Optimization of culture conditions for *Colletotrichum* sp. AP-12, an endophytic fungus from *Andrographis paniculata*, and its control of bacterial wilt in *Pogostemon cablin*

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Abstract

Current biocontrol research lacks sufficient investigation into the tripartite interaction mechanisms among "pathogen-biocontrol agent-host." To establish a research system integrating "fermentation process optimization-control efficacy validation-physiological regulation" and systematically elucidate the interactions among the three components, this study used the endophytic fungus AP-12 from Andrographis paniculata as the research object. The fermentation process of AP-12 was optimized through single-factor experiments combined with orthogonal experiments, and pot experiments were conducted to evaluate its control effect against bacterial wilt of Pogostemon cablin, as well as its effects on the physiological and biochemical indexes and active component contents of Pogostemon cablin under Ralstonia solanacearum stress. The results showed that: (1) the optimal fermentation process for strain AP-12 was fructose 20g·L-1, yeast extract 20g·L-1, pH6.0, liquid volume 400mL, rotation speed 150r·min-1, temperature 28℃, and fermentation time 8d; after optimization, the antibacterial activity increased by 83.41% compared with the initial conditions. (2) Pot experiments showed that the control efficacies of AP-12 fermentation broth and sterilized fermentation broth against bacterial wilt of Pogostemon cablin were 31.45% and 21.58%, respectively, confirming that its active substances possessed thermal stability. (3) AP-12 treatment delayed the decreases in chlorophyll content and nitrogen content in Pogostemon cablin leaves under Ralstonia solanacearum stress, and reduced catalase, peroxidase, and superoxide dismutase activities as well as malondialdehyde content ($P<0.05$). (4) AP-12 treatment alleviated the decreases in pogostone content in Pogostemon cablin leaves and root activity under Ralstonia solanacearum stress ($P<0.05$). In summary, the endophytic fungus AP-12 significantly enhanced antibacterial activity through fermentation process optimization and, via a synergistic mechanism of "antibacterial action-stress resistance-quality improvement," improved the antioxidant capacity of Pogostemon cablin, reduced oxidative damage, alleviated the decline in active component content, and significantly enhanced its resistance to Ralstonia solanacearum. This study not only provides an efficient microbial resource for the biological control of bacterial wilt in Pogostemon cablin, but also offers a new theoretical basis and technical support for the application of plant endophytic fungi in the green control of agricultural diseases.

Full Text

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Optimization of culture conditions for *Colletotrichum* sp. AP-12, an endophytic fungus from *Andrographis paniculata*, and its control of bacterial wilt in *Pogostemon cablin*

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Abstract: In view of the current insufficiency in biocontrol research concerning the tripartite interaction mechanism among "pathogen–biocontrol agent–host," this study aimed to construct a research system of "fermentation-process optimization–efficacy verification–physiological regulation" and to systematically analyze the interactions among the three. Using the endophytic fungus AP-12 from *Andrographis paniculata* as the research object, the fermentation process of AP-12 was optimized through single-factor experiments combined with orthogonal experiments. Pot experiments were used to determine its control effect against bacterial wilt of *Pogostemon cablin*, as well as its effects on the physiological and biochemical indices of *P. cablin* under the stress of the wilt pathogen and on the contents of active constituents. The results showed that: (1) the optimal fermentation conditions for strain AP-12 were fructose 20 g·L^-1, yeast powder 20 g·L^-1, pH 6.0, loading volume 400 mL, rotational speed 150 r·min^-1, temperature 28 °C, and fermentation time 8 d; after optimization, antibacterial activity increased by 83.41% compared with the initial conditions. (2) Pot experiments showed that the control effects of AP-12 fermentation broth and sterilized fermentation broth against bacterial wilt of *P. cablin* were 31.45% and 21.58%, respectively, confirming that its active substances possess thermal stability. (3) AP-12 treatment delayed the decreases in chlorophyll content and nitrogen content in *P. cablin* leaves under the stress of the wilt pathogen, and reduced the activities of peroxidase, polyphenol oxidase, and superoxide dismutase, as well as the malondialdehyde content ($P<0.05$). (4) AP-12 treatment alleviated the decreases in patchouli ketone content and root activity in *P. cablin* leaves under the stress of the wilt pathogen ($P<0.05$). In summary, the endophytic fungus AP-12 significantly enhanced antibacterial activity through optimization of the fermentation process and, through the synergistic mechanism of "bacteriostasis–stress resistance–quality improvement," strengthened the antioxidant capacity of *P. cablin*, reduced oxidative damage, alleviated the decline in active-constituent content, and significantly improved the resistance of *P. cablin* to the wilt pathogen. This study not only provides efficient microbial resources for the biological control of bacterial wilt of *P. cablin*, but also offers new theoretical evidence and technical support for the application of plant endophytic fungi in the green control of agricultural diseases.

Keywords: bacterial wilt of *Pogostemon cablin*; endophytic fungus AP-12 from

Andrographis paniculata; fermentation-process optimization; synergistic control; oxidative defense

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Abstract: To address the limited understanding of tripartite interactions among pathogen, biocontrol agent, and host in current biological control research, this study established an integrated research framework of "fermentation optimization-efficacy evaluation-physiological regulation" to systematically elucidate their interplay. Using endophytic fungus AP-12 from *Andrographis paniculata*, its fermentation process was optimized through single-factor and orthogonal tests. The biocontrol efficacy of AP-12 against *Ralstonia solanacearum*-induced wilt in *Pogostemon cablin* was evaluated via pot experiments, alongside its effects on physiological, biochemical, and bioactive compound levels. The results were as follows: (1) Optimal fermentation conditions (20 g · L^-1 fructose, 20 g · L^-1 yeast extract, pH 6.0, 400 mL broth volume, 150 r · min^-1, 28 °C, 8 days) enhanced AP-12' s antibacterial activity by 83.41%. (2) AP-12 fermented broth and sterilized broth exhibited

