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BIOCONTROL OF EGGPLANT ROOT ROT: ISOLATION AND FERMENTATION OPTIMIZATION OF AN ANTAGONISTIC BACTERIAL STRAIN X-104

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Abstract

Eggplant Root rot, caused by *Fusarium solani*, is a prevalent and devastating disease. To expand the resources of high-quality antagonistic bacteria against *F. solani,* the bacteria strains were isolated from soil and screened via a dual-culture plate assay. Among the isolates, strain X-104 exhibited superior antagonistic activity, achieving an inhibition rate of 66.70%. The inhibitory effect was determined as the mycelial growth inhibition rate (%). Based on morphological characteristics and molecular analysis, the strain was identified as *Bacillus stercoris.* To maximize the antagonistic efficacy of strain X-104, the fermentation medium and culture conditions were systematically optimized. Through a sequential approach involving single-factor experiments, orthogonal tests, the composition of glucose 20.88 g/L and ammonium fluoride 13.66 g/L, $ZnSO_4 \cdot 7H_2O$ 10.74 g/L was finally determined. Modeling via Design Expert software predicted inhibition rate of 82.53%. The actual inhibition rate after validation was 85.42%, closely matching the predicted value. The optimal fermentation conditions were 100 mL (in a 250 mL conical flask), 32°C temperature and 12 h of fermentation. A low concentration of the fermentation broth (diluted 1:50 with water) promoted both seed germination and plant growth. Specifically, it significantly enhanced key agronomic traits in eggplants, including plant height, root length, stem diameter, and both fresh and dry weight. Furthermore, significant increase in germination potential and germination rate was observed.

Key words: Eggplant Root rot; Biocontrol bacteria; *Bacillus stercoris*; Response surface methodology

Introduction

Eggplant or aubergine (*Solanum melongena* L.) is originated in Southeast Asia, with the earliest cultivation in ancient India. It is a major vegetable across Asia and the Mediterranean, ranking fourth in global vegetable production according to FAO statistics. Rich in carbohydrates, fats, vitamins, calcium, phosphorus, and iron, its regular consumption may support cardiovascular health (Bai *et al.,* 2016).

The primary pathogen responsible for eggplant root rot is *F. solani.* The fungal disease causes significant plant mortality. Disease development is exacerbated by high temperature and humidity, as well as by continuous cropping, low-lying land and heavy sticky soils. As a resilient soil-borne pathogen, the pathogen can survive in the soil for up to a decade in the form of mycelia, thick-walled spores, or sclerotia (Zhang *et al.,* 2019). Post-infection, the xylem of the plant develops brown discoloration, and the epidermis at the base of the stems and the main root is also believed to have brown discoloration and rot, as well as fewer lateral roots (Bao *et al.,* 2018). Eventually, the plant wilts and dies. When humidity is present at the onset of the disease, a white fungal layer supporting the pathogen appears on the plant. (Yang *et al.,* 2015). Furthermore, many *Fusarium* species generate numerous secondary metabolites, including fumonisins, which can accumulate in both grain and feed, subsequently posing a high risk of disease, such as liver failure and cancer, to both humans and livestock (Zhang *et al.,* 2005). Biocontrol bacteria are highly capable of survival and growth. They can produce antimicrobial metabolites and compete with plant pathogens for resources or space. Additionally, they can be produced on a large scale through fermentation. Therefore, biocontrol has become an environmentally sound and cost-effective approach to pest management (Zheng *et al.,* 1998). Antagonistic microbes comprise chiefly bacteria, actinomycetes, and fungi (Zhan *et al.,* 2014; Scannell *et al.,* 2000; Velthuis *et al.,* 1995). Biocontrol bacteria are highly diverse and successful in reproduction and survival. Their rapid growth allows them to colonize nutrient and ecological niches efficiently, becoming the predominant organisms in the microbiota. This principle of competitive exclusion is one way to reduce the number of pathogens on a plant's surface, thereby minimizing the likelihood of pathogen establishment in plant tissues Zhao *et al.,* (2018) and Wang *et al.,* (2024) discovered 4 strains of *Bacillus* spp. and 1 strain of *Pseudomonas* sp. located in the rhizosphere where soil was collected; all were noted to have inhibition greater than 60% with regard to *Fusarium* spp. Antagonistic bacteria isolated from the root rot disease area of onion (Karim *et al.,* 2021), as well as those isolated from the root rot pathogen of wolfberry (Zhu *et al.,* 2023), exhibited similar antagonistic activity. This current study involved finding an antagonistic bacteria source to utilize to treat the root rot of eggplant, followed by the isolation of the strains of antagonistic bacteria from vegetable cultivation soil and assays against the pathogen that causes this disease. Once identified, fermentation media and cultivation conditions were optimized for the isolated strains of antagonistic bacteria. Following this step, low concentrations of fermentation broth provided a much greater level of vegetative growth of

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eggplant plants. Parameters of yield (plant height, root length, stem diameter, biomass) were observed to be greater for treated plants than untreated plants. Additionally, seed germination parameters were also improved for treated seeds versus untreated seeds (germination rate, germination vigor). This work provides novel microbial resources for the biological management of this disease.

Materials and Methods

Test materials

Strain X-104 (*B. stercoris*), *F. solani*
Potato dextrose agar (PDA) medium: Potato 200 g/L, Dextrose 20 g/L, agar 15 g/L;
Luria-Bertani (LB) medium: Tryptone10.0 g/L, Yeast extract 5.0 g/L, Sodium chloride 10.0 g/L;
Luria-Agar (LA) medium: Tryptone10.0 g/L, Yeast extract g/L, Sodium chloride 10.0 g, agar 15 g/L;
Media for carbon source test: Carbon source (Glucose / Sucrose / Soluble starch / Dextrin / Maltose / Corn protein / Lactose / Mannitol / Fructose / Not add) 5 g/L;

In order to systematically isolate the best nutritional and environmental drivers, we have formulated a customized screening matrix. In the nitrogen source evaluation, we add various nitrogen-containing candidates of 10 g/L to the basic culture medium (containing 5 g/L glucose) - specifically including protein, yeast extract, beef extract, urea, potassium nitrate, ammonium chloride, peanut meal, bran and ammonium phosphate - at the same time Set a control group without any nitrogen sources added. On this basis, the inorganic salt test adopts an improved core culture medium (including 20 g/L glucose and 10 g/L predetermined optimal nitrogen source), and independently evaluates the addition of 10 g/L different mineral salts ($CuSO_4 \cdot 5H_2O$, $ZnSO_4 \cdot 7H_2O$, $MgSO_4 \cdot 7H_2O$, $FeSO_4 \cdot 7H_2O$, $CaCO_3$, $K_2HPO_4 \cdot 3H_2O$, KH_2PO_4 and zero addition baseline). Finally, in order to determine the ideal initial acidity, the standard LB liquid medium was adjusted to pH values of 5.0, 6.0, 7.0, 8.0, and 9.0 using 1.0 M HCl or 1.0 M NaOH.

