RDA, *Bacteroidota, Acidobacteriota* and *Verrucomicrobiota* in bacterial communities significantly correlated to soil variables except for SOC ($p < 0.05$; Figure 4.8A and Figure 4.9A). In the fungal community, however, only *Glomeromycota* showed a notable correlated to most soil variables, but its relative abundance was less than 1% ($p < 0.05$; Figure 4.8B and Figure 4.9B). In addition, the RDA also confirmed that soil variables had greater apparent influence on the bacterial communities of the different treatments compared to the fungal communities of the different treatments. A remarkable increase in soil factors (TN, TP, Olsen-P, and NO_3^--N) after HU2014 inoculation indicated that N and P availability improved wheat growth (Table 4.4). Native microbial communities could be affected by exogenous microbe (Li et al., 2017). In this study, HU2014 inoculation remarkably decreased bacterial diversity, but had no

notable effect on fungal diversity in wheat rhizosphere (Figure 4.6). The result showed that HU2014 inoculation is profitless to the bacterial diversity at the seedling stage of wheat, which was consistent with *Streptomyces pactum* strain Act12 inoculation in a pot–herb mustard planting (Wang L. L. et al., 2022). Another study showed that bacterial diversity decreased first and then increased, while fungal diversity decreased gradually after *Streptomyces alfalfa* XY25(T) inoculation during Chinese cabbage growth (Hu et al., 2021). These results indicate that exogenous inoculant influences the stability of indigenous microbial diversity, which ultimately comes to a new dynamic equilibrium to assist in increasing plant resistance. The relative abundances of *Proteobacteria* and *Bacteroidota* increased, whereas the relative abundances of *Acidobacteriota*, *Chloroflexi*, *Planctomycetota*, and *Myxococcota* decreased (Table 4.5). The results hindered that HU2014 inoculation led to the changes of these bacterial taxa. Previous studies have reported that *Proteobacteria* (such as *Pseudomonas*) can assist plant growth and decrease disease incidences (Egamberdieva, 2010; Hu et al., 2016). Considering that *Proteobacteria* had the highest abundance in these bacterial taxa, HU2014 inoculation played a key role in the synergism of beneficial bacteria. The further hypothesis was that the increasing relative abundance of *Proteobacteria* and *Bacteroidota* could have connection with the decreasing relative abundance of the bacterial taxa mentioned above in this study. In fungal communities, the most abundant fungal taxon was *Ascomycota* in the different treatments (Table 4.6), which is a key driver in carbon and nitrogen cycling (Green et al., 2008). In the RDA analyses, we found that the changes of microbial communities subsequently affected soil factors. the increase of key bacterial taxa changed the content of soil nutrients, while the key fungal taxa was not strongly correlated with soil variables in the wheat rhizosphere (Figure 4.8 and Figure 4.9). Therefore, despite the decrease in microbial diversity, the abundance of beneficial bacteria increased with HU2014 inoculation, which promoted wheat growth. On the other hand, the microbial operational taxonomic units found in soils of both experiments (greenhouse and field) were distinct (Araujo et al., 2020). In this study, we conducted the pot experiments, but the field experiments need to be verified, and then found the difference between pots and fields. It is also critical to

understand the microbial networks formed in rhizosphere soils to clarify the impact and effects of potential biocontrol strains (Poudel et al., 2016; Araujo et al., 2019). Overall, the selection of biocontrol agents becomes complex owing to their diversity and interactions with hosts (Moto et al., 2017).

Conclusions to Chapter 4

The results of this section show that HU2014 could provide nutrition and cope with other beneficial microbes to promote the growth of wheat.

Firstly, we assessed the allelopathy of HU2014 metabolites on wheat and green bristlegrass Four fractions (F2, F4, F6, and F8) from the fermentation broth of HU2014 cultures had the negative allelopathic effects on the seedling height and root length of two tested plants except for F2 at 1 mg/mL promoting the seedling height of wheat. Thus, at the early stage of the plant growth, the low concentration of allelochemicals produced by HU2014 could promote wheat growth, whereas the allelochemicals without the limitation of concentration inhibit green bristlegrass growth, which will provide a theoretical basis for the application of this strain in agriculture.

Then, in the experiment of promoting growth, HU2014 produced indoleacetic acid, siderophores, extracellular enzymes, and solubilized phosphate in vitro, and the properties of promoting growth were verified in pot trials. Shoot length, shoot and root dry weight, and chlorophyll content of wheat plant increased.

At last, in the experiments of the effect of this bacterium on soil and native soil microorganisms, HU2014 increased the malondialdehyde content of wheat leave, while decreased catalase activity, peroxidase activity. With HU2014 inoculation, the concentrations of total nitrogen, nitrate nitrogen, total phosphorus, and Olsen-phosphorus in the wheat rhizosphere increased. The diversity of rhizosphere bacteria, but not fungi clearly decreased by HU2014 inoculation. The compositions of bacterial and fungal community differed after HU2014 inoculation with *Proteobacteria*, *Acidobacteriota*, and *Ascomycota* being the dominant phyla in all treatments.

Thus, we concluded that HU2014 could be a valuable microbial resource for growth promotion of wheat and biological herbicide.

CHAPTER 5
STUDY ON FERMENTATION PROCESSING OF HU2014

Spectrophotometric analysis of EF at different growth periods

The yield of bioactive metabolites from *Streptomyces* spp. is crucial for related research and application. Furthermore, it is also necessary to determine the various factors that regulate the production of secondary metabolites with antifungal properties. Therefore, in this part, we conducted the experiments of optimization conditions. First, we investigated the metabolites of HU2014 during the growth using spectrophotometric analysis, through which we could know the maximum production of secondary metabolites at a certain time. The results were presented in Figure 5.1.

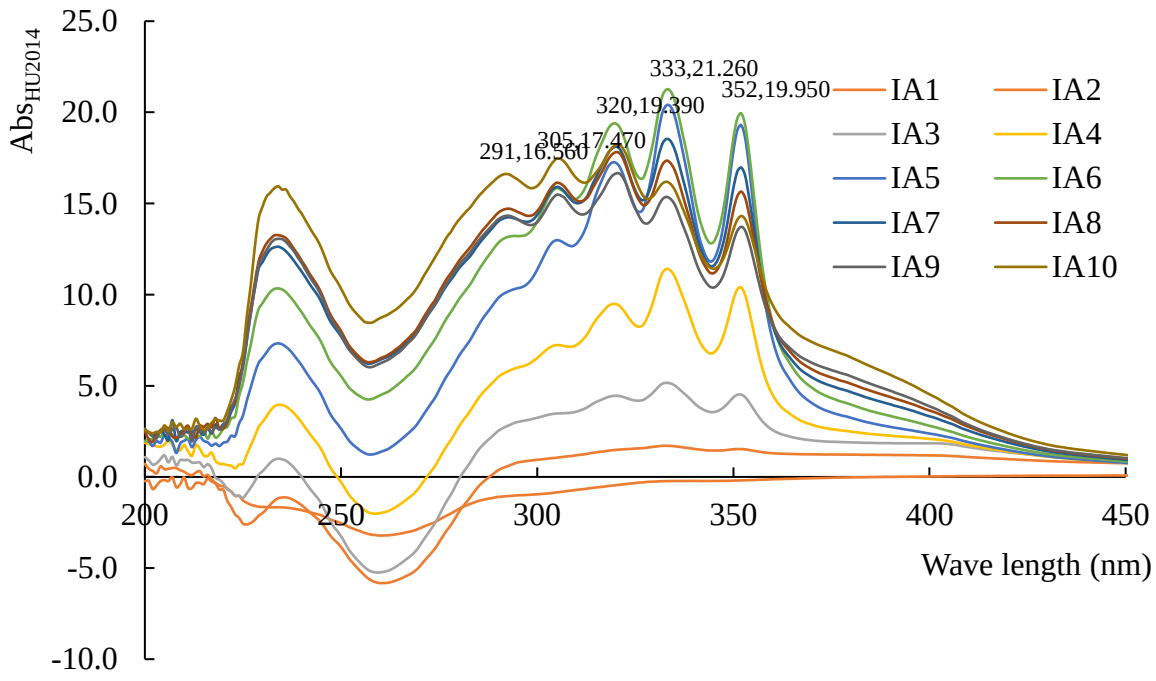


Figure 5.1. Scanning broad-spectrum of the EF from HU2014 broth by spectrophotometer (HERCCGE, HIST, 2022). IA1-10 represent the Abs lines of different sampling times.