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disease control efficiencies of 31.45% and 21.58%, respectively, confirming thermostability of active metabolites. (3) AP-12 treatment mitigated chlorophyll

and nitrogen loss in *Pogostemon cablin* under pathogen stress, while reducing activities of catalase (CAT), peroxidase (POD), superoxide dismutase (SOD), and malondialdehyde (MDA) content ($P<0.05$). (4) AP-12 alleviated declines in pogostone content and root vitality ($P<0.05$). Our results show AP-12 works through three mechanisms: directly suppressing pathogens, enhancing host resistance, and preserving bioactive compounds. This study provides an effective biocontrol agent for bacterial wilt in *Pogostemon cablin* and supports using endophytic fungi in sustainable agriculture.
Key words: bacterial wilt in *Pogostemon cablin*, endophytic fungus AP-12 from *Andrographis paniculata*, fermentation process optimization, synergistic biocontrol, oxidative defense mechanism

Guanghuoxiang is the dried aerial part of *Pogostemon cablin*, a plant in the family Lamiaceae. It has the effects of resolving turbidity with its aroma, harmonizing the middle and stopping vomiting, and releasing the exterior to relieve summerheat (Chinese Pharmacopoeia Commission, 2020). It is listed as one of China' s traditional "Ten Major Guang Medicines." However, bacterial wilt is a devastating soil-borne disease of this crop and is caused by *Ralstonia solanacearum* (abbreviated as the bacterial wilt pathogen). As one of the most destructive plant-pathogenic bacteria worldwide (Mansfield et al., 2012), *R. solanacearum* can invade plants through wounds in the root system, proliferate rapidly, and block vascular bundles, thereby impeding water transport and causing wilting and death (Xu Ran et al., 2013). Studies have found that *R. solanacearum* strains infecting *P. cablin* exhibit high genetic diversity and host specialization (Yang Yuxiu et al., 2013), and can survive for long periods (>5 years) in soil or water in the absence of a host. In addition, this pathogen can spread through soil cross-transmission involving rotation hosts such as peanut, potato, and tomato, posing severe challenges for its control (Yu Xiaoman and He Zifu, 2020).

At present, the prevention and control of bacterial wilt mainly rely on chemical agents and agricultural measures. Spraying chemical agents is the most convenient and efficient method for controlling bacterial wilt, but long-term use leads to pesticide residues and increased pathogen resistance (Wu Sixuan et al., 2023). Therefore, developing safe and efficient biological control technologies has become a research focus. With the deepening of research on biological control of plant diseases, some biocontrol fungi have shown potential in controlling bacterial wilt in crops such as tobacco and tomato. For example, Liu Xianliang (2014) found that inoculation with arbuscular mycorrhizal fungi could reduce the incidence and disease index of tobacco bacterial wilt, with a control efficacy of 9.70%; Zhao Jiangyuan et al. (2022) found that *Trichoderma yunnanense* 2-14F2 and *Beauveria pseudobassiana* 2-8F2 achieved control efficacies of 51.36% and 50.27% against tomato bacterial wilt. In recent years, studies on plant–microbe interactions have further revealed the systemic mechanisms of action of biocontrol microorganisms. For instance, Zhao Pei et al. (2019) found that cotton endophytic fungi promoted the upregulated expression of plant disease-resistance-related genes in cotton (POD, PAL, PPO,

etc.), thereby improving cotton stress resistance. In addition, endophytic fungi have received considerable attention in plant disease control because of their abundant secondary metabolites and antibacterial activity (Bu Xuanyin and Yang Weili, 2021). For example, *Phyllosticta* sp. Gbh45 isolated from ginkgo by Zhang Zujiao et al. (2018) showed good inhibitory effects against the ginger bacterial wilt pathogen and exhibited genetic stability; Hou Caixia et al. (2023) found that applying the culture broth of the wolfberry endophytic fungus *Alternaria alternata* 7 d in advance could effectively control wolfberry root rot, with a relative control efficacy of 50%. However, existing studies have obvious limitations: most work has focused on the direct effects of biocontrol agents on disease index and control efficacy, while insufficient attention has been paid to their effects on host physiological status and accumulation of active constituents. In particular, research on the application of strains after fermentation-process optimization in the control of bacterial wilt of *P. cablin* remains absent, which seriously restricts the promotion and application of biocontrol technologies.

Xu Zhizhou et al. (2019) showed that, because the secondary metabolites produced by biocontrol microorganisms are unstable, screening for the optimal fermentation formula and fermentation conditions is the primary task for improving the inhibitory effects of biocontrol microorganisms against plant pathogens and enabling their more efficient use in agricultural production. However, fungal biocontrol resources for bacterial wilt of *P. cablin* are still extremely scarce, especially strains that combine highly efficient antibacterial activity with the ability to improve plant physiological traits. Previous studies have shown that the endophytic fungus AP-12 from *Andrographis paniculata* has significant inhibitory effects against a variety of pathogens, such as *Staphylococcus aureus* and *Bacillus subtilis*, and can synthesize diterpene lactone constituents of *A. paniculata* with important medicinal value, demonstrating good antibacterial activity and metabolite diversity (Li et al., 2022). However, AP-12 is prone to degeneration during cultivation, which severely constrains its development and utilization. In addition, the application potential of AP-12 in plant disease control has not yet been fully explored; in particular, no relevant studies have been reported on bacterial wilt of *P. cablin*, a soil-borne disease that seriously threatens the production of Chinese medicinal materials.

In view of the current bottlenecks in applying biocontrol microorganisms for the control of bacterial wilt of *P. cablin*—namely, unstable control efficacy and unclear effects on host physiology—this study focuses on investigating: (1) how to improve the stability of the antibacterial activity of AP-12 by optimizing the fermentation-medium formula (carbon source and nitrogen source) and fermentation conditions (liquid volume, pH, and fermentation time); (2) the actual control efficacy and mode of action of AP-12 fermentation broth against bacterial wilt of *P. cablin*; and (3) the mechanisms by which AP-12 treatment affects the physiological and biochemical responses of *P. cablin* plants and the accumulation of medicinal constituents. By establishing a research system of "fermentation-process optimization—control-efficacy evaluation—physiological regulation," this study aims to clarify the multiple roles of endophytic fungi in plant disease

control

mechanism of action.