Test Methods

Isolation, purification and preservation of soil bacteria: Using a systematic five-point spatial sampling procedure, a total of 36 different samples of soil material were obtained from the greenhouse facilities at Jilin Agricultural University and the cultivated plots of vegetables at Hunan Agricultural University. Prior to the processing steps, the samples were initially preserved at 4°C. A 1.0 g sample of the processed material was then used to prepare the microbial samples in sterile aqueous media, creating a ten-fold gradient dilution series from 10^1 to 10^6. Thereafter, specific quantities of the gradient suspensions were uniformly distributed onto LA using a standard surface-spreading procedure. After incubation, distinct single colonies exhibiting diverse morphologies (including variations in color, luster, and size) were selected, purified, and subcultured for 1 to 2 days prior to preservation(Zhou *et al.,* 2020).

Screening of antagonistic bacteria: The quantitative analysis of in vitro antagonism adopted the standard countermeasure test scheme. Specifically, the standard 8 mm agar round piece inoculated with the target eggplant root rot pathogen was placed in the center of the fresh PDA culture medium. Subsequently, the candidate biological control strains were inoculated along the biaxial symmetry, and the radial distance of 2 cm from the core of the pathogenic bacteria was strictly maintained. After designated culture under the best environmental parameters, the size of the mycelium growth inhibition circle was measured to evaluate the biological prevention and control effect.

Identification of antagonistic bacteria: The morphological characteristics of bacterial colonies (their form, size, colour, opacity and texture) were observed and recorded.

Following the extraction of genomic DNA from the *B. stercoris* isolate, specific genes were amplified through polymerase chain reaction. The reaction involved a universally applied primer pair consisting of 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3'). The thermocycling process started with a 5-minute pre-denaturation step at 94°C, followed by 35 cycles of DNA amplification consisting of 94°C for 45 seconds, 55°C for 30 seconds, and 72°C for 45 seconds. The process was concluded with a final elongation step at 72°C for 5 minutes (Visagie *et al.,* 2014). Sequencing and Evolutionary Assessment: The results of the specific gene amplification were checked for DNA fragment size using 1% agarose gel electrophoresis. The results were later purified and accurately sequenced by Sangon Biotech (Shanghai, China). The results were later assessed for homology using a BLAST search in the NCBI database. Finally, a dendrogram illustrating the evolutionary relationships of the isolated organism was constructed using MEGA 7.0 software.

Screening of optimal components for fermentation medium: Bacterial cakes (8 mm in diameter) of strain X-104 were transferred into the 32 different test media, which varied in carbon sources, nitrogen sources, inorganic salts, and pH. Each treatment was performed in triplicate. A control was prepared by mixing sterile water with PDA medium at a 1:5 ratio. After 12 h 28°C 180 rpm of incubation with shaking, the inhibition rate of this filtrate against *F. solani* was then calculated using the formula below. All data were presented as mean ± SD from three independent replicates. Statistical comparisons among groups were performed using one-way analysis of variance followed by Tukey's post hoc test. A $p<0.05$ was considered statistically significant. All analyses were performed using SPSS.

$$I = \frac{C - T}{C} \times 100\% \quad (1)$$

where, I = Bacteriostatic rate, C = Growth in control, T = Growth in treatment.

Using carbon source, nitrogen source, and inorganic salt, and pH as the four factors, and the three most

significant components identified for each factor in the respective levels, the optimal combination of components was determined through an L_9 (3^4) orthogonal experimental design (Table 1) and subsequent range analysis.

Table 1. Factors and levels of orthogonal experiment.

Level	Factor			
	A Carbon source	B Nitrogen source	C Inorganic salt	D pH
1	Glucose	Peanut meal	$ZnSO_4 \cdot 7H_2O$	5
2	Not added	Ammonium chloride	$CuSO_4 \cdot 5H_2O$	6
3	Mannitol	Bran	$FeSO_4 \cdot 7H_2O$	7

Optimization of medium component concentrations: After preliminary orthogonal screening, we used the response surface method (RSM) framework to fine-tune the ratio of macronutrients and micronutrients through mathematical methods. In the end, we built a customized liquid medium whose formula aims to raise the fermentation yield of strain X-104 to the theoretical limit.

In order to determine the optimal substrate threshold, we conducted a series of single-variable analysis with inhibition activity as the core indicator. Unlike the enumerated discrete points, this study systematically titrated specific nutrients: the concentration range of glucose and ammonium chloride was 5 to 25 g/L, and the increment was 5 g/L; while the concentration range of zinc sulfate heptahydrate was 2.5 to 12.5 g/L, with an increment of 2.5 g/L. At the same time, the physical biological reaction environment was strictly locked at 28°C, the rotational shaking speed was 180 rpm, the initial acidity pH 6.0 and the 12-hour processing window.

Subsequently, based on the single-factor experimental results, the parameter ranges for the addition levels of the fermentation medium components for strain X-104 were determined for the response surface methodology (RSM) experiment (Song *et al.,* 2018; Wei *et al.,* 2012).To achieve this, a three-factor, three-level Box-Behnken design (Shu *et al.,* 2016) was employed for the RSM experiment, aiming to obtain the optimal fermentation parameters for strain X-104.The resulting data were subsequently analyzed using Design-Expert software (version 8.0.6).

Optimization of fermentation conditions: To determine the optimal fermentation conditions, a range of medium volumes (50, 75, 100, 125, and 150 mL in 250 mL conical flasks), fermentation times (8, 12, 16, 20, and 24 h), and incubation temperatures (20, 24, 28, 32, and 36°C) were tested. The bacteriostatic rate of the fermentation broth was then determined in order to select the optimal fermentation conditions.