As figure 5.1 shown, the Abs_{HU2014} values of the EF were evaluated at 2-day intervals from day 2 to day 20. From figure 3.18, we concluded that the absorbance increased from day 2 (IA1) to day 12 (IA6) and then followed a decreasing trend from day 14 (IA7) to day 20 (IA10). The peak value of Abs_{HU2014} in IA6 (on day 12) was

20.7. The characteristic ultraviolet-visible absorption peaks of the supernatants were at 291 nm, 305 nm, 320 nm, 333 nm and 352 nm. The curve trend of different fermentation times revealed that the contents of bioactive metabolites changed from pre-fermentation to post-fermentation.

Screening of basal culture medium and fermentation conditions

Second, HU2014 was inoculated in 11 different media to screen the best culture medium for achieving higher production of antifungal metabolites. Subsequently, the fermentation conditions were optimized. The results were in Table 5.1 and Figure 5.2.

Table 5.1

The inhibition rate of different media inoculated by *Streptomyces* sp. HU2014 against *R. solani* YL-3 at 3th day (HERCCGE, HIST, 2022)

Medium	Inhibition rate（%）
PD	80.91±0.46b
GPY	100.00±0.00a
Gause's No.1	32.04±1.83c
Czapek's	21.04±4.58d
ISP1	82.85±2.29b
ISP2	96.44±3.20a
ISP3	23.95±1.37d
ISP4	29.45±0.92c
ISP5	3.88±2.29e
ISP6	89.32±0.46b
ISP7	5.83±2.29e

Note: Each treatment was done three times with three biological replicates, and the data showed mean ± SD. Different lowercase letters in the same column show values that are significantly different at the $p < 0.05$ level by least significant difference (LSD) test.

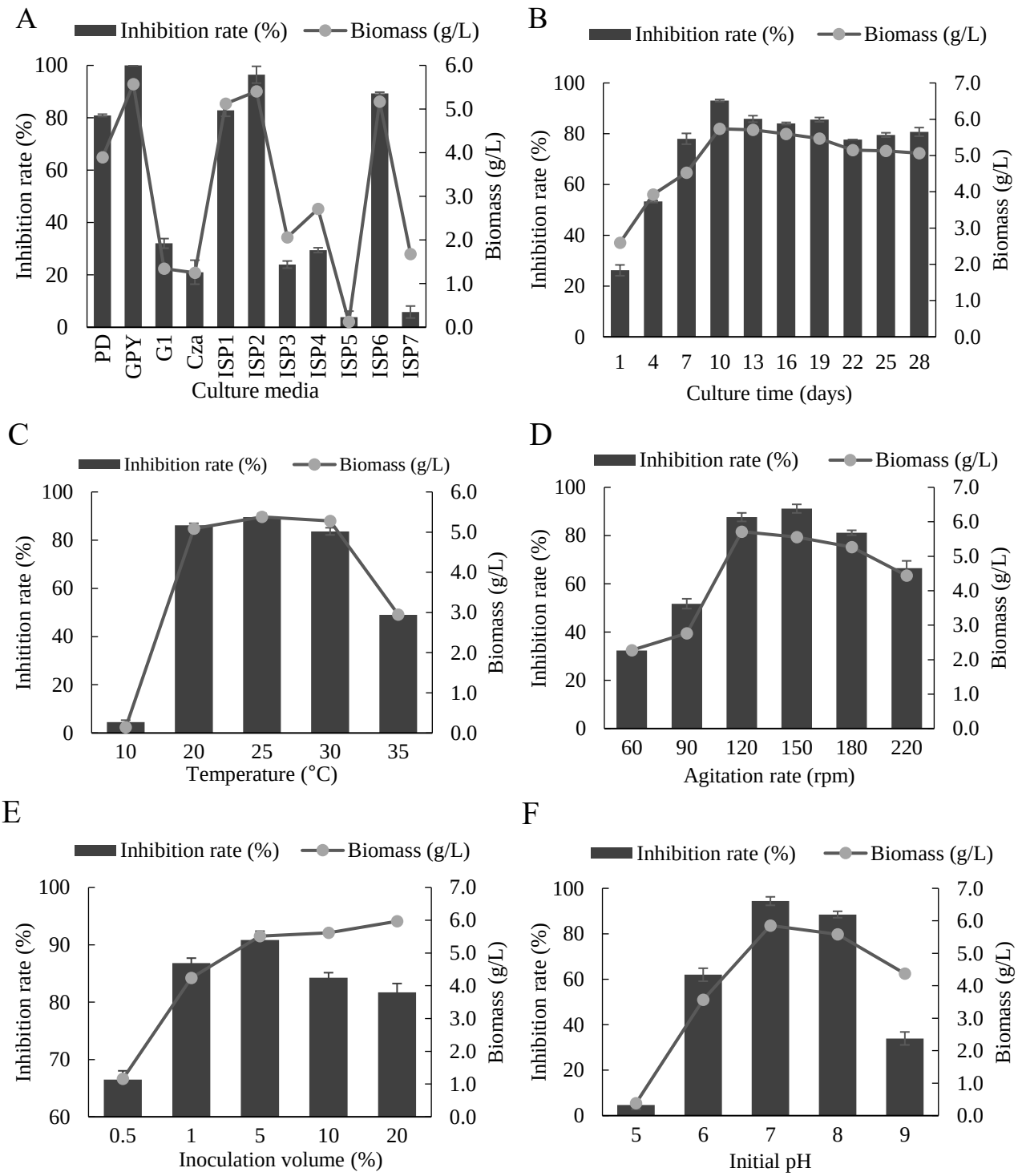


Figure 5.2. The antifungal activities of cell-free filtrates of HU2014 culture broth against *R. solani* YL-3 and biomass growth of HU2014 in different conditions (Different basal media (A) and different culture conditions (B-F), (HERCCGE, HIST, 2022)). Each treatment was done three times with three biological replicates, and the data show Means ± SD. G1 represents Gause's No.1medium. Cza represents Czapek's medium.