1 Materials and Methods

1.1 Samples

Seeds of *Andrographis paniculata* were provided by the Guigang planting base in Guangxi and were identified by Professor Du Qin of the Teaching and Research Section of Medicinal Botany, Guangzhou University of Chinese Medicine, as mature seeds of *Andrographis paniculata*. The seeds were sown in seedling trays and, when four true leaves appeared, transplanted to the experimental field for cultivation into plants. The endophytic fungus AP-12 was isolated from leaves by this research group at an earlier stage (Xu et al., 2023) and preserved; it was identified as a fungus of the genus *Colletotrichum*, *Colletotrichum* sp. (GenBank ID: OL477581.1).

The bacterial wilt strain GDMCC 1.71 was purchased from the Guangdong Microbial Culture Collection Center.

Leaves of patchouli were collected from the Yezhenshan medicinal garden of Guangzhou University of Chinese Medicine. Patchouli tissue-culture seedlings were propagated by tissue-culture techniques and transplanted into indoor flowerpots, where they were grown to more than 10 cm.

1.2 Culture Media

Potato dextrose broth (PDB) medium, nutrient agar (NA) medium, and nutrient broth (NB) medium were purchased from Guangdong Huankai Microbial Technology Co., Ltd.

Callus induction medium: MS basal medium 4.74 g, sucrose 30 g, 6-BA 0.2 mg·L^-1, NAA 0.1 mg·L^-1, agar 7 g, distilled water 1000 mL, pH 5.8. Rooting medium: 1/2MS basal medium 2.47 g, sucrose 30 g, IBA 0.2 mg·L^-1, agar 7 g, distilled water 1000 mL, pH 5.8.

1.3 Main Reagents and Instruments

Reagents: malondialdehyde (MDA) assay kit, superoxide dismutase (SOD) assay kit, peroxidase (POD) assay kit, and catalase (CAT) assay kit were purchased from Nanjing Jiancheng Bioengineering Institute; pogostone reference standard was purchased from Beijing Solarbio Science & Technology Co., Ltd.; red tetrazolium was purchased from Shanghai Macklin Biochemical Technology Co., Ltd.; ethyl acetate and methanol were purchased from Tianjin Zhiyuan Chemical Reagent Co., Ltd.

Instruments: CT-884 fully automatic vertical steam sterilizer (Shanghai Chitong Instrument Co., Ltd.); HZQ-X300 constant-temperature shaking incubator (Shanghai Yiheng Scientific Instruments Co., Ltd.); RE-52AA rotary evaporator (Shanghai Yarong Biochemical Instrument Factory); BGPX-300 light incubator (Shandong Boke Scientific Instrument Co., Ltd.); 7890B gas chromatograph-mass spectrometer (Agilent Technologies, USA); OK-Y104 plant nutrient analyzer (Zhengzhou Oukeqi Instrument Manufacturing Co., Ltd.); RT-2100C microplate reader (Rayto Life and Analytical Sciences Co., Ltd.).

1.4 Optimization of Culture Conditions for Strain AP-12

1.4.1 Screening of Carbon Sources in the Fermentation Medium

Using potato extract as the basal medium, 400 mL was dispensed into 500 mL conical flasks. Different carbon sources (glucose, sucrose, maltose, and fructose) were added separately at a mass concentration of 20 $g \cdot L^{\wedge}-1$. With a 6 mm sterile punch, three AP-12 mycelial plugs were taken and inoculated into the medium, followed by incubation for 10 d on a shaker at 28 °C and 150 $r \cdot min^{\wedge}-1$.

The fermentation product was vacuum-filtered to obtain the fermentation broth. The fermentation broth was concentrated to 100 mL on a rotary evaporator at 45 °C and 55 $r \cdot min^{\wedge}-1$, and extracted by mixing at $V_{\text{ethyl acetate}} : V_{\text{fermentation broth}} = 1 : 1$ until the ethyl acetate layer was colorless. The ethyl acetate layers were combined and evaporated to dryness on the rotary evaporator under the same conditions. Methanol was added to dissolve the residue and prepare a 20 $mg \cdot mL^{\wedge}-1$ fermentation extract. The bacterial wilt strain was made into a bacterial suspension with sterile water, and the concentration of the bacterial suspension was adjusted to 1×10^6 $CFU \cdot mL^{\wedge}-1$ for later use. The Oxford cup method (Zhu Jun et al., 2023) was used to determine the antibacterial ability of the fermentation extract against the bacterial wilt strain, and the optimum carbon source was determined according to the size of the inhibition zone.

On the basis of the screened optimal carbon source, the optimal carbon source was set at mass concentrations of 10, 20, 30, 40, and 50 $g \cdot L^{\wedge}-1$, and the optimal addition amount of the carbon source was determined by comparing antibacterial activity.

1.4.2 Screening of Nitrogen Sources in the Fermentation Medium

To the medium optimized for the carbon source, different nitrogen sources (yeast powder, peptone, beef extract, and soybean meal) were separately added at a mass concentration of 10 $g \cdot L^{\wedge}-1$. The method was the same as in 1.4.1, and the optimal type of nitrogen source was screened.

The optimal nitrogen source was then set at mass concentrations of 10, 15, 20, 25, and 30 $g \cdot L^{\wedge}-1$ to determine the optimal addition amount of the nitrogen

source. Subsequent experiments used the optimal fermentation medium after optimization of the carbon and nitrogen sources.

1.4.3 Effect of Liquid Loading Volume on the Antibacterial Activity of Strain AP-12

AP-12 mycelial plugs were inoculated into the optimal fermentation medium with liquid loading volumes of 100, 200, 300, and 400 mL, and fermented in a constant-temperature shaker for 10 d (28 °C, 150 r • min^-1). The method was the same as in 1.4.1, and the optimal liquid loading volume was determined.