Growth-promoting effect of strain X-104 fermentation broth: To investigate the effect of strain X-104 fermentation broth on germination, 350 plump and uniform eggplant seeds were selected for a Petri dish assay. The seeds were treated with the fermentation broth diluted at ratios of 1:5, 1:10, 1:20, 1:50, 1:75, and 1:100 (broth: water). Germination potential and germination rate were calculated using standard formulas. Subsequently, germinated seeds were transplanted into seedling pots and grown under controlled conditions with a day/night temperature of 28/20°C a photoperiod of 12 h, and a relative humidity of 65±5%. After a 30-day growth period, agronomic traits-including plant height, root length, stem diameter, and both fresh and dry weights-were measured. Statistical analysis was performed as described in Section 2.2.4.

$$GP = \frac{N_p}{N_t} \times 100\% \quad (2)$$

where, GP = Germination potential, N_P = Number of seeds germinated at the peak period, N_t = Total number of seeds

$$GR = \frac{N_g}{N_t} \times 100\% \quad (3)$$

where, GR = Germination rate, N_g = Number of germinated seeds, N_t = Total number of seeds

Results

Isolation and screening of antagonistic bacteria: From the collected soil matrix, 182 different strains of bacteria were successfully isolated and purified. The subsequent in vitro screening showed that 7 of the candidate strains - N16, N08, M33, M86, X-104, X-02 and A04 showed significant antagonism against eggplant root rot pathogens. These interactions are characterized by the formation of an obvious transparent circle, indicating that the pathogenic bacteria inhibition rate of each strain exceeds the threshold of 50%. Figure 1 shows a typical visualization model, which illustrates this powerful biological prevention and control ability.

The inhibitory zone widths and inhibition rates of the 7 selected bacterial strains against the eggplant root rot fungus are summarized in Table 2.

Table 2. Anti-efficacy of 7 soil bacterial strains against *F. solani*.

Antagonistic bacterial	Inhibition zone (mm)	Inhibition rate of sterile fermented broth (%)
N 16	4.23 ± 0.1101^{c}	57.94 ± 0.3297^{b}
M 86	5.91 ± 0.3435^{b}	45.72 ± 0.5237^{c}
X-104	6.87 ± 0.3262^{a}	66.70 ± 0.1853^{a}
X-02	6.22 ± 0.0208^{ab}	64.54 ± 0.8352^{e}
M 33	6.41 ± 0.2338^{ab}	61.85 ± 0.7416^{f}
N 08	4.75 ± 0.3319^{c}	65.04 ± 0.3658^{d}
A 04	4.09 ± 0.1440^{c}	50.47 ± 0.5732^{b}

Note: different lowercase letters indicate significant differences of different antagonistic bacteria at $p<0.05$ level

Based on the comparative analysis of inhibition rates, strain X-104 was selected for further investigation. Microscopic examination of the eggplant root rot pathogen (*Fusarium solani*) revealed that the untreated hyphae exhibited intact morphology and uniform diameter. On the other hand, the hyphae treated with the fermentation broth of strain X-104 showed notable morphological changes. As shown in Fig. 2, the blank control group showed a normal morphology for the hyphae, while the treated group showed abnormal deformation in the tips, which were swollen or constricted. This shows that the bioactive compounds in the fermentation broth of X-104 affect the morphology of the pathogenic hyphae, thus preventing mycelial growth.

Identification of antagonistic strains

Morphological identification: On lysogeny broth (LB) agar medium, the strain X-104 formed large-sized opaque yellowish-colored colonies with rough surface features, irregularities, folding, and glossy edges. The representative morphologies of the bacterial colonies are given in Fig. 3a and 3b.

Molecular biological characterization: To establish precise taxonomic affiliations, the acquired 16S rRNA amplicon data were subjected to rigorous homology alignments against reference nucleotide archives housed within the GenBank repository via the BLAST analytical tool. After validating the sequences, a neighbour-joining dendrogram was generated to map the evolutionary topology of the isolate using the MEGA 7.0 suite. *Pseudomonas viridiflava* was used as an outgroup. The strain X-104 that is antagonistic positioned itself in a corresponding clade to the B. The strain was *Bacillus stercoris*, based on the 16S rRNA sequences similarities of the study and reference strains. The tree showing evolutionary genetic relationship is seen in Fig. 4.

Screening of optimal fermentation medium components: The antagonistic effectiveness of metabolites secreted by cell-free X-104 isolate was highly dependent on the medium. This was dependent on the initial acidity with the specific provision of carbon, nitrogen, and inorganic salts. The maximum inhibition was exhibited by mannitol (72.35%) followed by unsupplemented (67.38%) and glucose (66.93%) after systematic screening of nine carbon additives against carbon-deficient control. Statistical validation confirmed that this optimized carbon regimen yielded a substantially enhanced bacteriostatic response relative to the alternative carbon conditions evaluated ($p<0.05$) (Fig. 5).

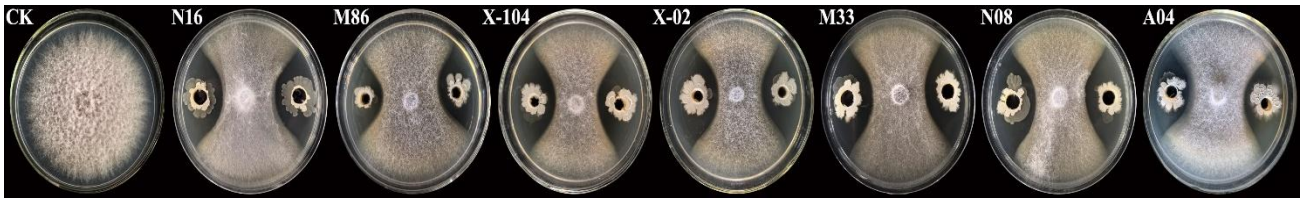

Fig. 1. Antifungal effects of 7 soil bacterial strains.

Fig. 2. Morphological alterations of *Fusarium solani* hyphae induced by the fermentation broth of strain X-104.
(a) Untreated control: *Fusarium solani* hyphae exhibiting typical filamentous morphology. **(b)** Treated hyphae: showing hyphal expansion. (c) Treated hyphae: demonstrating hyphal constriction.

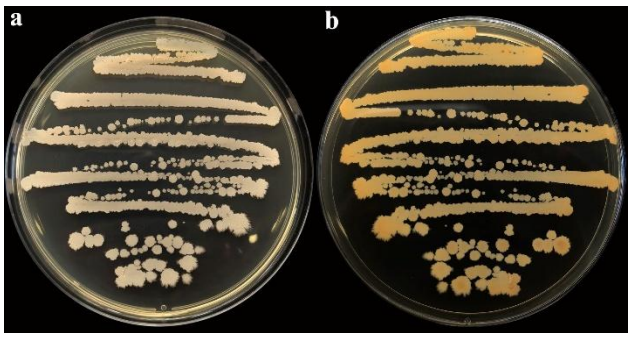

Fig. 3. Colony morphology and microscopic morphology of strain X-104
a: positive picture of strain b: reverse picture of strain

Eight nitrogen sources, along with a control lacking any added nitrogen source, were screened. The results showed that the three nitrogen sources exhibiting the highest inhibition rates were: Peanut meal (80.27%) >Ammonium chloride (77.29%)>Bran (76.16%). The bacteriostatic rate was significantly higher than those of the other nitrogen sources tested ($p<0.05$).