From these charts, it is clear that GPY medium presented the highest *R. solani*

YL-3 mycelial growth inhibition rate (100%), then the ISP2 (96.44%) (Table 5.1). Correspondingly, the biomass of the strain was approximately 5.6 g/L in GPY medium, followed by the ISP2 medium with 5.4 g/L (Figure 5.2A). Therefore, GPY, as the basal medium, was used in the next experiments. The biomass growth of HU2014 reached a maximum on day 10, which was coincided with the highest *R. solani* YL-3 mycelial growth inhibition rate (Figure 5.2B). Temperature was another important factor that affected the metabolic activities of HU2014, and the *R. solani* YL-3 mycelial growth inhibition rate increased from 10°C to 25°C and then decreased, with the highest *R. solani* YL-3 inhibition rate (89.6%) and the maximum HU2014 biomass (5.4 g/L) being observed at 25°C (Figure 5.2C). Similarly, the maximum *R. solani* YL-3 mycelial growth inhibition rate and corresponding HU2014 biomass were achieved at an agitation speed of 150 rpm, inoculum size of 5%, and pH of 7.0 (Figure 5.2 D–F).

Optimization of medium components

Although GPY was as the basal medium, the antifungal effects of HU2014 significantly varied in different the types and concentrations of carbon sources, nitrogen sources and inorganic salts, which were presumed to have crucial influence on the *R. solani* YL-3 mycelial growth inhibition rate. To obtain the higher metabolites production, we further conducted the optimization experiment of medium components. The results were shown as follows (Figure 5.3).

In this figure, when DEX was as a sole carbon source in the medium, the growth of HU2014 with a maximum biomass of 6.4 g/L and the highest *R. solani* YL-3 mycelial growth inhibition rate of 86.7%, followed by ARA (at 5.6 g/L and 83.0%, respectively) (Figure 5.3 A). Therefore, DEX was selected as the optimal carbon source for HU2014 to produce antifungal substances. As shown in Figure 5.3 D, the concentration of DEX was optimized, and 40.0 g/L DEX was the concentration that supported HU2014 to induce the highest *R. solani* YL-3 mycelial growth inhibition rate.

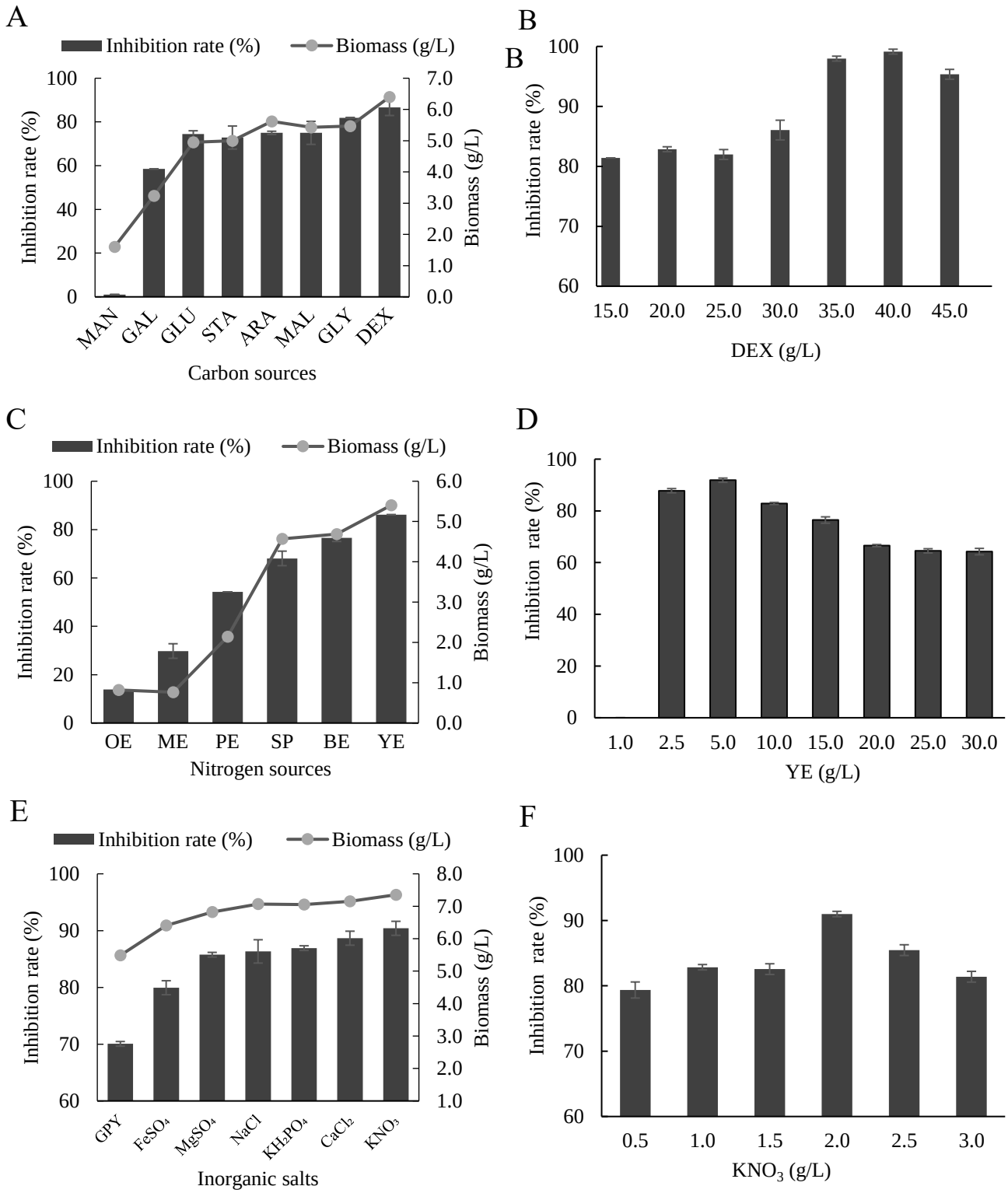


Figure 5.3. The antifungal activities of cell-free filtrates of HU2014 culture broth with different medium components against *R. solani* YL-3 and the biomass of the strain (ACE), and variation of the *R. solani* YL-3 (BDF) inhibition rate with different concentration of medium components (HERCCGE, HIST, 2022).

Similarly, screening of nitrogen sources revealed YE as the optimal nitrogen

source, which supported the growth of HU2014 with a maximum biomass of 5.4 g/L and the highest *R. solani* YL-3 mycelial growth inhibition rate of 86.2% (Figure 5.3 B). Furthermore, the optimal concentration of YE was 5.0 g/L, at which HU2014 produced the highest mycelial growth inhibition rate (Figure 5.3 E). Likewise, screening of inorganic salts revealed KNO_3 as the optimal inorganic salt, which supported the growth of HU2014 with a maximum biomass of 7.4 g/L and the highest *R. solani* YL-3 mycelial growth inhibition rate of 90.4% (Figure 5.3 C), and the inhibition rate was the highest at 2.0 g/L KNO_3 (Figure 5.3 F).

Optimization of main factors by PB design and FCCD

PB design can quickly and effectively screen out the most important factors with minimum number of tests. After PB design, the screen important factors were optimized using FCCD. First, the results of PB design were presented in Table 5.2 and Figure 5.4.