1.4.4 Effect of pH on the Antibacterial Activity of Strain AP-12

The pH of the optimal fermentation medium was adjusted to 5.0, 6.0, 7.0, and 8.0, respectively. The medium was fermented in a constant-temperature shaker for 10 d (28°C, 150 r · min^{-1}). AP-12 fungal plugs were inoculated into the optimal fermentation medium at the above-determined optimum liquid volume; the method was the same as in 1.4.1, and the optimal pH value was determined.

1.4.5 Effect of fermentation time on the antibacterial activity of strain AP-12 The pH of the optimal fermentation medium was adjusted to the optimal pH. AP-12 fungal plugs were inoculated into the optimal fermentation medium at the above-determined optimum liquid volume, and fermentation was carried out in a constant-temperature shaker at 28°C and 150 r · min^{-1} for 6, 8, 10, and 12 d. The method was the same as in 1.4.1, and the optimal fermentation time was determined.

1.4.6 Orthogonal experimental design for strain AP-12 On the basis of the determined optimal carbon source and nitrogen source, four factors were selected for optimization experiments: liquid volume (A), optimal carbon-source concentration (B), optimal nitrogen-source concentration (C), and pH (D). Three levels were set for each factor, forming a four-factor, three-level experiment. SPSS was used for the orthogonal experimental design, and the combination schemes were determined for testing. The nine treatments were fermented for 8 d (28°C, 150 r · min^{-1}) under the corresponding culture conditions. The method was the same as in 1.4.1. Antibacterial activity was measured, with three replicates. Range analysis and analysis of variance were performed on the orthogonal results, and the optimal fermentation combination was determined through comprehensive analysis.

1.5 Preparation of the *Ralstonia solanacearum* suspension and the fermentation broth of endophytic fungus AP-12

A single colony of *R. solanacearum* was selected and inoculated into 150 mL of NB medium in a 250 mL conical flask. After shaking culture for 12 h at 30°C and

200 r · min^{-1}, the bacterial suspension concentration was adjusted with sterile water to $OD_{600} = 1.5$.

With reference to the method of Xu et al. (2023), the endophytic fungus AP-12 from lotus seedpod was cut into fungal plugs and cultured under the optimal fermentation process obtained above. After vacuum filtration to remove the mycelia, the fermentation broth was obtained. The fermentation broth was divided into two parts: one part was left untreated, and the other part was sterilized at 121°C under high pressure for 20 min. The two parts were each diluted twofold with sterile water, yielding the fermentation broth and the sterilized fermentation broth, respectively, for use in subsequent experiments.

1.6 Pot control-efficacy experiment

With reference to the method of Cao Shangxiao et al. (2011), *Pogostemon cablin* seedlings were obtained by leaf induction. When the plants reached 5–6 cm in height, they were hardened and then transplanted into flowerpots containing nutrient soil. After one week of continuous watering culture, they were used for subsequent inoculation experiments.

A mixed substrate of peat soil:vermiculite:perlite (3:1:1) was used and sterilized at 121°C for 20 min. Four treatments were established in the experiment: (1) CK: 20 mL of sterile water was added to each pot; (2) R: 20 mL of *R. solanacearum* suspension ($OD_{600} = 1.5$) was applied to each pot using the root-injury irrigation method; (3) FR: 20 mL of fermentation broth was added to each pot for 12 h of pretreatment, followed by inoculation with 20 mL of *R. solanacearum* suspension; (4) MF: 20 mL of sterilized fermentation broth was added to each pot for 12 h of pretreatment, followed by inoculation with 20 mL of *R. solanacearum* suspension. Each treatment comprised 9 pots. Plant culture conditions were 30°C, 60% RH, and 12 h · d^{-1} light. The status of the roots, stems, and leaves of *P. cablin* was observed 7 d after disease onset, and the morphology of stem cross sections was observed under a stereomicroscope. The pot experiment lasted 7 d; disease incidence was investigated within 7 d after infection, and the disease index and control efficacy were calculated. The disease grading standard for *P. cablin* bacterial wilt referred to the National Standard of the People' s Republic of China GBT-23222-2008 (Li Xinzhong et al., 2019). The formulas for the disease index and control efficacy were as follows:

$$\text{Disease index}(\%) = \sum \frac{\text{number of diseased plants at each grade} \times \text{severity of each grade}}{\text{total number of plants investigated} \times \text{highest severity}} \times 100;$$

$$\text{Control efficacy}(\%) = \frac{\text{disease index of the control group} - \text{disease index of the treatment group}}{\text{disease index of the control group}} \times 100.$$

1.7 Determination of physiological and biochemical indicators and active-ingredient contents in *Pogostemon cablin* plants

1.7.1 Leaf physiological indicators At 3 and 7 d after application of the *R. solanacearum* suspension, a plant nutrition analyzer was used to determine the chlorophyll content and nitrogen content of the second pair of leaves from top to bottom in the four treatment groups (CK, R, FR, and MF).

1.7.2 Leaf CAT, POD, and SOD enzyme activities and MDA At 3 and 7 d after application of the *R. solanacearum* suspension, CAT enzyme activity was determined using the ammonium molybdate method, POD enzyme activity using the guaiacol method, SOD enzyme activity using the nitroblue tetrazolium reduction method, and MDA content using the thiobarbituric acid method.

1.7.3 Active ingredients in leaves With reference to the method of Wang et al. (2023), at 3 and 7 d after application of the *R. solanacearum* suspension, the content of pogostone in *P. cablin* leaves was determined by gas chromatography-mass spectrometry using an HP-5MS chromatographic column (30 m × 0.25 mm × 0.25 μm).

1.7.4 Root activity With reference to the method of Li Hesheng et al. (2000), at 3 and 7 d after application of the *R. solanacearum* suspension, root activity of *P. cablin* was determined using the TTC reduction method.

