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Research on the prevention and control of plant diseases by antagonistic microorganisms

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Abstract:

Plant diseases, often caused by bacterial infections, pose significant challenges to human society due to their widespread and harmful consequences, resulting in substantial losses. Although traditional chemical prevention and control methods have shown effectiveness, they still pose serious environmental and other risks. Therefore, developing biological control is crucial for future plant disease prevention and management. In this study, we investigated the efficacy of antagonistic microorganisms. We conducted four related experiments and eventually reaffirmed the substantial inhibitory effect of antagonistic microorganisms on the growth of plant pathogens. Our findings are of great significance for further expanding the application scope and popularization of antagonistic bacteria and also enriching the specific application of antagonistic microorganisms for specific plant control.

Keywords: Antagonistic Microorganisms, Plant Diseases, Biological Control, Antagonistic Effect

Top of Form

I. Introduction

Antagonistic microorganisms refer to the phenomenon where microorganisms produce certain metabolites or alter environmental conditions during their life activities, thus inhibiting the growth and reproduction of other microorganisms and even causing their death. In the growth process of many crops, various external environmental factors can lead to diseases, resulting in poor plant growth, which ultimately affects agricultural production and causes significant losses for businesses. Chemical prevention and control methods have traditionally been used, offering advantages such as rapid effectiveness, high efficiency, and wide-scale application. However, chemical prevention and control also have drawbacks, including excessive environmental pollution and disruption of ecosystem balance. [1] Biological control offers important benefits in protecting the environment, reducing chemical pesticides, maintaining ecological balance, providing healthy food, and ensuring agricultural sustainability. Biological prevention and control have gained attention in mainstream plant control research and have been successfully implemented in rice, wheat, corn, and vegetable crops. [2] However, plant diseases still pose challenges to economic value and healthy ecosystems. A study conducted in the Huanchao Lake Wetland Group revealed that plant diseases greatly impact biodiversity and result in substantial economic loss in plant production. [3] Therefore, the research on the prevention and control of plant diseases by antagonistic microorganisms is crucial and worthy of further in-depth study.

Zhang Lingyin employed the flat panel confrontation method to isolate and screen banana wilt bacteria (Cuba specialized No.4 physiological small species) from banana wilt suppressive soil. [4] Li Chaonan and other researchers from Hebei Agricultural University identified Bacillus, which can prevent and control the root rot of Cangshu, thereby providing rich microbial resources for the biological prevention and control of Cangshu root rot. [5] Xing Jieshuai isolated a high-yielding protease from the wheat rhizosphere soil, demonstrating its antagonistic effect on plant pathogenic fungi. [6] Qiao Xinlei found that strain Z-5 showed significant antagonistic activity on Scythex spores. [7] Drawing upon the accumulated experience of numerous predecessors, this article outlines the study of antagonism and disease prevention mechanisms within one aspect of biological prevention and control. It aims to provide a reference basis for studying bacteriostatic mechanisms and the effective use of anti-bacteriological strains and provide technical support for using beneficial microorganisms in preventing and controlling plant diseases.

This article summarizes research on the mechanism of antagonism in microbial disease prevention within biological prevention and control. Antagonistic microorganisms

primarily control the occurrence and development of diseases by introducing beneficial microorganisms with antagonistic effects to compete with pathogenic microorganisms for nutritional, space, and physiological resources and by inhibiting the growth and infection of pathogenic microorganisms. Many studies on antagonism have greatly improved the prevention and control of plant diseases, such as Bacillus and wood mold. [8] Compared with chemical prevention and control, antagonistic microorganisms are safer, more stable, and entail less loss.

II. Methods and materials

1.1 Experiment 1: Antibacterial and pathogenic bacteria antagonism experiment

1.1.1 Experimental principle

Bacillus is a genus of bacteria that can form spores (endospores). They are resistant to external harmful factors, widely distributed, and capable of producing antibacterial substances that can prevent and control various plant diseases. F. oxysporum is a globally distributed soil-borne pathogenic fungus that can cause a variety of plant blight. Bacillus and F. oxysporum spores exhibit antagonism when they grow. Antagonism is when a microorganism inhibits or kills another microorganism by producing a metabolic substance.

1.1.2 Experimental materials

Bacillus, F. oxysporum

1.1.3 Experimental steps

1.1.3.1 Experimental design

This experiment set up two groups: control and experimental groups. Control group 1 was inoculated with Bacillus and F. oxysporum, along with LB culture medium solution at the inoculation site, respectively. Control group 2 was inoculated with Bacillus and LB culture medium solution at the inoculation site. The experimental group underwent inoculation with Bacillus and F.oxysporum at the inoculation site, respectively.