Table 5.2

ANOVA for the experimental result of PB design

Source	DF	SS	MS	*F* value	*P* value
Model	8	646.284	80.785	23.74	0.012
Linear	8	646.284	80.785	23.74	0.012
Dextrin	1	126.425	126.425	37.15	0.009
Yeast extract	1	439.593	439.593	129.19	0.001
KNO3	1	57.685	57.685	16.95	0.026
pH	1	3.597	3.597	1.06	0.380
Inoculum size	1	6.035	6.035	1.77	0.275
Temperature	1	0.304	0.304	0.09	0.785
Agitation speed	1	3.050	3.050	0.90	0.414
Incubation time	1	9.594	9.594	2.82	0.192
Error	3	10.208	3.403		
Total	11	656.492			

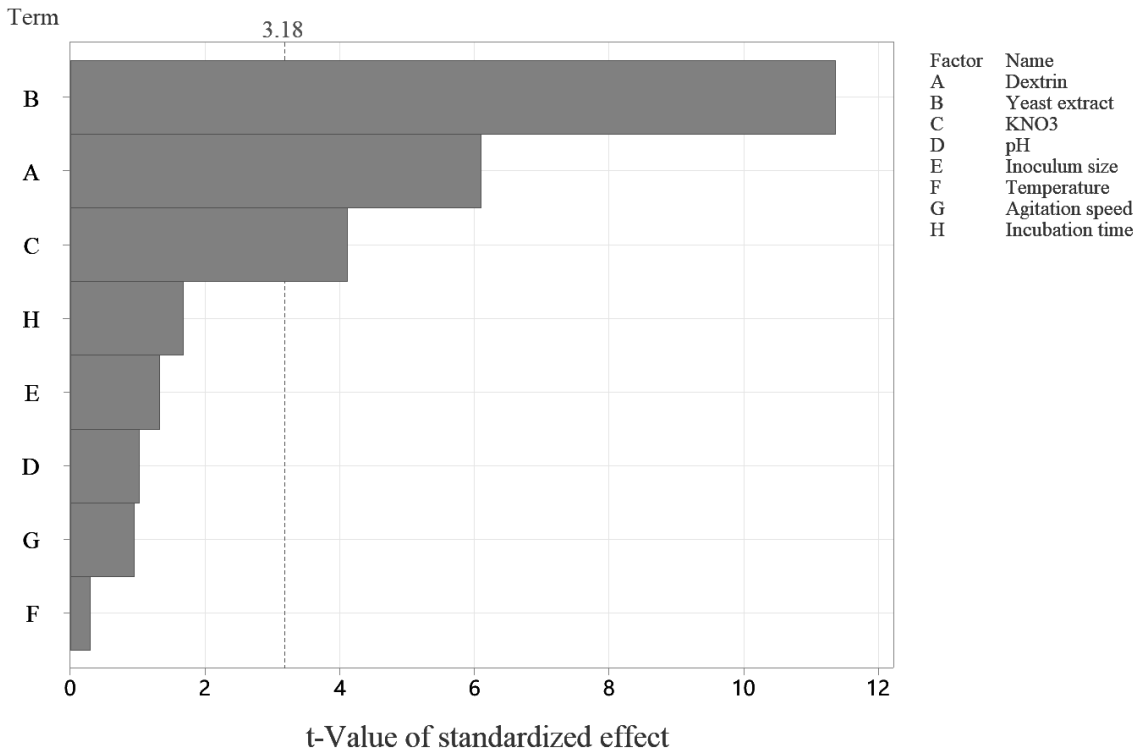


Figure 5.4. Pareto chart of the standardized effects for eight medium factors on the antifungal activity of the EF.

As the presentation by the charts, the influence of eight factors, namely DEX, YE, KNO_3, pH, inoculum size, temperature, agitation speed and incubation time, on the antifungal activities of HU2014 against *R. solani* YL-3 was studied using PB design. The results of the standardized effects and ANOVA showed that DEX, YE and KNO_3 were the most significant factors ($p < 0.05$). The t-values of standardized effect of them were more than 3.18. The other factors that had less significant effects ($p < 0.05$) on the *R. solani* YL-3 mycelial growth inhibition rate were omitted in the subsequent optimization experiments using FCCD, for the t-values of standardized effect of them were less than 3.18.

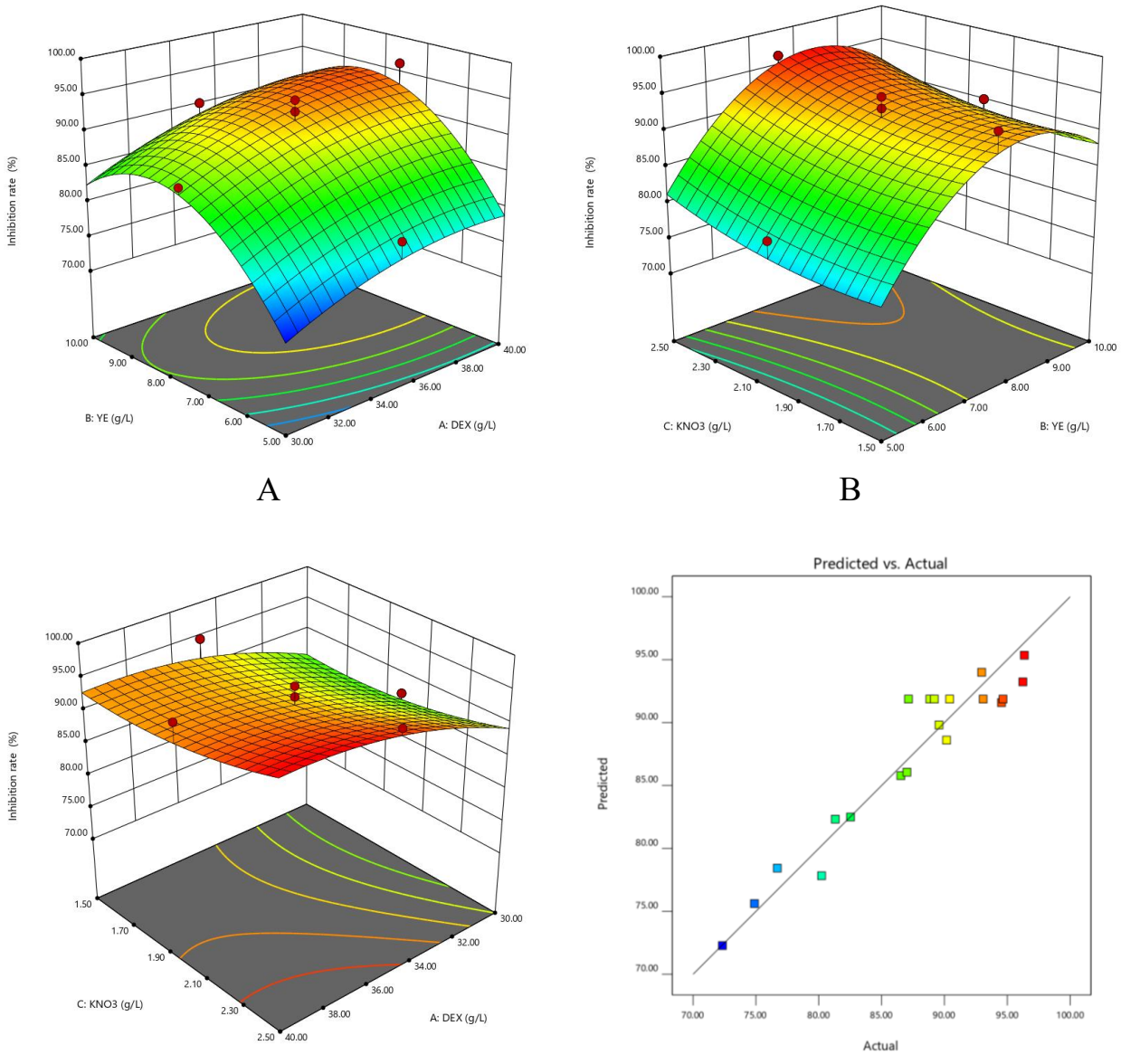
After PB design, ANOVA for quadratic model was employed for the optimization of medium components. The regression equation can be given as follows:

$$Y = 91.87 + 3.59 \times A + 5.39 \times B + 1.88 \times C + 0.3337\,(A \times B) + 0.1820\,(A \times C) + 0.0303\,(B \times C) - 2.22 \times A^2 - 8.66 \times B^2 + 1.60 \times C^2,$$

where Y represents the inhibition rate, and A, B and C denote DEX, YE and KNO_3, respectively. The significance of the model for the inhibition rate response was validated by $p < 0.0001$. The ANOVA and regression showed that the lack of fit was