Eight different inorganic salts, along with a control group without any added inorganic salts, were screened. The results showed that the three inorganic salts exhibiting the highest inhibition rates were: $CuSO_4 \cdot 5H_2O$ (76.83%) >$FeSO_4 \cdot 7H_2O$ (75.40%) >$ZnSO_4 \cdot 7H_2O$ (72.70%). The bacteriostatic rate was significantly higher than those with the addition of other inorganic salts (Fig. 6).

In the pH optimization test, the fermentation broth exhibited the highest bacteriostatic activity at pH 5, 6, and 7.

Range analysis of the orthogonal test results revealed that factor B exhibited the highest range (R = 9.15), identifying it as the primary factor influencing the inhibition rate. This was followed by factors D (R = 8.99) and C (R = 8.26). In contrast, factor A had a considerably smaller range (R = 2.42), indicating a lesser effect. This result confirmed that the nitrogen source (Factor B) was the most significant factor affecting the antibacterial activity. The order of significance for the tested factors was: nitrogen source > pH > inorganic salt > carbon source (Table 4). The optimal combination of fermentation medium components, as determined by intuitive analysis, was $A_3B_3C_3D_1$. This combination corresponds to glucose as the carbon source, ammonium chloride as the

nitrogen source, $ZnSO_4 \cdot 7H_2O$ as the inorganic salt, and an initial pH of 5.0. Fermentation with this optimized medium achieved the highest inhibition rate of 81.10% (Table 3).

Single-factor experiments: Figure 7 illustrated that the glucose concentration in the medium (tested range: 15 to 25 g/L) significantly influenced the antagonistic activity of strain X-104 against the eggplant root rot pathogen. The highest inhibition rate (78.39%) was achieved at a glucose concentration of 20 g/L. Notably, higher concentrations (up to 25 g/L) resulted in reduced efficacy, indicating that excessive glucose was suboptimal for the fermentation process of strain X-104. Similarly, ammonium chloride concentration markedly affected the antibacterial activity of the fermentation filtrate (tested range: 10 to 20 g/L). Regarding the nitrogen provision, strictly regulating the ammonium chloride dosage at 15 g/L culminated in a peak inhibitory response of 77.99%. Likewise, changes in the analyzed trace mineral window ($ZnSO_4 \cdot 7H_2O$ at the level of 7.50 to 12.50 g/L) had an important effect on the antagonistic activity, where 10 g/L obtained an 80.75% efficacy. Upon synthesis of these univariate findings, the final baseline formulation of the liquid broth consisted of glucose (20 g/L), ammonium chloride (15 g/L) and $ZnSO_4 \cdot 7H_2O$ (10 g/L).

Response surface optimization tests: Medium 1 acted as the baseline matrix for constructing the prediction model and estimating their statistical significance. The dependent response was the inhibitory efficacy (Y) within this optimization framework. Meanwhile, the simultaneous supplemented dosages including glusose (X_1), ammonium chloride (X_2), and $ZnSO_4 \cdot 7H_2O$ (X_3) are the independent operational factors (Chen *et al.,* 2018; Wang *et al.,* 2018). In order to determine the best possible combination of nutrients, a three variable three-level experimental design matrix was constructed using Design-Expert software (version 8.0.6). The detailed configurations of this response surface array along with the observed results are given in Table 5.

Applying multiple regression analysis to the empirical datasets from Table 5 yielded the following second-order polynomial model: $Y = 82.36 + 0.039X_1 + 0.13X_2 + 1.00X_3 - 0.33X_1X_2 + 0.31X_1X_3 - 0.74X_2X_3 - 0.63X_1^2 - 0.76X_2^2 - 2.10X_3^2$. Evaluating the quantitative magnitude of the linear coefficients in this case, the subsequent decreasing order of the individual nutrient factors on the antagonistic response is as follows: the $ZnSO_4 \cdot 7H_2O$ dosage had the most profound effect, followed by the nitrogen source dosage provision, while the specified carbon source had the most meager effect.

Analysis of variance (Table 7) supports the importance of the regression model due to the high F-value calculated as 27.08 (P = 0.0001). The analysis of the particular coefficients of the polynomials showed that the variable squared X_3^2 had a very heavy impact ($p<0.05$). The mathematical reliability of the derived equation was confirmed by a non-significant lack of fit to pure error (F = 4.64; P = 0.0860 > 0.05), indicating non-presence of systematics. Evaluation of goodness-of-fit was performed using the determination coefficient (R^2 = 0.9721) along with the adjusted R^2 which relies on 0.9362 confirming little variance in experiment.

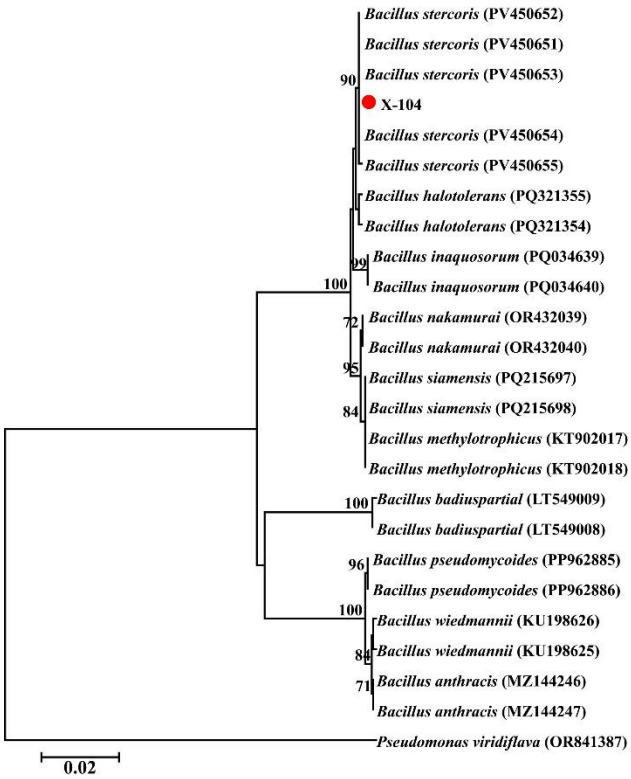

Fig. 4. Phylogenetic tree.

Table 3. Intuitive analysis of orthogonal experiments.