1.1.3.2 Strain culture

There are two steps involved: bacterial inoculation and bacterial culture. Bacterial inoculation involved taking the cooled and solidified LB plate medium in the ultra-clean workbench. A straight ruler was used to draw a straight line at the bottom of the plate, and the center of the circle at about 2cm from each end of the straight line was marked as the bacterial inoculation points. The control group plate was placed flat on the table with the plate cover opened, and 2 micro-liters of F. oxysporum culture broth(or culture medium solution) were added at two inoculation points about 2cm from the center point of the control group. The plate cover was then covered, and the solution was allowed to dry. Additionally, control group 2 was placed flat on the table, the plate lid was opened, and 2 microliters of Bacillus culture broth (or culture medium solution) was added, and then the solution was left to dry. Then, the experimental group was then inoculated with Bacillus and F. oxysporum at the inoculation sites.

Bacterial culture involves transferring the vaccinated plate to the incubator and culturing it at 37 degrees Celsius for 48 hours.

1.2 Experiment 2: Western blot

1.2.1 Experimental principle

Under the electric field conditions, after SDS-PAGE separates the protein in the polyacrylamide gel, the protein is fixed on the gel and then transferred to the NC membrane. After sealing the protein and adding antibodies, it is finally placed under the developer to detect its location through a specific antibody for a specific amino acid sequence as a probe. The function of this technology is to show the protein's location and identify a specific protein in the protein mixture.

1.2.2 Experimental materials

Protein samples, protein inhibitors, polypropylamide gel, SDS-PAGE, Tris-Gly, NC membrane, 5% skim milk, HA rabbit-derived antibodies.

1.2.3 Experimental steps

1. Protein extraction: The protein extract and protease inhibitor (PMSF or Cocktail) were added to the sample, followed by centrifugation. The upper protein sample was collected, and the precipitated plant residue was discarded.
2. SDS-PAGE electrophoresis: Electrodes were installed, and a polyacrylamide gel was placed into the electrode clip for SDS-PAGE. Tris-Gly was added to overflow, and the comb was carefully removed. Buffer was added to each hole of the polypropamide gel, followed by the addition of 20μl of the protein sample. The switch was turned on, and electrophoresis was performed.
3. Transfer to membrane: The excess part of the gel was carefully removed and soaked in the transfer liquid. Foam paper, gel, and NC film were layered on the black surface of the clip, ensuring each layer was wet with transfer liquid to prevent bubbles. The assembly was placed into the electrode tank with an ice bag, and ice cubes were added around the tank.
4. Closed protein: The NC membrane was removed, and 5% skimmed milk was added. It was placed on the shaker and shaken evenly for 1 hour.

5. Antibody incubation: 5% skim milk was poured, and HA rabbit-derived antibody was added. It was placed on a rocking bed and shaken evenly for 3 hours.
6. Detection: Substrates A and B (1ml each) were added, and the NC film was evenly coated using a gun. The eBlot was then activated, and the results were observed after the self-examination.

1.3 Experiment 3: DNA extraction and PCR amplification of plants

1.3.1 Experimental principle

Plant genome DNA extraction uses chemical and physical methods to disrupt the cell and nuclear membranes, releasing DNA in the nucleus. Plant DNA extraction yields samples for subsequent PCR amplification, which relies on DNA replication to produce a large amount of target DNA through repeated DNA degeneration, primer binding, and DNA amplification. PCR technology has extensive applications in biomedical research, medical diagnosis, and forensics.

1.3.2 Experimental materials

Plant leaves, centrifuge tubes, centrifuges, small steel beads, DNA extracts (Tris, EDTA-2Na, NaCl, SDS, PVP), tissue crusher, isopropyl alcohol, 75% ethanol, ddH2O, Mix (DNA polymerase, DNTP, Mg ions, dyes), F+R pirs, DNA samples, ddH2O, PCR amplifier, agar gel electrophoresis, nanodrop2000.