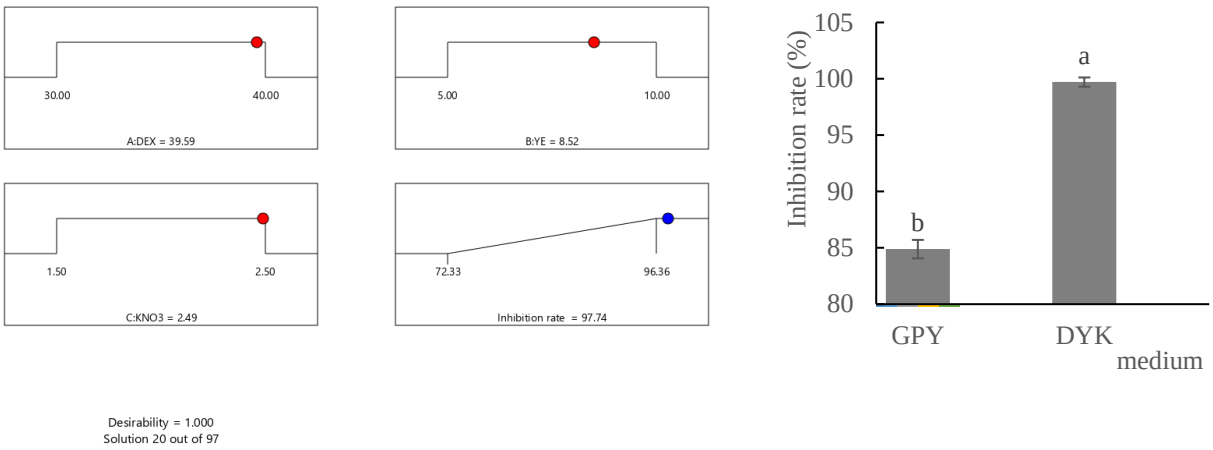
insignificant for this model.

Second, the effects of different factors on the antifungal activity of bioactive metabolites from HU2014 were analyzed using FCCD. The above theoretical Optimization data needs to be validated, thus proving the feasibility of the theoretical values. The screened medium condition was conducted, compared to GPY medium. The results were shown as follows (Figure 5.5 and Figure 5.6).



A B C D

Figure 5.5. 3D surface plot for the inhibition rate (HERCCGE, HIST, 2022). (A) Nitrogen source and Carbon source; (B) Nitrogen source and inorganic salt; (C) Carbon source and inorganic salt. (D) Actual vs. predicted values plot for the inhibition rate. Abbreviation: DEX represents dextrin, YE represents yeast extract.



A B

Figure 5.6. Optimization result for the inhibition rate using RSM (A) and the inhibition rate of the filtrated supernatants of *Streptomyces* sp. HU2014 cultured in GPY and DYK to *R. solani* YL-3 (B) (HERCCGE, HIST, 2022). DEX represents dextrin, YE represents yeast extract; DYK represents the optimized medium (dextrin, yeast extract and KNO_3).

From the above figures, the contour plot of the *R. solani* YL-3 mycelial growth inhibition rate in the presence of DEX and YE, with a significant interaction, denoted that the carbon and nitrogen sources had the highest effect on the mycelial growth inhibition rates (Figure 5.5 A). KNO_3 had a smaller effect on the *R. solani* YL-3 mycelial growth inhibition rate, when compared with DEX and YE, with an increase in concentration from 1.5 to 2.5 g/L. Similarly, the combined effects of DEX with KNO_3 and YE with KNO_3 on the *R. solani* YL-3 mycelial growth inhibition rate were assessed using 3D plots (Figure 5.5 B and C). Plotting the actual vs. predicted values for *R. solani* YL-3 mycelial growth inhibition rate (Figure 5.5 D) showed close adherence between these values along the line, thus validating the RSM model.

The RSM model suggested 97 optimal solutions, of which No. 20 was the best optimal solution. The maximum *R. solani* YL-3 mycelial growth inhibition rate was 97.7% in the presence of 39.6 g/L DEX, 8.5 g/L YE and 2.5 g/L KNO_3 (Figure 5.6 A). The validation experiments showed that the mycelial growth inhibition rate induced by the cell-free filtrates in the optimized medium against *R. solani* YL-3 increased by 14.9%, when compared with that in basal medium (GPY) (Figure 5.6 B).

Some widely used commercial BCAs, such as *Bacillus* spp., *Pseudomonas* spp.

and *Streptomyces* spp., have been applied in controlling phytopathogenic fungal diseases (Khan & Doohan, 2009; Moharam et al., 2018; Lagogianni & Tsitsigiannis, 2019; Martinez-Diz et al., 2021). As important members of Actinomycetes, *Streptomyces* spp. are well known for the production of bioactive metabolites (Latha et al., 2017). Because the active metabolite production by microorganisms is affected by fermentation conditions and medium composition (Son et al., 2001; Ratnam et al., 2003), the addition of suitable nutrients can promote metabolite synthesis, cell growth, antibiotic fermentation units and antibiotic extraction processes (Kiers et al., 2000). As microbial fermentation is a large-networked, nonuniform and complex process, optimization of the culture medium is particularly important. RSM is an effective tool to improve the production of bioactive metabolites through optimization of cultural characteristics and physicochemical conditions.

Many factors, such as incubation time, temperature, pH, carbon source, inorganic salt content and nitrogen source, can have a significant influence on the microbial metabolites production efficiency during fermentation (Oskay, 2009; El-Naggar et al., 2013b; Ganesan et al., 2017; Sabu et al., 2017), and a Pareto chart can intuitively reflect the important factors of multiple vertical factors. Statistical optimization of different parameters affecting the antifungal activity of the bioactive metabolites from HU2014 revealed that DEX as a carbon source, YE as a nitrogen source and KNO_3 as an inorganic salt could significantly influence the productivity of antifungal metabolites. It must be noted that the nutritional demand of *Streptomyces* spp. plays a vital role in the synthesis of secondary metabolites (Romero-Rodriguez et al., 2018), and the analysis of optimal media components and cultural characteristics is correlated with the suitability of the number of carbon and nitrogen sources in the fermentation medium. Some *Streptomyces* spp. have been reported to produce high yield of secondary metabolites in the presence of DEX as the carbon source (Ng et al., 2014; Abdelwahed et al., 2017; Feng et al., 2021). Likewise, the nitrogen source in the culture medium has been found to be related to the yield of antibiotic metabolites in the course of fermentation. For instance, moisture, YE and $CaCl_2$ have been reported to significantly influence the antibiotics production by *Streptomyces* sp. AS4 (Al Farraj

et al., 2020). Nevertheless, it is necessary to seek further cheaper alternative nitrogen sources for mass production of bioactive metabolites.