Test number	Factor				Inhibition rate (%)
	A	B	C	D	
1 ($A_1B_1C_1D_1$)	Mannitol	Peanut meal	$CuSO_4 \cdot 5H_2O$	5	77.74 ± 1.1227^{ab}
2 ($A_1B_2C_3D_2$)	Mannitol	Bran	$ZnSO_4 \cdot 7H_2O$	6	70.03 ± 0.8172^{bc}
3 ($A_1B_3C_2D_3$)	Mannitol	Ammonium chloride	$FeSO_4 \cdot 7H_2O$	7	78.12 ± 0.3501^{bc}
4 ($A_2B_1C_3D_3$)	0	Peanut meal	$ZnSO_4 \cdot 7H_2O$	7	67.44 ± 1.7144^{ab}
5 ($A_2B_2C_2D_1$)	0	Bran	$FeSO_4 \cdot 7H_2O$	5	76.16 ± 1.5633^{b}
6 ($A_2B_3C_1D_2$)	0	Ammonium chloride	$CuSO_4 \cdot 5H_2O$	6	65.32 ± 0.6697^{cd}
7 ($A_3B_1C_2D_2$)	Glucose	Peanut meal	$FeSO_4 \cdot 7H_2O$	6	80.25 ± 0.3593^{ab}
8 ($A_3B_2C_1D_3$)	Glucose	Bran	$CuSO_4 \cdot 5H_2O$	7	62.48 ± 0.3100^{d}
9 ($A_3B_3C_3D_1$)	Glucose	Ammonium chloride	$ZnSO_4 \cdot 7H_2O$	5	81.10 ± 0.7813^{a}

Note: 0 means not added; different lowercase letters indicate significant differences at the $p<0.05$

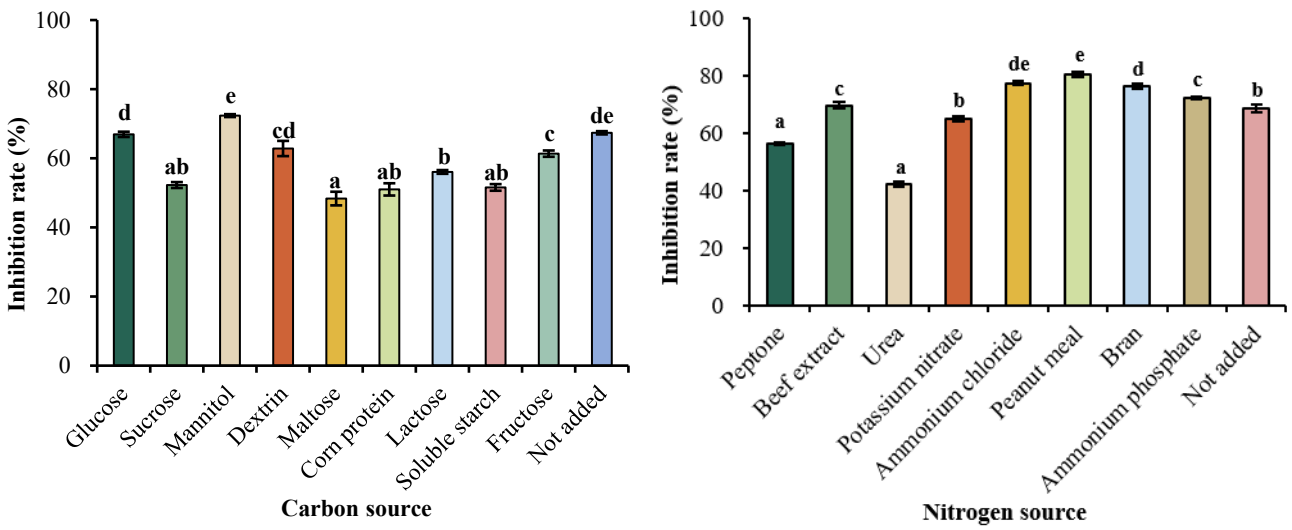

Fig. 5. Inhibition rate of fermentation with different carbon and nitrogen sources.
Different lowercase letters indicate statistically significant differences between groups (p<0.05, one-way ANOVA with Tukey's test).

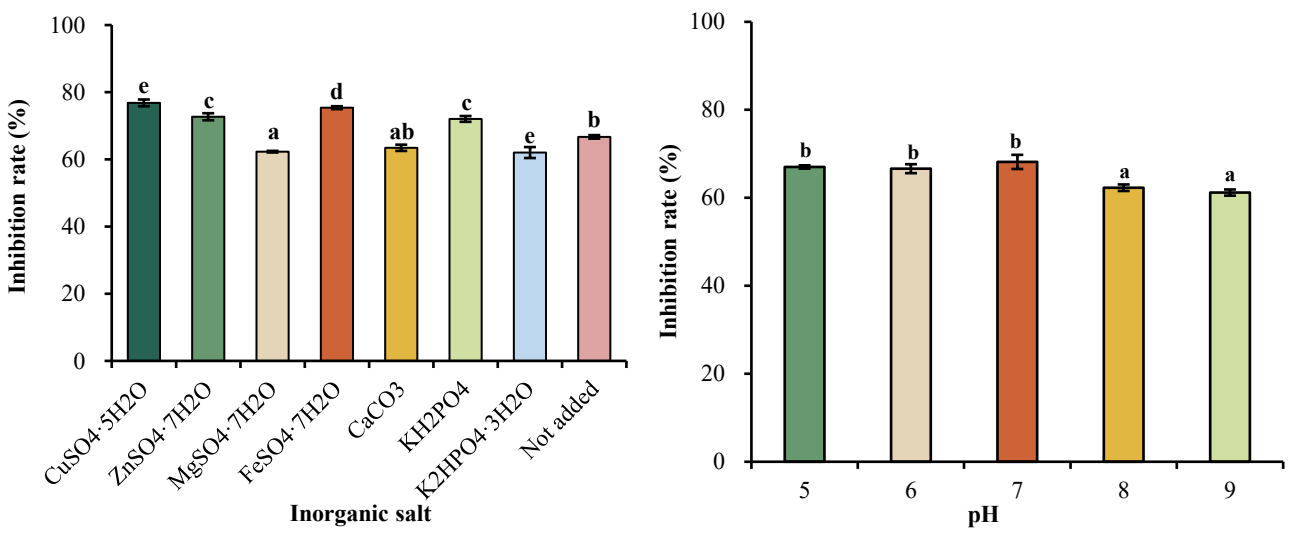

Fig. 6. Inhibition rate of fermentation with different inorganic salt and pH.
Different lowercase letters indicate statistically significant differences between groups (p< 0.05, one-way ANOVA with Tukey's test).

Table 4. Results of range analysis for each factor.