1.8 Data processing

The experimental data were statistically analyzed using SPSS 26.0 software. Data conforming to a normal distribution were described as mean ± standard deviation ($\bar{x} \pm s$).

measurement data with a normal distribution. Range analysis, variance analysis, and significance analysis were performed on the orthogonal test results. GraphPad Prism 9.5 was used for graphing.

2 Results and Analysis

2.1 Optimization of Culture Conditions for Strain AP-12

2.1.1 Effects of Carbon and Nitrogen Sources on the Bacteriostatic Activity of Strain AP-12 As shown in Fig. 1A, different types of carbon sources were separately added to the potato leachate. When fructose was used as the carbon source, strain AP-12 had the strongest bacteriostatic activity against *Botrytis cinerea*, with an inhibition zone diameter of 12.18 mm, significantly higher than those with sucrose, glucose, and maltose; therefore, fructose was selected as the optimal carbon source. Fructose was then added at different mass concentrations. As shown in Fig. 1B, the inhibition zone diameter first increased and then decreased as the fructose concentration increased. The bacteriostatic

activity was greatest at a concentration of 40 g · L^-1, reaching 13.15 mm, and was lowest at 10 g · L^-1; therefore, a mass concentration of 40 g · L^-1 was selected as the optimal carbon-source concentration.

Different types of nitrogen sources were separately added to the culture medium optimized for carbon source. The inhibition zone diameters of the media supplemented with yeast powder, peptone, and soybean flour were significantly higher than that of the medium without an added nitrogen source, whereas the medium supplemented with beef paste showed no significant difference from the medium without a nitrogen source. The inhibition zone diameter with yeast powder was the largest (13.85 mm), an increase of 1.9 mm compared with the nitrogen-source-free medium (11.96 mm) (Fig. 1C). Therefore, yeast powder was selected as the optimal nitrogen source. Yeast powder was then added at different mass concentrations. As shown in Fig. 1D, the inhibition zone diameter at 20 g · L^-1 was significantly higher than those at 10 g · L^-1 and 15 g · L^-1. As the concentration continued to increase, the increase in bacteriostatic activity became slower, reaching a maximum of 14.68 mm at a mass concentration of 25 g · L^-1. Thereafter, the bacteriostatic activity of the strain no longer increased; moreover, as the residual amount of nitrogen source in the culture medium increased, the fermentation broth became overly viscous, thereby increasing the difficulty of processing the fermentation broth during subsequent metabolite separation and purification. Therefore, a mass concentration of 25 g · L^-1 was selected as the optimal nitrogen-source concentration.

Figure 1, panel labels and axes:
A Carbon source type; legend: Glucose, Maltose, Sucrose, Fructose; y-axis: Inhibition zone diameter (mm).
B Carbon source concentration (g · L^-1); y-axis: Inhibition zone diameter (mm).
C Nitrogen source type; legend: No nitrogen source, Yeast powder, Peptone, Beef paste, Soya flour; y-axis: Inhibition zone diameter (mm).
D Nitrogen source concentration (g · L^-1); y-axis: Inhibition zone diameter (mm).

A. Carbon source species; **B.** Carbon source concentration; **C.** Nitrogen source species; **D.** Nitrogen source concentration. Different lowercase letters indicate significant differences in Duncan' s test (P<0.05). The same below.

Fig. 1 Effects of carbon and nitrogen sources on the bacteriostatic activity of strain AP-12

2.1.2 Effects of Loading Volume, pH, and Fermentation Time on the Bacteriostatic Activity of Strain AP-12 Optimized culture medium at volumes of 100, 200, 300, and 400 mL was separately added to 500 mL conical flasks. As shown in Fig. 2A, when the loading volumes were 300 and 400 mL, the inhibition zone diameters were 15.17 and 15.00 mm, respectively, and the difference between the two inhibition zone diameters was not significant,

indicating that within this range, the enhancement of the strain' s antibacterial activity by increasing the loading volume had reached saturation. However, these values were significantly higher than those at loading volumes of 100 and 200 mL. Therefore, 300 mL was selected as the optimal loading volume for the subsequent culture medium.

The pH of the optimized culture medium at a loading volume of 300 mL was separately adjusted to 5.0, 6.0, 7.0, and 8.0. As shown in Fig. 2B, this strain

The strain was able to maintain its antibacterial activity in environments ranging from acidic (pH 5.0) to weakly alkaline (pH 8.0), indicating that it had strong tolerance to changes in environmental pH. At pH 6.0, the inhibition zone diameter was the largest, reaching 16.33 mm; therefore, pH 6.0 was selected as the optimal pH of the culture medium.

Under the basic fermentation culture conditions, the optimized medium with a liquid filling volume of 300 mL and pH 6 was cultured for 6, 8, 10, and 12 d, respectively. As shown in Fig. 2C, the strain exhibited antibacterial activity after fermentation for 6–12 d. At 8 d of fermentation, the inhibition zone diameter was the largest, at 16.50 mm, showing a significant difference from the other fermentation times. The smallest value occurred at 6 d of fermentation, with an inhibition zone diameter of 14.23 mm. Therefore, 8 d of fermentation was selected as the optimal fermentation time for the strain.

A. Loading volume; B. pH; C. Fermentation time.

Fig. 2 Effects of fermentation conditions on the bacteriostatic activity of strain AP-12

2.1.3 Results of orthogonal optimization of strain AP-12

On the basis of the single-factor test results determining the suitable components of the fermentation medium and fermentation conditions for strain AP-12, fructose, yeast powder, liquid filling volume, and pH were used as the investigated factors, and the inhibition zone diameter was used as the evaluation index. An $L_9(3^4)$ orthogonal design was adopted, and the factor levels are shown in Table 1.