Inorganic salts play a role in the production of active metabolites during the process of microbial fermentation. It has been reported that the regulation of pigment biosynthesis pathway in *Serratia* sp. S2B is affected by inorganic micronutrients, particularly, sulfate ions (Rastegari & Karbalaei-Heidari, 2016). Furthermore, KNO_3 has been found to exert a significant positive effect on the production of antimicrobial agents by *Streptomyces* sp. NEAE-1 (El-Naggar et al., 2013a). In the present study, addition of most of the tested inorganic salts to the basal medium (GPY) significantly increased the *R. solani* YL-3 mycelial growth inhibition rate of HU2014. In particular, KNO_3 was needed to produce the highest mycelial growth inhibition rate and the maximum HU2014 biomass, which could be owing to its advantage of being an inorganic salt and a nitrogen source. Based on the PB design, DEX, YE and KNO_3 were selected for further FCCD. The quadratic model with different concentrations of medium components was investigated by RSM, and the 3D response surface plots showed the interaction effects of individual factors. The medium components, especially the DEX and YE ratios, significantly affected the growth of HU2014 and its production of bioactive metabolites. However, although RSM has some advantages in many aspects, it still presents certain limitations (Chaudhary et al., 2021), and other more powerful techniques are necessary to overcome these constraints.

Conclusions to Chapter 5

The results of this section show that the antifungal components productivity of HU2014 had significantly increased after optimizing the fermentation conditions. Spectrophotometry and bioassay revealed that F6 and F8 fractions of the culture supernatant exhibited higher antifungal components. Subsequently, single-factor experiments were conducted using glucose-peptone-yeast extract medium as basal medium, and the effects of different parameters on bioactive metabolites production by HU2014 were evaluated and optimized using shake-flask experiments under the following conditions: incubation time, 10 days; temperature, 25°C; agitation speed,

150 rpm; inoculum size, 5% (v/v); initial pH, 7.0; and medium composition, dextrin, yeast extract, and KNO_3. The central composite face-centered design from response surface methodology predicted 39.59 g/L dextrin, 8.52 g/L yeast extract, and 2.49 g/L KNO_3 as the optimum medium composition, which was confirmed by experiments in which *Rhizoctonia solani* YL-3 mycelial growth inhibition rate increased by 14.87% in the optimum medium, when compared with that in basal medium. A good correlation between measured and predicted values of the model validated both the responses. Thus, the present study used statistical optimization method to improve bioactive metabolites productivity in HU2014.

CHAPTER 6
FIELD APPLICATION OF HU2014 IN WHEAT PLANTS

Investigation on physiological indicators of wheat by HU2014 inoculation

In the previous co-culture and pot experiments, we found that HU2014 has antifungal and promoting growth effects on wheat. Many reports have showed that the performance of microbes acting on plants in pots and in field was different. In order to verify the effect of this strain on wheat in the field environment, we conducted the experiments. The results were shown as follows (Figure 6.1, Table 6.1 and Table 6.2)

Figure 6.1. ZM22 growth at tiller stage in 2023 (Experimental field, HIST, 2023). CK represents the soil without any treatment, S represents the soil with HU2014 inoculation, (R+J) P represents the soil with *R. cerealis* G11 infection, (S+J) SP represents the soil with HU2014 and *R. cerealis* G11 treatments.

Table 6.1

Traits of plant vegetative and generative development at the wheat tiller stage (HERCCGE, HIST, 2022)

Treatment		Shoot height (cm)	Number of tillers	Chlorophyll content (nmol/g)	MDA (nmol/g)	Root activity (ug/h/g)
2022	CK	45.7±2.34	5.4±1.67	15.88±0.94	33.45±4.73	30.68±6.11
	S	50.0±1.97	5.1±0.35	17.72±0.74	25.56±0.57	39.31±1.49
2023	CK	56.2±1.94	6.2±0.31	14.83±2.69	31.08±5.65	29.43±4.08
	S	57.8±2.07	6.3±0.30	15.24±1.88	25.24±0.68	38.84±5.66
	P	46.5±2.16	5.5±0.45	13.41±1.54	39.41±5.89	27.19±0.73
	SP	54.1±1.34	7.0±0.64	14.93±0.49	32.20±3.57	41.33±2.29

Note: CK represents the soil without other treatment, S represents the soil with HU2014 inoculation, P represents the soil with *R. cerealis* G11 infection, SP represents the soil with HU2014 and *R. cerealis* G11 treatments.

Table 6.2

Traits of plant vegetative and generative development at the wheat maturation stage (HERCCGE, HIST, 2022)

Treatment		Spike length (cm)	Number of seeds per spike (pcs)	1000 grain weight (g)	Yield per cell (g)
2022	CK	6.3±0.22	38.1±2.23	49.66±1.92	1002.85
	S	6.4±0.35	41.1±2.89	52.34±2.01	1098.22
2023	CK	8.4±0.55	45.3±3.39	55.76±2.15	1415.67
	S	8.8±0.99	50.5±3.93	57.89±1.78	1750.60
	P	8.1±1.78	45.2±8.92	60.31±2.88	360.98
	SP	8.1±0.93	44.1±5.13	48.74±1.86	1280.62

Note: CK represents the soil without any treatment, S represents the soil with HU2014 inoculation, P represents the soil with *R. cerealis* G11 infection, SP represents the soil with HU2014 and *R. cerealis* G11 treatments.

The test in 2023 showed that wheat growth was affected by *R. cerealis* G11 infection, with the shoot height reduced 3.74-17.36% in the P and SP treatments. Except the CK and S treatments, the number of tillers had clear increase in the SP treatment but decrease in the P treatment. The chlorophyll content was not obviously

different in all treatments. The P treatment had the highest MDA value, while the S treatment showed the lowest. All the treatments with HU2014 inoculation increased the root activity (Figure 6.1 and Table 6.1). The S treatment showed the highest spike length and number of grains per spike. Yield per cell increased by 23.66% in the S treatment and decreased by 9.50% in the SP treatment, but that in the P treatment decreased by 74.50% (Table 6.2).

On the whole, HU2014 inoculation promoted wheat growth. Comparing with the pot experiments, the amplification of shoot length and chlorophyll content was different. Therefore, the plant-beneficial bioactivities of microorganisms in the environment may differ substantially from laboratory observations (Vurukonda et al., 2018). The reason could be that the microbial operational taxonomic units found in soils of biocontrol experiments were distinct between the greenhouse and field (Araujo et al., 2020). It should be noted that the wheat planting area experienced a catastrophic flood disaster in July 2021, which had an obvious impact on the wheat yield. Overall, the selection of biocontrol agents becomes complex, mainly owing to their diversity and interactions with hosts (Moto et al., 2017) and climates.

Conclusions to Chapter 6

The results of this section show that HU2014 could promote wheat growth and effectively suppress WSE in the co-culture conditions in field. In 2022 and 2023, the most promoting growth indicators after HU2014 inoculation all showed increasing, but the amplification of them in field was less than in pot experiments. In 2023, the yield per cell increased by 23.66% in the S treatment and decreased by 9.5% in the SP treatment, but that in the P treatment decreased by 74.50% compared to the CK treatment.