Index	Factor			
	A	B	C	D
K_1	215.21	236.11	205.54	235.00
K_2	219.60	208.67	229.25	215.60
K_3	223.83	213.86	223.85	208.04
k_1	71.74	78.70	68.51	78.33
k_2	73.20	69.55	76.41	71.86
k_3	74.61	71.29	74.62	69.34
R	2.42	9.15	8.26	8.99

Note: K_1, K_2 and K_3represent the sum of the experimental results at each level for a given factor; k_1, k_2 and k_3 represent the corresponding mean values; and R is the range (the difference between the maximum and minimum k values)

Figure 8 converts the predictive polynomial model into a visual format, and projects complex three-way dynamics (especially the dynamics between glucose, ammonium chloride and $ZnSO_4 \cdot 7H_2O$) onto the three-dimensional topographic structure and its plane contour equivalents, thus clarifying how these precise nutrient intersections determine the final antagonistic performance. Specifically, the upper panel of Fig. 8 captures the synergistic cross-interaction between the specified carbon and nitrogen sources, modeled under the boundary condition where the $ZnSO_4 \cdot 7H_2O$ dosage is anchored at its median coordinate (10.0 g/L).The inhibition rate initially increased but subsequently decreased as the concentrations of both glusose and ammonium chloride increased, indicating a clear optimal range. Figure 8-middle demonstrates the interaction between glusose and $ZnSO_4 \cdot 7H_2O$ at the central level of ammonium chloride (15.0 g/L). A similar nonlinear relationship was observed, where the inhibition rate reached a maximum before declining with further increases in the concentration of either component. Figure 8-below examines the interaction between ammonium chloride and $ZnSO_4 \cdot 7H_2O$ at the central level of glusose (20 g/L). The interaction of the two factor also showed a peak inhibition rate confirming that the relationship of each factor and the response is not linear but is rather quadratic in the range tested.

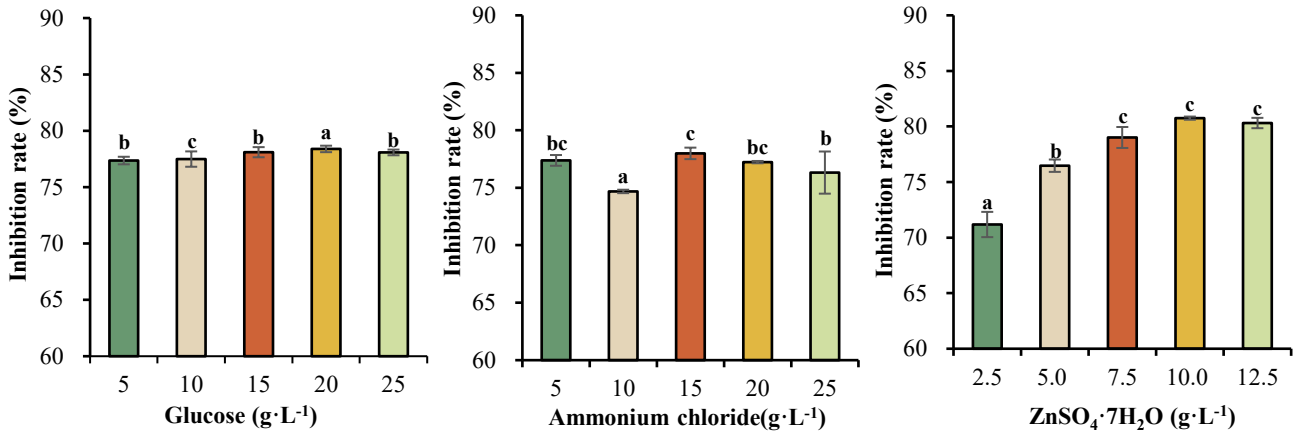

Fig. 7. Effects of glucose, ammonium chloride and $ZnSO_4 \cdot 7H_2O$ addition on the inhibition rate.
Different lowercase letters indicate statistically significant differences between groups ($p<0.05$).