Extreme range analysis was performed on the orthogonal test results (Table 2). The results showed that $R_A > R_B > R_C > R_D$, indicating that the effects of the medium formulation and fermentation conditions on the antibacterial activity of strain AP-12 followed the order: liquid filling volume > fructose > yeast powder > pH. Analysis of variance and significance analysis (Table 3) showed that only the liquid filling volume had a significant effect on the test results ($P < 0.05$), whereas fructose, yeast powder, and pH did not produce significant differences in the results. This indicates that liquid filling volume was the main factor affecting the test results. The optimal combination for strain AP-12 was finally determined to be $A_3B_1C_1D_1$, numbered 10; that is, the carbon source was fructose at a mass concentration of 20 $g \cdot L^{-1}$, the nitrogen source was

yeast powder at a mass concentration of 20 $g \cdot L^{-1}$, the pH value was 6.0, the liquid filling volume was 400 mL, the rotation speed was 150 $r \cdot min^{-1}$, and cultivation was carried out at 28 °C for 8 d. Strain AP-12 was fermented under the optimal combination conditions and under the initial culture conditions described in Section 1.4.1 (with PDB as the fermentation medium), respectively. The results showed that, under the optimal fermentation process conditions, the inhibition zone diameter of strain AP-12 against the bacterial wilt pathogen was (19.57±0.47) mm, which was 83.41% higher than that before optimization. This result verified the rationality of the optimal fermentation process conditions.

Table 1 Factor levels for $L_9(3^4)$ orthogonal test

Level	A: Liquid filling volume (mL)	B: Fructose ($g \cdot L^{-1}$)	C: Yeast powder ($g \cdot L^{-1}$)	D: pH
1	300	20	20	6.0
2	350	30	25	6.5
3	400	40	30	7.0

Table 2 Orthogonal test results and extreme variance analysis

Number	A	B	C	D	Inhibition circle diameter (mm)
1	3(400)	3(40)	2(25)	1(6.0)	18.13
2	3	2(30)	1(20)	2(6.5)	18.97
3	3	1(20)	3(30)	3(7.0)	18.43
4	2(350)	2	3	1	17.20
5	2	3	1	3	16.67
6	2	1	2	2	17.33
7	1(300)	3	3	2	16.43
8	1	2	2	3	16.97
9	1	1	1	1	18.73
K_1	17.378	18.167	18.122	18.022	
K_2	17.067	17.711	17.478	17.578	
K_3	18.511	17.078	17.356	17.356	
R	1.444	1.089	0.767	0.667	

Table 3 Analysis of variance of orthogonal test results

Table 3 Analysis of variance of orthogonal test results

Source	Sum of square	Degree of freedom	Mean square	F	P
Liquid filling volume	10.403	2	5.201	7.555	0.003
Fructose	5.383	2	2	2.691	2.998
Yeast	3.054	2	1.527	1.535	0.236
pH	2.074	2	1.037	1.001	0.382

2.2 Pot experiment control effect of strain AP-12

2.2.1 Symptoms of bacterial wilt in *Pogostemon cablin*

In healthy *Pogostemon cablin*, the leaves are dark green, and the rhizomes are green, thick, and free of disease spots. Under a stereomicroscope, the rhizome sections are green and show no signs of decay. After infection with bacterial wilt, the leaves remain green, but they droop slightly and curl, showing obvious dehydration symptoms; distinct lesions appear on the rhizomes, the roots turn black and rot, and under a stereomicroscope the transverse sections of the rhizomes are broken and show browning caused by decay from the outside inward (Fig. 3).

A, B, C. Leaves, rhizomes, and rhizome sections of healthy *Pogostemon cablin*; D, E, F. Corresponding tissues of diseased *P. cablin*.

Fig. 3 Symptoms of *Pogostemon cablin* 7 d after inoculation with *Ralstonia solanacearum*

2.2.2 Control effect of fermentation broth of the *Andrographis paniculata* endophytic fungus AP-12 against bacterial wilt of *Pogostemon cablin*

Over time, the disease index of bacterial wilt in *Pogostemon cablin* gradually increased in all treatment groups to which the bacterial wilt suspension was applied (R, FR, and MF). At 1, 3, 5, and 7 d after inoculation, the disease index consistently followed the order R > MF > FR. In the CK group, plant stems were upright, and the leaves were expanded and dark green. At 3 d, the stems of *Pogostemon cablin* in the bacterial-wilt treatment group (R) were obviously bent, and the lower leaves were wilted and dehydrated in appearance while still green; in the fermentation-broth treatment group (FR) and the sterilized fermentation-broth treatment group (MF), only slight bending of the stems was observed. At 7 d, the stems of *Pogostemon cablin* in the R group were severely lodged, the lower leaves were yellow and withered, and leaf drooping and abscission were severe; the stems of *Pogostemon cablin* in the FR and MF groups were both bent

and the bending amplitude in the MF group was greater; only the lower leaves became dehydrated and curled, whereas the upper leaves remained expanded (Fig. 4 and Table 4). At 7 d, the disease indices of all treatment groups reached their maximum values. The bacterial wilt disease indices in the FR and MF

groups were 26.80% and 18.39% lower than that in the R group, respectively, and the differences were significant ($P < 0.05$). The control efficacy of the fermentation broth against bacterial wilt reached 31.45%, whereas the efficacy of the sterilized fermentation broth was lower than that of the fermentation broth, reaching 21.58% (Table 5). These results indicate that both the fermentation broth and the sterilized fermentation broth delayed the occurrence of bacterial wilt in *Pogostemon cablin* and reduced its severity, with the fermentation broth showing better efficacy than the sterilized fermentation broth.

CK. Control group; **R.** bacterial wilt pathogen; **FR.** fermentation broth + bacterial wilt pathogen; **MF.** sterilized fermentation broth + bacterial wilt pathogen. The same below.

CK. Control group; **R.** *Ralstonia solanacearum*; **FR.** Fermentation broth + *R. solanacearum*; **MF.** Sterilised fermentation broth + *R. solanacearum*. The same below.

