A high-throughput screening method for endophytic bacteria with antagonistic activity against *Magnaporthe oryzae* in rice (*Oryza sativa* L.) seeds

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Abstract
Screening for microorganisms with antagonistic activity against crop pathogens is essential for microbial antagonist preparation, crop disease control, and sustainable agricultural development. This study established a rapid, efficient, one-step high-throughput method for screening endophytic bacteria displaying antagonistic activity against *Magnaporthe oryzae* (M. oryzae) ACCC36020 in rice seeds. The experimental results indicated that these bacteria could be directly screened from six sample groups using this method. This technique can achieve simultaneous screening and purification, identify endophytic bacteria displaying antifungal activity in rice seeds, and save significant time.

Keywords Rice seed · Endophytes · High-throughput screening · Antifungal activity · Biological control

Introduction
Rice blast caused by *Magnaporthe oryzae* (M. oryzae) is a rice disease that causes significant annual losses in rice yield worldwide (Feng et al. 2019). Rice blast is primarily controlled via fungicides, field management reinforcement, the breeding of disease-resistant varieties, and biological methods. However, it has been proven that increasing the use of fungicides or rice varieties resistant to rice blast in field conditions is ineffective for the long-term control of the disease (Deng and Naqvi 2019). Furthermore, the excessive use of fungicides and pesticide residues pose a substantial threat to food safety and the environment and are likely to cause pathogen drug resistance, presenting unpredictable ecological risks. Therefore, identifying a new, safe, environmentally-friendly, cost-efficient fungicide against *M. oryzae* has become essential for rice production.

As a type of microbial resource found in the tissues or organs of healthy plants, plant endophytes have shown potential as microbial pesticides, production-increasing bacteria, or biocontrol carrier bacteria (Liu et al. 2017, 2018; Afzal et al. 2017; de Almeida Lopes et al. 2018; Huang et al. 2021). Studies have shown that endophytic bacteria isolated from plant seeds have a strong antagonistic effect on plant pathogens and can be used to prepare microbial agents for plant disease control (Shameer and Prasad 2018; Rangjaroen et al. 2019; Matsumoto et al. 2021). Endophytic bacteria isolated from rice seeds also showed strong antagonism against *M. oryzae* (Jing et al. 2020). Therefore, isolating and screening microbial resources from rice tissues or the environment that display antifungal activity is vital for the biological control of pathogenic fungi.

Currently, the most commonly used methods to screen for microorganisms in plants displaying antibacterial activity are the plate confrontation method (Jing et al. 2020; Degani and Dor 2021) and the Oxford cup method (Sun et al. 2018). However, these techniques involve the integration of separate cultures, purification, and screening. The synchronous growth and coordination between different strains must be maintained while the operation is tedious and protracted and
the flux is low. This study selected six rice varieties with different resistance levels to rice blast to establish a high-throughput screening method for endophytic bacteria exhibiting antagonistic activity against *M. oryzae* in rice seeds.

### Materials and methods

#### The source of rice seeds and *M. oryzae*

The six rice seed samples were provided by the Hunan Hybrid Rice Research Center. Table 1 shows all the sample-related information. The seed samples were collected over several years and preserved at the Changsha Comprehensive Test Base of Hunan Hybrid Rice Research Center. The samples were transferred to sterile bags, sealed, and stored at 4 °C until used (it can be preserved for extended periods in a sealed environment).

*M. oryzae* ACCC36020 represented the pathogenic rice blast strain used in this study and was provided by Professor Xiaoxia Zhang at the Agricultural Culture Collection of China (ACCC). This pathogenic strain is well documented for causing rice blast (in the Strain Catalogue of the ACCC).

#### Sample surface sterilization and treatment

The surface sterilization of the rice seeds was mainly accomplished using sterile water and alcohol. The specific steps were as follows: Here, 1 g of rice seeds were weighed and washed with sterile water to remove impurities from the seed surfaces, such as dust. The seeds were transferred to alcohol and soaked for about 30 s, after which they were washed again six times with sterile water to completely remove any residual alcohol that might affect follow-up experiments. The surface sterilization was validated by pressing the seeds into LB medium (LUQIAO) using sterile tweezers, after which the samples were incubated at 32 °C for 72 h.

#### Enrichment culture of endophytic bacteria in rice seeds

The surface-sterilized seeds were ground into a powder using a sterile mortar. One in three powder was placed in 1.5 mL aseptic centrifuge tubes, after which 1 mL of LB liquid medium (LUQIAO) was added. All the samples were cultured at 32 °C for 8–12 h for endophytic bacterial enrichment.

#### *M. oryzae* activation

The *M. oryzae* was collected from the storage test tube using an inoculation needle, placed in solid PDA medium (Acme), and activated at 28 °C. Next, the spores were collected and mixed with sterile water. The *M. oryzae* spore suspension was prepared via re-suspension for at least 30 min in a shaker at 28 °C.

#### Screening of the endophytic bacteria displaying antagonistic activity against *M. oryzae*

The solid LB medium was sterilized at 121 °C, after which the *M. oryzae* spore suspension was added (200–300 µL spore suspension/100 mL LB medium, the concentration can be adjusted according to the specific experimental conditions) at a lower temperature (50–60°C). The mixture was rapidly poured into a Petri dish and left to solidify. After culture enrichment, the sample culture medium was diluted to $10^{-5}$ and $10^{-6}$ with aseptic water and mixed thoroughly. Then, 120–150 µL was collected using a pipettor and coated onto the solidified LB medium plate mixed with *M. oryzae*. Then all the samples were cultured at 32 °C for 1–2 days. Finally, the presence of endophytic bacteria displaying antagonistic activity against *M. oryzae* in the rice seeds of this variety was determined by establishing whether transparent circles were present on the plate. The diameters of the transparent circles reflected the antagonistic activity of the endophytic bacteria.