1.3.3 Experimental steps:

1. Sampling: A plant leaf was taken and placed into a 1.5ml centrifuge tube. Small steel balls and 500ml DNA extract were added, and the mixture was ground for 2 minutes with a tissue crusher at 50Hz.
2. DNA extraction: After completing the water bath at 65°C for 10 minutes, the mixture was centrifuged at 12000rpm for 15 minutes. Then, 400μl was taken and transferred to a new 1.5ml centrifuge tube. Isopropyl alcohol (0.6 times the volume) was added, mixed upside down, and centrifuged at 12000rpm for 10 minutes. The white DNA precipitation at the bottom of the tube was obtained after discarding the supernatant.
3. DNA washing: 700μl of 75% ethanol was added to the centrifuge tube, gently mixed, and the precipitation was washed. The tube was centrifuged at 12000rpm for 5 minutes, and the excess was poured out.
4. DNA recovery: The precipitation was placed in a ventilated place and blown dry for about 30 minutes. Then, the precipitation was re-dissolved with 20μl ddH2O.
5. DNA sample extraction: A DNA sample was obtained by extracting plant DNA.
6. DNA sample processing: 10μl of Mix, 0.8μl of F+R of each, 2μl of DNA samples, and 6.4μl of ddH2O were added to the DNA sample to process the PCR amplification of DNA samples.
7. PCR amplification: The processed sample was put into the instrument. The PCR amplification instrument had three steps: deformation, annealing, and 72-degree extension. Different procedures were set.
8. Running electrophoresis: The obtained PCR products were placed on agarose gel for electrophoresis analysis after running electrophoresis.
9. Concentration measurement: The agarose gel was cut at a ratio of 1:100μl for debugging, put into the centrifuge tube for centrifugation, and cleaned three times with 75% ethanol. Finally, the recovered DNA was obtained and then put into the instrument nanodrop2000 to measure the concentration for testing the concentration of DNA and purity.

1.4 Experiment 4: Taipan blue dyeing

1.4.1 Experimental Principle

Taipanlan can penetrate the cell membranes of dead cells and color the DNA of dead cells.

1.4.2 Experimental materials

Arabidopsis seedling leaves, Taipan blue dye, lactophenol, 2.5g/ml hydrated chloral solution, 50% glycerin, 50% glycerin.

1.4.2 Experimental Steps

1. Sample collection: Arabidopsis seedling leaves were collected and centrifuged.
2. Making mother liquor: Taipan blue dye solution (100mg) was dissolved in 40ml lactophenol solution to create 10x mother liquor.
3. Prepare Taiwan Panlan: 10x Taiwan Panlan mother liquor was diluted to a 1x2 using lacteol for each concentration.
4. Staining: Arabidopsis tissue samples were immersed in the Taipan blue staining solution, boiled for 2 minutes, and then cooling to room temperature.
5. Decolorization: Stained plant material was decolorized in a 2.5g/ml hydrated chloral solution for 24h, with solution replacement twice.
6. Storage: Decolorized samples were stored in 50% glycerin at 4°C and mounted on 1% agarose gel.
7. Making slides: Decolored Arabidopsis samples were used to make disposable slides.
8. Observation: Prepared slides were observed under a light microscope and recorded staining patterns.
Data analysis: Staining data was processed using ImageJ software.

1.5 Experiment 5: Carrier Construction

1.5.1 Experimental principle

After the target gene and vector DNA are properly cut and modified, the two are connected and then imported into the host cell to achieve the correct expression of the target gene in the host cell.

1.5.2 Experimental materials

Escherichia coli DH5a receptor cells, gfp carrier, LB liquid culture medium, LB solid culture medium containing carboxypenicillin.

1.5.3 Specific steps

1. Carrier selection: Plasmid was chosen as the carrier. Arabidopsis leaves were crushed for DNA extraction and subsequent PCR amplification. The OMEGA glue recycling kit was used to recover target gene fragments.
2. Gene Insertion: E. coli cells expanded in a low-temperature, low-permeability calcium ion solution, altering the permeability of the cell membrane. This facilitated the adhesion of exogenous DNA, forming hydroxy-calcium phosphate complexes. At 42℃, disturbances and gaps formed in the cell membrane, allowing attached DNA to enter the cell through the membrane channel to achieve transformation.
3. Transformation of Escherichia coli: Resuscitated E. coli cultures were coated on the LB medium containing carboxyl penicillin and incubated. Positive transformer screening and identification of the transfected cells were performed.
4. Analysis: The cultured Escherichia coli plates were analyzed using a confocal microscope after culture completion.

III. Experimental results and analysis

1.1 Experiment 1: Antibacterial and pathogenic bacteria antagonism experiment

Figure 1 illustrates a clear difference in growth between the control group and the experimental groups 1 and 2. GraphPad software analysis revealed a significant difference in the growth radius between the control and experimental groups 1 and 2. The difference is attributed to the unhindered growth of F.oxysporum in the control group, whereas experimental groups 1 and 2 exhibited inhibition, indicating that Bacillus had an antagonistic effect on the fungus.