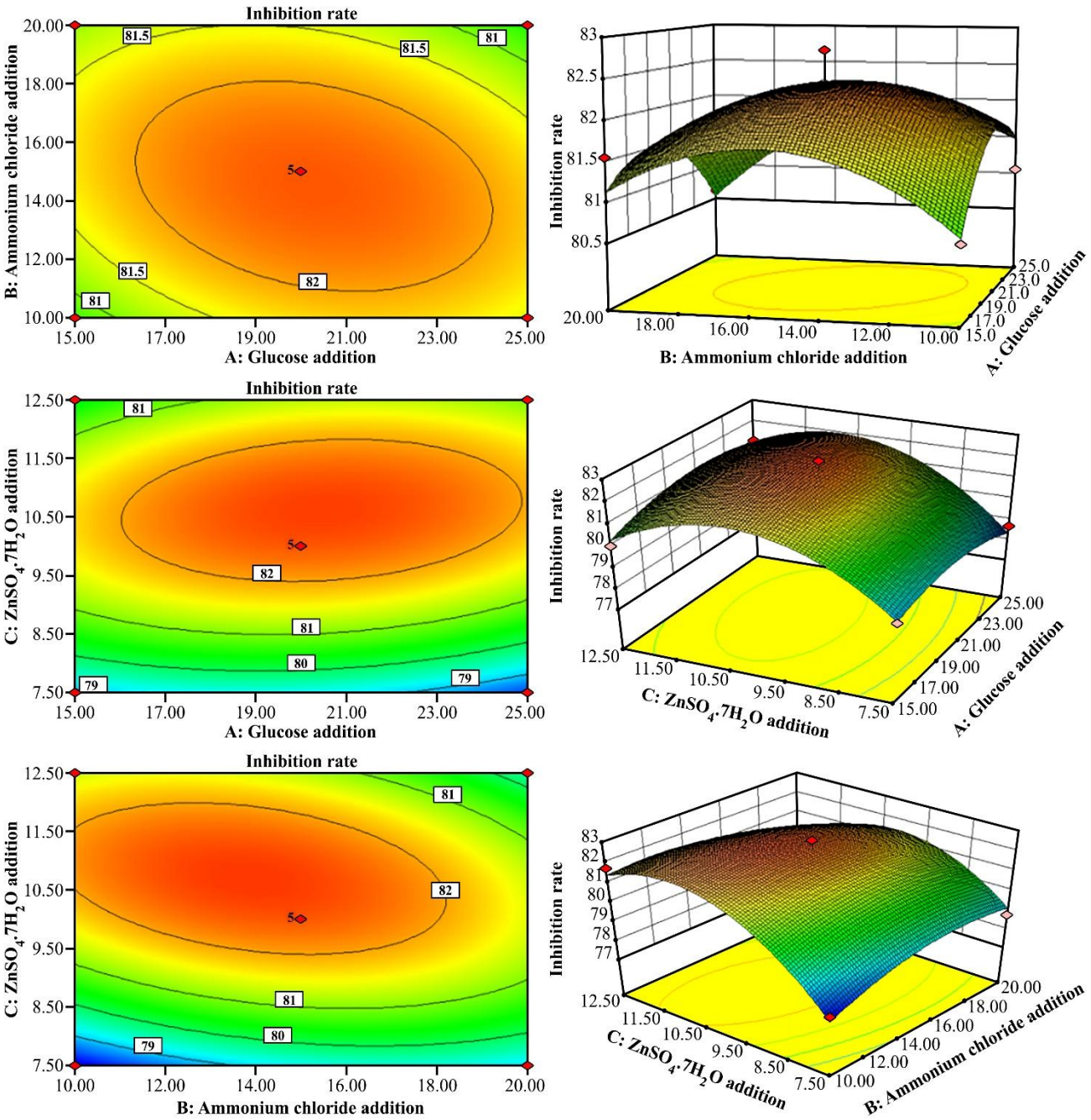

Fig. 8. Contour and 3D surface maps of interactions between factors.

Table 5. Variables and level of Box-Behnkendesign.

Level	Factor		
	X_1 Glusose addition ($g \cdot L^{-1}$)	X_2 Ammonium chloride addition ($g \cdot L^{-1}$)	X_3 $ZnSO_4 \cdot 7H_2O$ addition ($g \cdot L^{-1}$)
-1	15	10	7.5
0	20	15	10
1	25	20	12.5

Table 6. Results of response surface optimization experiment for X-104 strain fermentation.

Code	Factors and levels			Y Inhibition rate (%)
	X_1 ($g \cdot L^{-1}$)	X_2 ($g \cdot L^{-1}$)	X_3 ($g \cdot L^{-1}$)	
1	-1	-1	0	80.66
2	1	-1	0	81.05
3	-1	1	0	81.55
4	1	1	0	80.61
5	-1	0	-1	78.82
6	1	0	-1	78.62
7	-1	0	1	80.00
8	1	0	1	81.06
9	0	-1	-1	78.05
10	0	1	-1	78.77
11	0	-1	1	81.70
12	0	1	1	79.47
13	0	0	0	82.21
14	0	0	0	82.17
15	0	0	0	82.32
16	0	0	0	82.35
17	0	0	0	82.77

The model predicted an optimum inhibition rate of 82.53% under the following conditions: glucose at 20.88 g/L, ammonium chloride at 13.66 g/L, and $ZnSO_4 \cdot 7H_2O$ at 10.74 g/L. A verification experiment conducted under these optimized conditions yielded an inhibition rate of 85.42%, which is in close agreement with the predicted value (Fig. 9).

Optimization of fermentation conditions: The physical cultivation parameters of chronological fermentation length, working liquid volume and thermal conditions were systematically mapped to reaffirm that eggplant root rot can be suppressed. The antagonistic effectiveness showed a distinct parabolic pattern for all the parameters measured. According to results, a 12 hours of incubation appeared to be the optimal duration as it attained a maximum inhibition of 79.43% which was found superior to other time points (8, 16, 20 and 24 hours). Concerning the aeration dynamics in a 250mL Erlenmeyer vessel, a volume of 100mL liquid broth gave maximum inhibitory response at 81.69%. The performance of the 100 mL setup was statistically similar to 125 mL and 150 mL setups. However, it had a significant edge over 50 mL and 75 mL setups. Thermally, controlling the incubation environment at 32°C gave rise to a maximum inhibition rate of 82.61%, which was statistically different than all other thermal treatments. Based on these proofs of concept, the optimal physical protocol for maximum biocontrol should be a 12-hour kinetic incubation at 32°C in 100 mL broth in a 250 mL flask (Fig. 10).

Table 8. Influence of X-104 fermentation broth on seed germination.

Code	Fermentation broth : Water	Germination potential (%)	Germination rate (%)
A	1:5	18	66
B	1:10	24	72
C	1:25	28	78
D	1:50	52	94
E	1:75	46	90
F	1:100	42	82
G	CK	44	88

Effects of strain X-104 fermentation broth on eggplant seed germination and seedling growth: The findings showed that strain X-104 fermentation broth was concentration-dependent (high at low concentration and low at high concentration). Optimal effects were observed at dilutions of 1:50 and 1:75, with germination vigor and rate peaking at 52%, 94% and 46%, 90%, respectively, both significantly higher than the control (water with fresh water) (44% and 88%, as detailed in Table 8).

The fermentation broth of strain X-104 exerted a biphasic, concentration-dependent effect on eggplant seedling growth after 30 days, with promotion at low concentrations and inhibition at high concentrations. Optimal growth was observed at 1:50 and 1:75 dilutions, which significantly enhanced multiple parameters. The 1:50 dilution was particularly effective, maximizing height (4.3%), root length (30.74%), fresh weight (15.66%), and dry weight (34.29%), while the 1:75 dilution produced the largest stem diameter (16.46%). Inhibition at high concentrations is potentially due to phytotoxic effects of antimicrobial metabolites in the broth. For detailed results, see Table 9 and Fig. 11.

Table 7. Analysis of variance for response surface experiment results.

Source	Sum of squares	df	Mean square	F-Value	*P*-value Prob > F	Sig.
Model	35.64	9	3.96	27.08	0.0001	**
X_1	0.012	1	0.012	0.082	0.7827	
X_2	0.14	1	0.14	0.96	0.3597	
X_3	7.94	1	7.94	54.30	0.0002	*
X_1X_2	0.44	1	0.44	3.02	0.1256	
X_1X_3	0.40	1	0.40	2.71	0.1435	
X_2X_3	2.18	1	2.18	14.88	0.0062	*
X_1^2	1.70	1	1.70	11.59	0.0114	*
X_2^2	2.44	1	2.44	16.72	0.0046	*
X_3^2	18.65	1	18.65	127.52	<0.0001	**
Residual	1.02	7	0.15			
Lack of Fit	0.80	3	0.27	4.64	0.0860	
Pure Error	0.23	4	0.057			
Cor Total	36.66	16				