Figure 4 Effects of the fermentation broth of endophytic fungus AP-12 from *Andrographis paniculata* on bacterial wilt in *Pogostemon cablin*

Fig. 4 Effects of the fermentation broth of endophytic fungi AP-12 from *Andrographis paniculata* on the bacterial wilt in *Pogostemon cablin*

Table 4 Effect of the fermentation broth of endophytic fungus AP-12 from *Andrographis paniculata* on the disease index of bacterial wilt in *Pogostemon cablin*

Table 4 Effect of fermentation broth of endophytic fungi AP-12 from *Andrographis paniculata* on the disease index of bacterial wilt in *Pogostemon cablin*

Treatment	Disease index (%) 1 d	Disease index (%) 3 d	Disease index (%) 5 d	Disease index (%) 7 d
CK	0 c	0 d	0 c	0 c
R	40.55$±1.79a|64.38±2.28a|81.33±1.82a|85.22±1.79a||FR|25.31±2.83b|38.99±1.71c|51.21±1.70c|5 b			

Note: Different lowercase letters within the same column indicate significant differences ($P < 0.05$). The same below.

Table 5 Control efficacy of the fermentation broth of endophytic fungus AP-12 from *Andrographis paniculata* against bacterial wilt in *Pogostemon cablin*

Table 5 Effectiveness of fermentation broth of endophytic fungi AP-12 from *Andrographis paniculata* in the control of bacterial wilt in *Pogostemon cablin*

Treatment	Disease index (%)	Prevention effect (%)
CK	0 c	-
R	85.22$±1.79a| −	
	||FR|58.42±0.95c|31.45±1.12||MF|66.83±0.43b|21.58±$0.50	

2.3 Effects of strain AP-12 on physiological and biochemical indices and active component contents in *Pogostemon cablin* plants

2.3.1 Effects of the fermentation broth of endophytic fungus AP-12 from *Andrographis paniculata* on physiological indices of *Pogostemon cablin*

At 3 d and 7 d after infection with the bacterial wilt pathogen, the chlorophyll and nitrogen contents in the leaves of *Pogostemon cablin* showed the order CK > FR > MF > R.

When plants had been infected by the bacterial wilt pathogen for 7 d, the chlorophyll contents in the FR and MF groups were 1.29- and 1.27-fold those in the R group, respectively, with significant differences ($P < 0.05$) (Fig. 5A). The nitrogen contents in the FR and MF groups were 21.43% and 14.29% higher than that in the R group, respectively, reaching a significant level (Fig. 5B). Compared with the CK group, the chlorophyll content and nitrogen content in the R group decreased by 34.76% and 22.22%, respectively, whereas the FR and MF treatments significantly alleviated this downward trend. These results indicate that the AP-12 fermentation broth and the sterilized fermentation broth can effectively delay the decreases in chlorophyll content and nitrogen content caused by bacterial-wilt-pathogen stress.

Fig. 5 Effects of the fermentation broth of the endophytic fungus AP-12 from *Andrographis paniculata* on the physiological indexes of *Pogostemon cablin*

2.3.2 Effects of the fermentation broth of the endophytic fungus AP-12 from *Andrographis paniculata* on antioxidant enzyme activity and MDA content in *Pogostemon cablin*

As shown in Fig. 6, under different inoculation durations, the antioxidant enzyme activities and MDA content in the CK group were all the lowest, whereas those in the R group were all the highest. At 7 d after infection, compared with the R group, the FR and MF groups both significantly reduced antioxidant enzyme activities in *Pogostemon cablin* leaves ($P < 0.05$). The CAT, POD, and SOD activities in the FR group decreased by 24.43%, 10.71%, and 16.24%, respectively, compared with the R group, while those in the MF group decreased by 17.30%, 8.49%, and 14.19%, respectively. The MDA content in the R group was significantly higher than that in the CK group, indicating that *Pogostemon*

cablin had suffered a certain degree of oxidative damage; meanwhile, the MDA contents in the FR and MF groups were 23.75% and 13.64% lower than that in the R group, respectively. The greater the degree of stress-induced injury in plants, the higher their antioxidant enzyme activities and MDA content. This trend indicates that adding the fermentation broth and the sterilized fermentation broth can, to a certain extent, alleviate the degree of stress injury caused by the bacterial wilt pathogen in *Pogostemon cablin*, thereby exerting a certain disease-control effect.

Fig. 6 Effect of fermentation broth of the endophytic fungus AP-12 from *Andrographis paniculata* on defence enzyme activity and MDA content of *Pogostemon cablin*

2.3.3 Effects of the fermentation broth of the endophytic fungus AP-12 from *Andrographis paniculata* on the contents of active components and root vitality of *Pogostemon cablin*

Under different inoculation durations, the pogostone content in the CK group was significantly higher than that in the other treatment groups ($P < 0.05$). At 7 d after infection by the bacterial wilt pathogen

At the same time, the pogostone content in the R group was 45.32% lower than that in the CK group, with a significant difference, indicating that bacterial wilt stress inhibited the accumulation of pogostone in *Pogostemon cablin*. The pogostone contents in the FR and MF groups were 1.38 and 1.30 times that in the R group, respectively (Fig. 7A). These results indicate that the AP-12 fermentation broth and the sterilized fermentation broth effectively alleviated the inhibition of pogostone accumulation caused by bacterial wilt stress. At different days after inoculation, root activity in the CK, FR, and MF groups was significantly higher than that in the R group ($P < 0.05$). At 7 d after infection, root activity in the R group decreased by 83.14% compared with the CK group; the root activity values in the FR and MF groups were 3.35 and 2.98 times that in the R group, respectively, and the differences were significant ($P < 0.05$). Compared with 3 d after infection, the recovery effect at 7 d was more pronounced (Fig. 7B). This indicates that AP-12 treatment significantly reduced the damage caused by bacterial wilt to the roots and partially restored root physiological function.