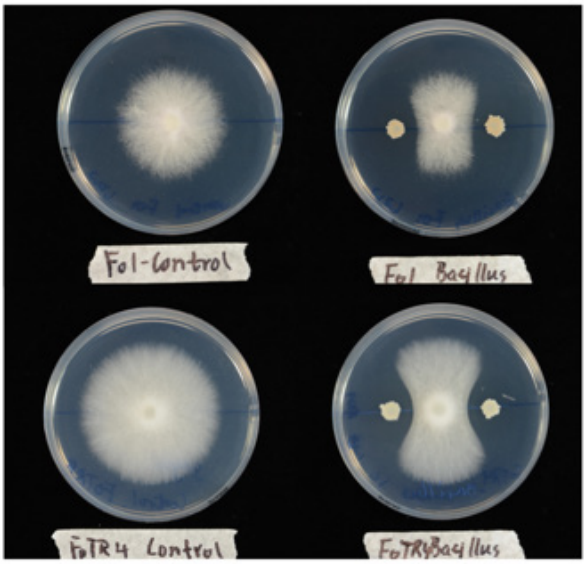


Figure 1: Comparison of the growth of F.oxysporum spore in the antagonistic experimental control group and the experimental group Proportion of cell death zones(%)

1.2 Experiment 2: Western Blot

After development, we found that the target protein was about 30kDa. As shown in Figure 2, the band size closely matches the ladder, indicating the successful isolation of our target protein. Hence, the experiment can be deemed successful.

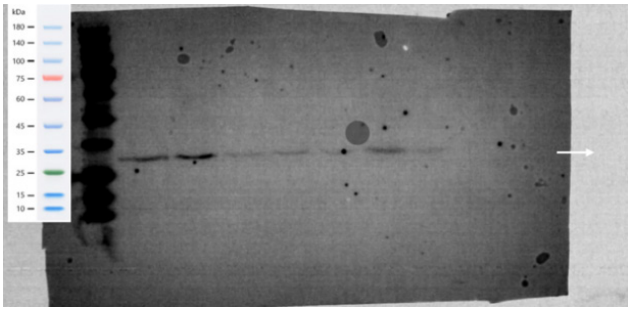


Figure 2: Protein running electrophoresis results

1.3 Experiment 3: Plant DNA extraction and PCR amplification

Experimental results and analysis: As shown in Figure 3, electrophoresis analysis revealed a strong DNA strip in sample 3, suggesting successful PCR amplification. However, this outcome was observed only in sample three, possibly due to several reasons: low DNA concentration, inappropriate annealing temperature, presence of impurities in the DNA, or inefficient enzyme efficiency. The low DNA concentration may be the main reason for the failure

of other sample experiments.

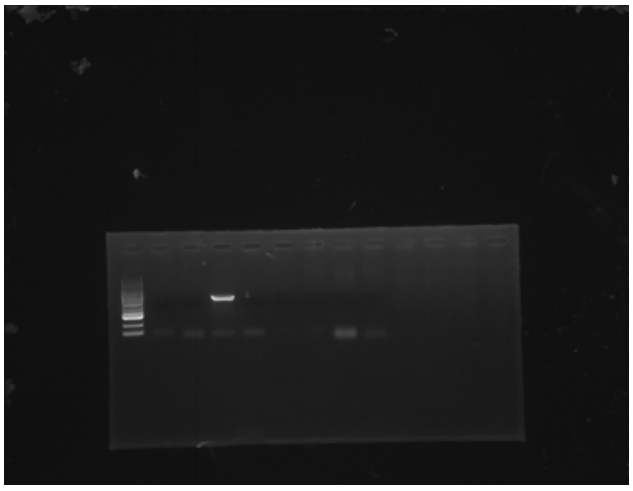


Figure 3: PCR amplification result diagram

1.4 Experiment 4: Taipan blue dyeing

We compared the controlled-treated Arabidopsis leaves with the bacillus-treated ones (Fig 4). The proportion of the dead area of Arabidopsis cells in the control group is lower than that of the bacillus group, which is abnormal. The experiment failed because anti-bacterial bacteria should inhibit the growth of pathogens and thus reduce cell mortality. The specific reasons may be that when using ImageJ software, it may be inaccurate in selecting the cell death area, and the chart shows that the highest values of both groups are much higher than the average value and the data. There is no significance, and this phenomenon also shows a great error in the experiment.