### Table 1 Statistical table of information on rice seed samples

| Sample ID | Variety names | Rice blast resistance | Rice blast resistance gene | Source |
|-----------|---------------|------------------------|---------------------------|--------|
| RBR01     | R900          | High infection         | –                         | Parents of super hybrid rice |
| RBR02     | W1404         | High resistance        | Pigm                      | Restorer line |
| RBR03     | C_liangyou_557| Moderate resistance    | P2                        | Hybrid breeding of C815S and R557 |
| RBR04     | R1137         | Resistance             | P2                        | Hybrid breeding of R900 and Wushansimiao |
| RBR05     | Y_liangyou_1137| Moderate resistance/Moderate infection | P2            | Hybrid breeding of Y58S and R1137 |
| RBR06     | Xingliangyou_1283| Moderate infection    | –                         | Hybrid breeding of Xing_88S and R1283 |
Fig. 1 High-throughput screening of endophytes with antagonistic activity against Magnaporthe oryzae in rice seeds.

Fig. 2 Screening of endophytic bacteria with antagonistic activity against M. oryzae in rice seeds by high throughput screening and activity verification of several strains selected in the RBR04.
Identification and functional verification of the screened strains

The endophytic bacterial colonies were obtained via the high-throughput screening method. The phylogenetic analysis of these colonies was performed using molecular biological identification methods involving 16 S rRNA and gyrA genes (Jing et al. 2020). The plate confrontation method was used to further verify the functional activity of these endophytic bacteria (Jing et al. 2020; Degani and Dor 2021).

Results and discussion

The results showed that the endophytic bacteria displaying M. oryzae ACCC 36020-resistant activity were successfully screened from the six rice seed samples using the experimental method designed in this study (Figs. 1 and 2). The RBR04 sample yielded the most endophytic bacteria, while the other five groups only exhibited one or two transparent circles (Fig. 2). To verify that the endophytic bacterial colony obtained via the high-throughput screening method was indeed a purified single bacterial colony, RBR04, displaying the most significant number of colonies, was used for further experiments. Four randomly selected single colonies in the RBR04 plate were identified via molecular biology, of which three were Bacillus mojavensis, and one was Bacillus subtilis (Fig. 3). Functional verification of the endophytic bacteria from the selected colonies showed that they indeed exhibited antagonistic activity against M. oryzae (Fig. 2). Therefore, the high-throughput method for screening endophytic bacteria established in this study can achieve the same result as the traditional method based on screening and plate confrontation experiments. Although the number of endophytic bacteria with antifungal activity was minimal in each rice variety, this data provides direct evidence for the feasibility of high-throughput endophytic bacterial screening in rice seeds.

The screening rate of the bacteria with antagonistic activity against corresponding pathogenic fungi generally has a great relationship with the species and concentration of endophytic bacteria inherent in the sample itself. Rice blast-resistant seed varieties were selected to increase the screening rate of the target strains while enriching the endophytic bacteria in the seed samples to avoid failure due to insufficient or low concentrations of endophytic bacteria. Previous studies used traditional methods to screen and explore endophytic bacteria exhibiting antagonistic activity against pathogenic fungi in plant seeds, requiring at least a week or more to complete the entire experimental process (Jing et al. 2020; Yang et al. 2020). The method descriptions in related reports indicated that ample time is needed from strain screening to the completion of the antagonistic experiments (Liu et al. 2016; de Almeida Lopes et al. 2018; Rangjaroen et al. 2019; Tian et al. 2020). Compared with traditional strain screening and plate confrontation experiments, the one-step high-throughput method used to screen endophytic bacteria displaying antagonistic activity against pathogenic fungi in rice seeds can complete all the required processes in 4–5 days. Therefore, this method simultaneously screened...
bacteria while accomplishing the antagonistic experiment, significantly improving the experimental efficiency.

Although the experimental method designed in this study is only aimed at the high-throughput screening of endophytic bacteria in rice seeds, it is also suitable for the high-throughput screening of endophytic bacteria in corn, sorghum, wheat, and other plant seeds by adjusting the experimental scheme. Since future research regarding endophytic bacteria in plant seeds is set to focus on characteristics, such as the high yield, high quality, and multi-resistance of the plant, the identification, and screening of endophytic growth-promoting bacteria and endophytic antagonistic bacteria are essential. Therefore, this study provides a one-step high-throughput screening method for the rapid and efficient identification of endophytic bacteria displaying antifungal activity in plant seeds.

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Author contributions WZS and WXY designed and participated in all experimental procedures, performed data analysis, and drafted the manuscript. LN participated in the samples collection, preparation, and cultivation. WWP and LY supervised the study and critically revised the manuscript. All authors read and approved the final manuscript.

Data availability All data generated or analyzed during this study are included in this published article.

Declarations

Conflict of interest The authors declare that they have no competing interests.

Ethical approval Not applicable.

Consent to participate Not applicable.

Consent for publication Not applicable.

Research involving human and/or animal participants This article does not contain any studies with human participants or animals performed by any of the authors.

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