Figure 7 Effects of the fermentation broth of the endophytic fungus AP-12 from *Andrographis paniculata* on pogostone content and root activity of *Pogostemon cablin*

Fig. 7 Effects of the fermentation broth of the endophytic fungus AP-12 from *Andrographis paniculata* on pogostone content and root activity of *Pogostemon cablin*

3 Discussion and Conclusion

In the process of biological control using antagonistic strains, increasing the yield of antimicrobial substances and improving inhibitory efficacy are key scientific issues (Lu Xiaohong et al., 2022). Previous studies have confirmed that optimizing medium composition and fermentation conditions can significantly enhance the production of active substances and the control efficacy of antagonistic microorganisms (Zhang Meijun et al., 2020). For example, after Zhang Ziqi et al. (2024) optimized the fermentation conditions of antagonistic bacteria against tobacco brown spot disease, the diameter of the inhibition zone increased by 5.10 mm; Cui Wenyan et al. (2024) increased the antibacterial activity of antagonistic bacteria against ginger bacterial wilt by 62.90%. Similarly, the present study found that the antibacterial activity of AP-12 increased by 83.41% after fermentation optimization. This prominent effect may be attributable to the following: fructose, as an efficient carbon source, promoted the synthesis of polyketide antimicrobial substances, while the abundant nitrogen source and growth factors provided by yeast extract significantly increased mycelial biomass (Zhang He et al., 2023). The resulting improvement in metabolic efficiency shortened the fermentation cycle to 8 d while maintaining high activity, providing an important advantage for large-scale application.

The development of biocontrol fungi is of great theoretical and practical significance in the field of biological control of plant diseases. At present, mycorrhizal fungi and *Trichoderma* spp. have been widely applied in the prevention and control of bacterial wilt (Liu Shuaikang et al., 2023). For example, Rahman et al. (2023) found that the synergistic use of two *Trichoderma harzianum* strains reduced the incidence of tomato bacterial wilt by 47.50%. In the present study, the control efficacy of AP-12 fermentation broth against bacterial wilt of *P. cablin* reached 31.45% (21.58% for the sterilized fermentation broth), comparable to the effect of endophytic fungi from *P. cablin* reported by Cheng Xiaoqing et al. (2023). Although its efficacy still lags behind that of some AMF or *Trichoderma* strains (Wang Qian et al., 2023), the sterilized fermentation broth still retained significant activity, suggesting that the active substances of AP-12 possess thermal stability and may involve andrographolide-type compounds or other heat-resistant metabolites. This characteristic is particularly important under high-temperature field conditions.

Photosynthesis is the basis of plant growth and development, and chlorophyll is the key pigment for photosynthesis (Arjenaki et al., 2012). This study found that bacterial wilt infection led to decreases in chlorophyll and nitrogen contents in *P. cablin*, which is directly associated with pathogen-induced vascular blockage, impeded water transport, and interference with photosynthesis and nitrogen assimilation (Guo et al., 2016). However, application of AP-12 fermentation broth and sterilized fermentation broth effectively restored chlorophyll and nitrogen contents. This result is consistent with the findings of Wang Xiaoqian et al. (2023), indicating that endophytic fungi can enhance plant resistance to adversity stress by maintaining pigment balance within the plant, thereby

exerting an important physiological regulatory role. Similarly, changes in the activities of antioxidant enzymes (CAT, POD, and SOD) and MDA content in *P. cablin* leaves showed that AP-12 can dynamically regulate oxidative stress responses: it may rapidly activate antioxidant defenses at the early stage by secreting small-molecule signaling substances such as SA to eliminate ROS bursts, and later restore the balance of enzyme activities. This temporally regulated pattern is consistent with the results of Hao Xiaohua et al. (2020), while the present study further reveals the specific response under bacterial wilt stress.

Pogostone is one of the main active constituents of *P. cablin*. Endophytic fungi can improve the ability of medicinal plants to resist diseases and pests, promote growth, and enhance the absorption and utilization of N and P (Li Biao et al., 2016). Studies have shown that inoculation of *Anoectochilus roxburghii* with endophytic fungi can alleviate the inhibitory effect of pathogenic bacteria on the growth of *A. roxburghii* and promote the accumulation of active components such as kinsenoside, rutin, and quercetin (Ye Bingzhu, 2019).

In this study, AP-12 treatment significantly alleviated the inhibition of pogostone synthesis by the bacterial-wilt pathogen. This effect may be achieved through two pathways: (1) reducing the pathogen' s direct destruction of host cells, thereby ensuring the supply of metabolic substrates; and (2) activating key enzymes in the phenylpropanoid metabolic pathway (Tao Jinhua et al., 2014), thereby promoting the synthesis of pogostone precursors.

Root activity comprehensively reflects a plant' s capacity for nutrient absorption and substance synthesis (Laliberte, 2017). The results show that AP-12 treatment can improve root function in *Pogostemon cablin* infected by the bacterial-wilt pathogen. In combination with the findings of Li Xiaoting et al. (2023), who added exogenous endophytic fungi to oat seedlings under drought stress, the possible mechanisms include: (1) secondary metabolites in the fermentation broth inhibited the growth of the bacterial-wilt pathogen and reduced its colonization rate in the root system; and (2) secondary metabolites promoted increases in root length, root volume, and root surface area, thereby improving nutrient-absorption function.

This study reveals a new pathway by which the endophytic fungus AP-12 from *Andrographis paniculata* enhances disease resistance in *Pogostemon cablin* through multiple mechanisms. The results indicate that AP-12 not only directly inhibits the bacterial-wilt pathogen, but, more importantly, enhances disease resistance through systemic regulation of the host's physiological state: it significantly restores chlorophyll synthesis, maintains nitrogen-metabolism balance, and improves root activity; at the same time, it effectively activates the antioxidant defense system and reduces membrane-lipid peroxidation damage. In addition, AP-12 treatment can specifically maintain the content of pogostone, a key medicinal component, which is of great significance for ensuring the quality of medicinal plants. These findings provide theoretical support for the development of novel biocontrol strategies based on the multi-target framework of "antibiosis–stress resistance–quality regulation," and demonstrate good ap-

plication prospects. Subsequent research will focus on elucidating the targets of the active components of AP-12 and evaluating the stability and synergistic measures of its field application.

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Note: Figure translations are in progress. See original paper for figures.