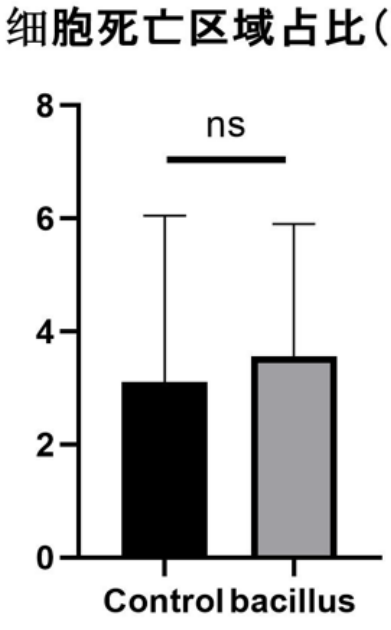
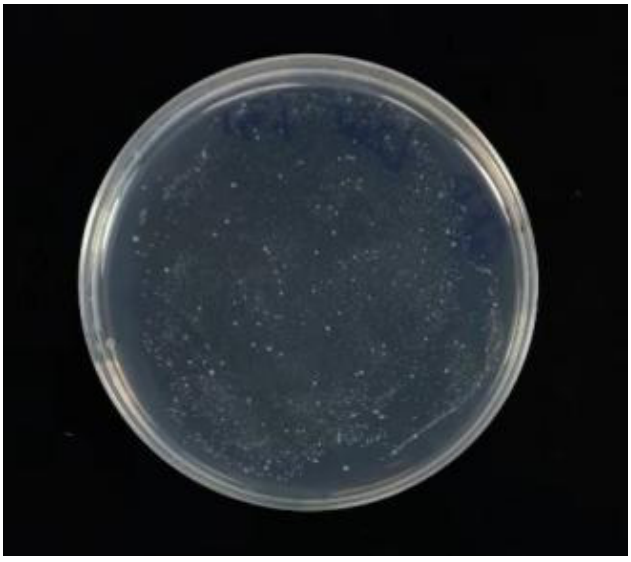


Figure 4: Taipan blue dyeing map

1.5 Experiment 5: Carrier Expansion

By assessing the images taken by the confocal microscope, two different results were found: fluorescence and non-fluorescence (Fig5) (Fig6). The appearance of fluorescent E. coli indicates successful transformation with the target DNA, confirming the incorporation of the GFP vector into Escherichia coli. After analysis, the image without fluorescence should be a false positive bacteria.



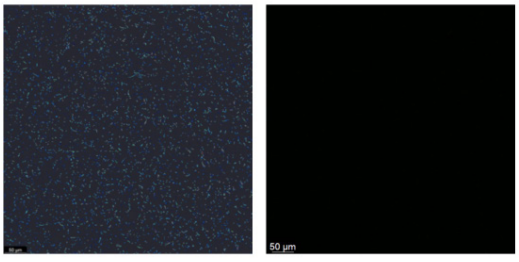


Figure 5: E. coli coated petri dish Figure 6: E. coli emits fluorescence

IV. Summary and Prospect

Our main research focuses on the role of antagonistic microorganisms in plant disease control. In this paper, we conducted four main experiments: antagonism experiments between anti-bacterial and pathogenic bacteria, Arabidopsis artificial hybridization tests with purification and mutant screening, carrier construction, and cell death phenotype observation of pathogenic bacteria infecting plants. We used the following four technologies in the experiment: Western Blot, PCR amplification, Taipan blue staining, and bacterial antagonism. The above experimental results demonstrate that antagonistic microorganisms can inhibit plant pathogens' growth, thus reducing plant

disease occurrence. Consequently, antagonistic microorganisms play a significant role in preventing and controlling plant diseases.

The prospects for microbial antagonism are promising. While Bacillus, as an antagonist, has an obvious antagonistic effect on plant diseases, the specific mechanism of antagonism for individual plants requires further exploration. In conclusion, delving into the bactericidal mechanism of plant-derived antagonistic bacteria on microorganisms can provide a deeper understanding of their antibacterial effects. This knowledge can offer theoretical support for applying antagonistic antibacterial agents and foster new insights into using antagonistic microorganisms for plant disease control. However, due to the immature screening and application technology of antagonistic bacteria, their effectiveness may be inconsistent and influenced by various factors. Studying the bactericidal mechanism of plant-derived antagonistic bacteria on microorganisms is of great significance for further improving the application effect and promotion of antagonistic antibacterial and the specific application of antagonistic microorganisms for specific plant control.

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