CONCLUSION

The determinants of the beneficial activity of *Streptomyces* sp. have been theoretically substantiated and experimentally confirmed. HU2014 and revealed its potential as a biocontrol agent through studies of its antifungal activity and ability to promote plant growth:

Through the investigation of the physiological, biochemical, phylogenetic and genomic characteristics, HU2014 indicates that it is a novel *Streptomyces* species most closely related to *Streptomyces albireticuli.*

1. In vitro and vivo traits, the strain exhibited strong antifungal activity against *R. cerealis* G11 and *R. solani* YL-3. In pot experiment, the drenching of wheat with spore suspension of HU2014 demonstrated a control efficiency of 65.1% against *R. cerealis* G11 (compared with 66.9% when treated by a 30% hymexazol aqueous solution).

2. F2, F4, F6, and F8 fractions extracted from the metabolites of HU2014 were verified the high antifungal activity against *R. cerealis* G11 and *R. solani* YL-3. The bioassay revealed that the F6 and F8 fractions could inhibit the growth of *R. solani* YL-3 with the maximum inhibition rates of 100%, followed by F4 and F2 (less than 60.50%). Spectrophotometric analysis was found that F6 and F8 had the same strong characteristic absorption peaks at 319 nm, 333 nm, and 351 nm. The main active components in the filtrated supernatants were determined to enrich in F6 and F8.

3. Ultra-performance liquid chromatography-mass spectrometry (UPLC-MS) on the four extracts from the EF of HU2014 identified 10 chemical constituents in the Natural Products Atlas with high match levels (more than 90%).

4. The activities of POD, PAL and PAL in wheat (AK58, BN307, and ZM22) drenching with EF of HU2014 could be significantly induced at low concentrations (F3). Six specific gene expression analyses were performed using QRT-PCR. The expression of *PR1, PR2, PR3, PR5, PAL*, and *LOX* were markedly induced.

5. HU2014 metabolites (F2, F4, F6, and F8 fractions) on wheat and green bristlegrass had the negative allelopathic effects on the seedling height and root length of two tested plants except for F2 at 1 mg/mL promoting the seedling height of wheat.

6. In vitro and pot experiments demonstrated that HU2014 produced indoleacetic acid, siderophores, extracellular enzymes, and solubilized phosphate, and it promoted the growth of three wheat cultivars. The shoot height increased significantly by 12.9% to 22.4% in comparison to the non-inoculated control in the three cultivars at 28 dpi ($P < 0.05$). Similarly, HU2014 was able to improve shoot dry weight in comparison to the non-inoculated control. The chlorophyll content increased by 27.3% to 87.1% compared with the non-inoculated control. The root dry weight increased significantly by 45.5% to 83.4% in comparison to the non-inoculated control at 60 dpi ($P < 0.05$).

7. In the experiments of the effect of this bacterium on soil and native soil microorganisms, HU2014 increased the malondialdehyde content of wheat leave, while decreased catalase activity, peroxidase activity. With HU2014 inoculation, the concentrations of total nitrogen, nitrate nitrogen, total phosphorus, and Olsen-phosphorus in the wheat rhizosphere increased. The diversity of rhizosphere bacteria, but not fungi clearly decreased by HU2014 inoculation. The compositions of bacterial and fungal community differed after HU2014 inoculation with *Proteobacteria*, *Acidobacteriota*, and *Ascomycota* being the dominant phyla in all treatments.

8. The effects of different parameters on the production of bioactive metabolites by *Streptomyces* sp. HU2014 were evaluated and optimized using shake-flask experiments as follows: incubation time, 10 days; temperature, 25°C; agitation speed, 150 rpm; inoculum size, 5% (*v/v*); initial pH, 7.0; and medium composition, dextrin, yeast extract and KNO_3. The central composite face-centered design from response surface methodology predicted 39.6 g/L dextrin, 8.5 g/L yeast extract and 2.5 g/L KNO_3 as the optimum medium composition. The validation experiment confirmed that a 50-fold dilution of the optimized filtered fermentation broth of the strain culture showed an increase of 14.9% in the inhibition of *Rhizoctonia solani* YL-3 when compared with the basal medium.

9. To verify the biocontrol ability of HU2014 against wheat sharp eyespot in field, the physiological parameters, as well as characteristic traits in promoting growth were assessed. In 2023, Yield per cell increased by 23.66% in the S treatment and decreased by 9.5% in the SP treatment, but that in the P treatment decreased by 74.50%

compared to the CK treatment. In 2022 and 2023, the most promoting growth indicators after HU2014 inoculation showed increasing, but the amplification of them in field was less than in pot experiments.

RECOMMENDATIONS FOR PRODUCTION

1. *Streptomyces* sp. HU2014 can be used for production of a new biological fungicide because it has antifungal activity against WSE caused by *R. cerealis* G11 and *R. solani* YL-3, and the properties of promoting wheat growth.

2. Optimal conditions for cultivation of the new strain HU2014 were developed: incubation time - 10 days; temperature - 25°C; agitation speed- 150 rpm; inoculum size - 5% (v/v); initial pH - 7.0; medium composition (39.6 g/L dextrin, 8.5 g/L yeast extract and 2.5 g/L KNO_3; and 50-fold dilution of the optimized filtered fermentation broth of the strain culture.

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APPENDIXES

Appendix A

The act of implementing the results of the doctoral dissertation in the production process

This act states that the results of the dissertation with the topic "Biocontrol mechanisms of *Streptomyces* sp. HU2014 against *Rhizoctonia* spp. caused wheat sharp eyespot" were found in Henan Engineering Research Center of Crop Genome Editing, Xinxiang, China. The dissertation was presented for the award of the Doctor Philosophy Degree.

These discoveries were used to the development of pesticide, plant and microbial fertilizer in the conditions of "Hebei Sunny Biotechnology Co., Ltd" (Hebei province, China).

Director of " Hebei Sunny Biotechnology Co., Ltd"

04/09/2023

Appendix B

"ЗАТВЕРДЖУЮ"
Проректор з науково-педагогічної
та навчальної роботи,
доктор біологічних наук,
професор

_____ Ігор КОВАЛЕНКО

від 12 вересня 2023 року

АКТ
про впровадження результатів дисертаційної роботи
в навчальний процес

Даним актом стверджується, що результати дисертаційної роботи Чжу Хунся на тему «Механізми біоконтролю Streptomyces sp. HU2014 проти Rhizoctonia spp., збудників ризоктоніозної гнилі пшениці» («Biocontrol mechanisms of Streptomyces sp. HU2014 against Rhizoctonia spp. caused wheat sharp eyespot») на здобуття наукового ступеня доктора філософії з галузі знань 20 Аграрні науки та продовольство філософії за спеціальністю 202 Захист і карантин рослин впроваджені у навчальний процес під час викладання дисциплін "Загальна фітопатологія", "Загальна мікологія", "Патологія насіння сільськогосподарських культур", "Сільськогосподарська фітопатологія", "Імунітет рослин" для підготовки студентів зі спеціальності 202 Захист і карантин рослин.

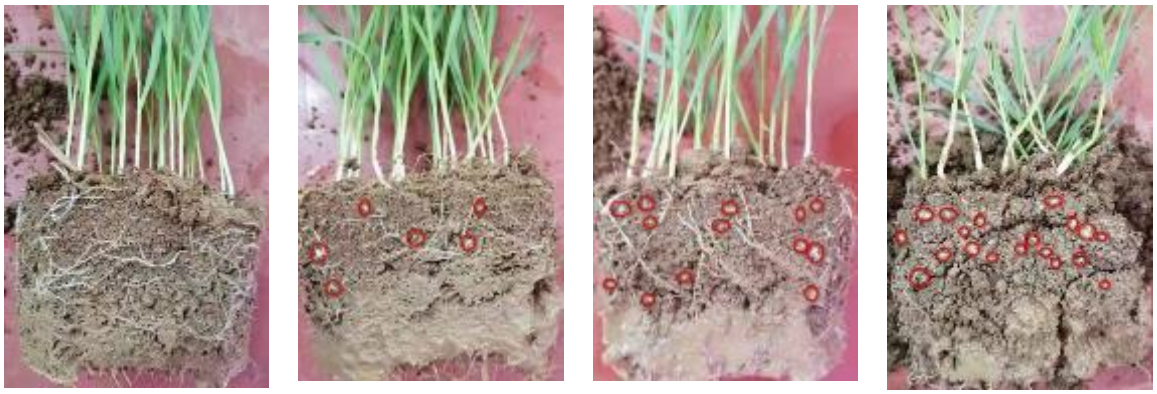
Розглянуто і схвалено на засідання кафедри захисту рослин ім. доц. А. К. Мішньова, протокол № 2 від 11 вересня 2023 року.

В. о. зав. кафедри захисту рослин
ім. доц. А. К. Мішньова,
доцент Валентина ТАТАРИНОВА

Декан факультету агротехнологій
та природокористування Ольга БАКУМЕНКО

Appendix C

A

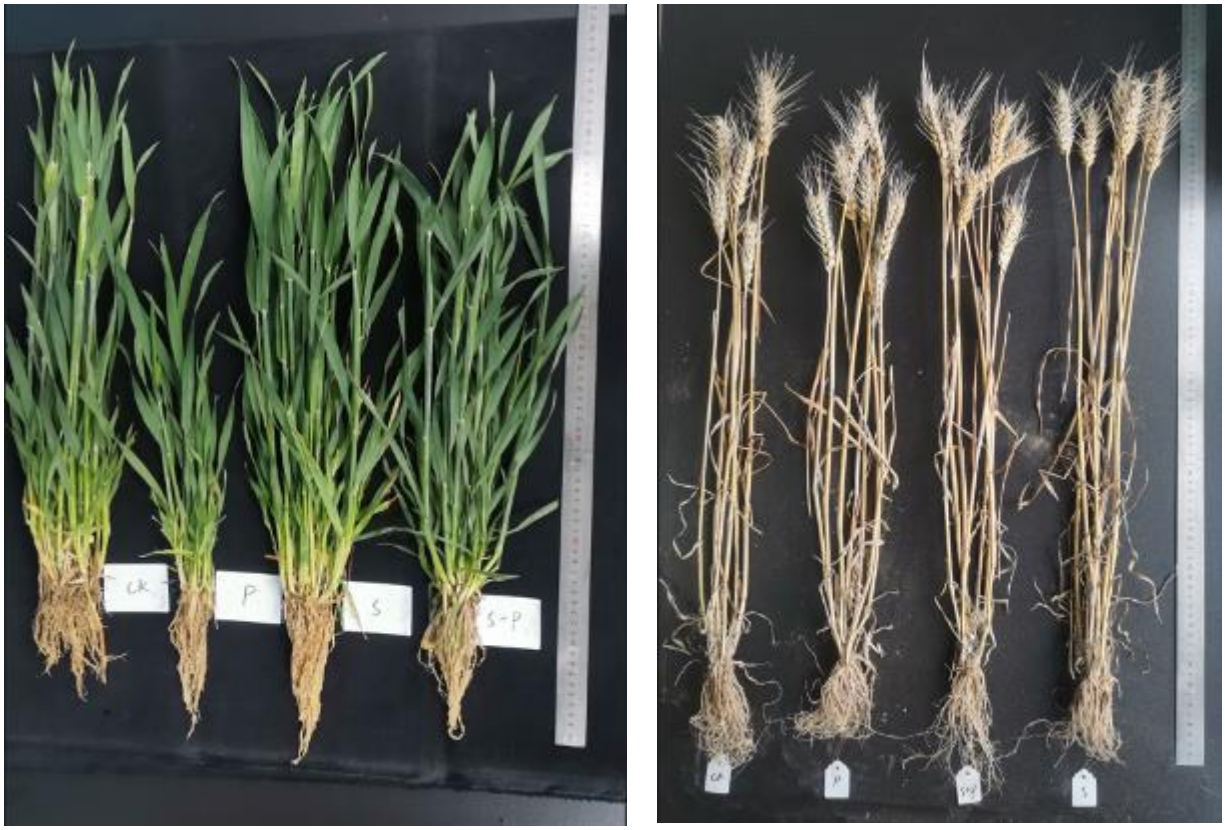


CK S1 S5 S15

B

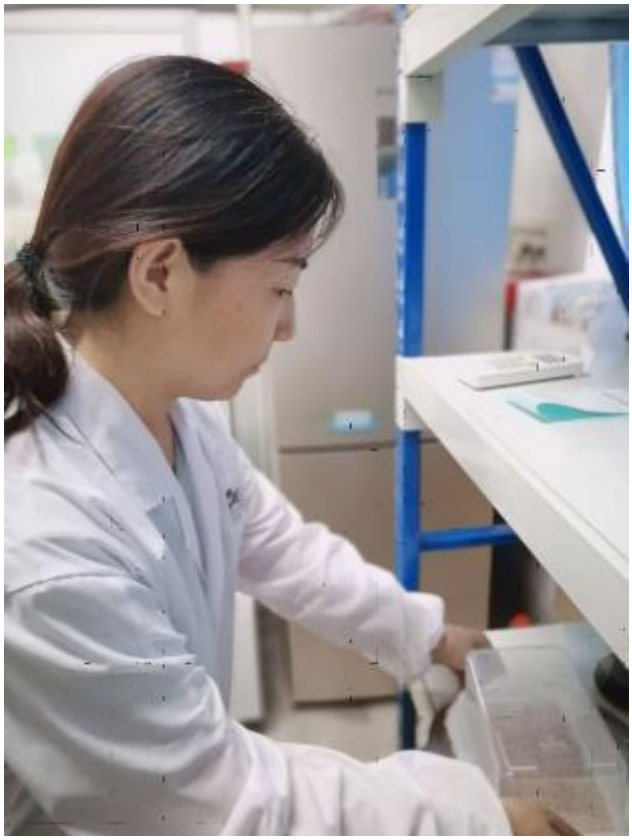
Fig.1. HU2014 colonization investigation (HERCCGE, HIST, 2022). HU2014 Colonization (A) and phenotype of HU2014 in LB media with ampicillin resistance (200μg/mL) (B) in pots. S1, S5 and S15 represent the different concentrations of HU2014 colonies mixed with soil. 100×3 represents 10^3-diluted suspension of soil samples with different HU2014 concentration.

Appendix D



A B

Fig.2. ZM22 growth at the tiller stage (A) and maturation stage (B) (Experimental field, HIST, 2023). The sequence of the treatments in A is CK, P, S, and SP; The sequence of the treatments in B is CK, P, SP, and S; CK represents the soil without any treatment, S represents the soil with HU2014 inoculation, P represents the soil with *R. cerealis* G11 infection, SP represents the soil with HU2014 and *R. cerealis* G11 treatments.



5

Fig.3. Photographs during the research progress.