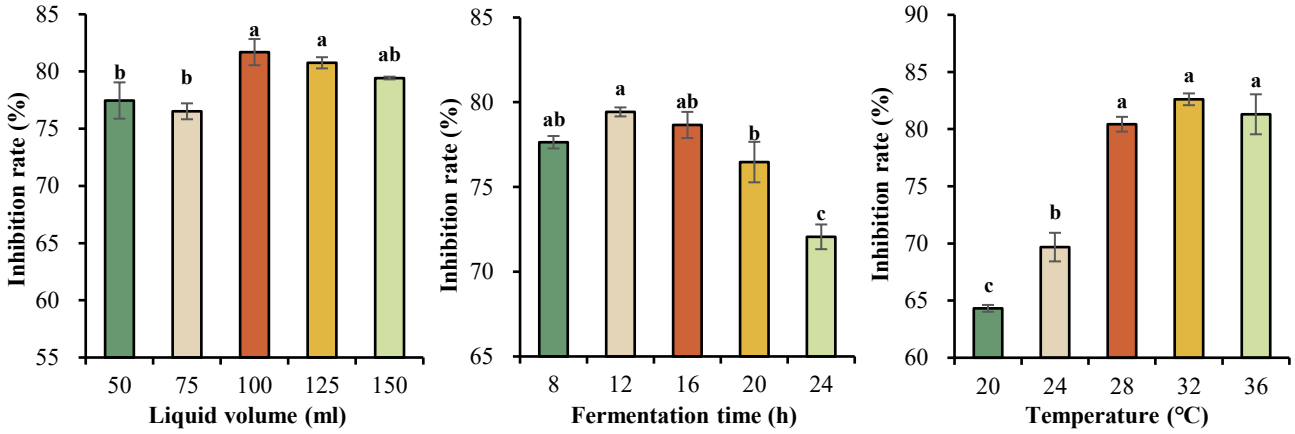

Fig. 10. Inhibition rate of fermentation broth under different fermentation times, liquid volumes, and temperatures. Different lowercase letters indicate statistically significant differences between groups (p<0.05).

Table 9. Influence of X-104 fermentation broth on eggplant seedlings.

Fermentation broth: Water	Plant height (mm)	Root length (mm)	Stem diameter (mm)	Fresh weight (mg)	Dry weight (mg)
1:100 (A)	128.00 ±1.61^{ab}	58.31 ± 3.48^{ab}	1.46 ± 0.04^{b}	410.56 ± 28.78^{bc}	32.31 ± 1.98^{cd}
1:75 (B)	135.05 ± 2.28^{a}	50.84 ± 3.09^{bc}	1.84 ± 0.06^{a}	550.55 ± 57.05^{ab}	43.43 ± 5.99^{bc}
1:50 (C)	139.31 ± 1.22^{a}	60.86 ± 0.95^{a}	1.74 ± 0.05^{a}	690.35 ± 78.06^{a}	65.99 ± 8.29^{a}
1:25 (D)	121.05 ± 1.77^{bc}	30.73 ± 2.32^{d}	1.27 ± 0.03^{c}	367.33 ± 8.73^{c}	27.58 ± 1.65^{cd}
1:10 (E)	113.95 ± 6.01^{cd}	26.26 ± 2.28^{d}	1.19 ± 0.06^{c}	307.06 ± 62.36^{c}	20.76 ± 4.19^{d}
1:5 (F)	103.24 ± 8.41^{d}	23.11 ± 4.08^{d}	1.12 ± 0.06^{c}	257.19 ± 3.51^{c}	17.84 ± 0.64^{d}
CK (G)	133.58 ± 1.52^{ab}	46.55 ± 0.40^{c}	1.58 ± 0.03^{b}	596.88 ± 60.62^{a}	49.14 ± 8.11^{b}

Note: Using SPSS for data analysis, different lowercase letters indicate significant differences at the p<0.05 level

Discussions

The bacteria in biocontrol possess high reproductive and survival potential. They are able to grow in large numbers, which enables them to occupy the available nutritional and ecological niches in a very fast manner. This enables them to dominate the microbiota and exclude the pathogens in a competitive manner, thereby reducing the chances of invasion in the plant tissue (Zhao *et al.,* 2018). Notably, *Bacillus* species synthesize a diverse array of antimicrobial metabolites, such as antimicrobial peptides (e.g., surfactin, ectoine, fengycin), cell wall-degrading enzymes (e.g., chitinases, glucanases, proteases), and volatile organic compounds (Caulier *et al.,* 2019). These metabolites can directly inhibit or lyse pathogenic structures such as mycelia, spores, and cells. In addition, the rapid proliferation and strong life cycle turnover inherent in Bacillus species enable it to dominate the specific plant microenvironment, especially between roots and leaves (Compant *et al.,* 2005). This space occupation will trigger a strong competitive exclusion mechanism, which will severely limit the spread of pathogen. In the initial environmental sampling, 182 different bacteria were successfully isolated and purified. Subsequently, several candidate strains that can significantly inhibit the disease were screened out in vitro counter-tests of *F. solani* as the pathogen. Based on its excellent antagonism index and other favorable biological prevention and control characteristics, we give priority to specific strains numbered X-104 for comprehensive follow-up characterization. Finally, combined with phenotypic analysis and precise molecular phylogenetic analysis (constructing an evolutionary tree diagram), we finally determined that this highly active strain X-104 was *B. stercoris*.The width of the inhibition zone of X-104 against *F. solani* was 6.87 mm. In a repeated experiment, the inhibition rate of strain X-104 against *F. solani* was confirmed to be 66.70%.

In order to elucidate the multivariate interactions which influence the development of X-104, response surface methodology (RSM) has been applied as a primary statistical modeling tool. This methodology has been recognized for its ability to optimize microbial bioprocesses effectively (Zhang *et al.,* 2021; Zhou *et al.,* 2023; Hassan *et al.,* 2020). Initially, univariate tests revealed the most important macronutrient variables which influence the development of X-104. Subsequently, an orthogonal array was applied to ascertain the optimal nutrient formulation. This revealed an optimal formulation consisting of glucose, ammonium chloride, and $ZnSO_4 \cdot 7H_2O$. The initial pH was kept at 5.0. A predictive matrix was produced through computational modeling in Design-Expert software. This revealed an optimal formulation consisting of 20.88 g/L glucose, 13.66 g/L ammonium chloride, and 10.74 g/L $ZnSO_4 \cdot 7H_2O$. This formulation has a maximum theoretical inhibition efficacy of 82.53%. Empirical verification at this precise formulation resulted in an inhibition rate of 85.53%In order to elucidate the multivariate interactions which influence the development of X-104, response surface methodology (RSM) has been applied as a primary statistical modeling tool. This methodology has been recognized for its ability to optimize microbial bioprocesses effectively (Zhang *et al.,* 2021; Zhou *et al.,* 2023; Hassan *et al.,* 2020). Initially, univariate tests revealed the most important macronutrient variables which influence the development of X-104. Subsequently, an orthogonal array was applied to ascertain the optimal nutrient formulation. This revealed an optimal formulation consisting of glucose, ammonium chloride, and $ZnSO_4 \cdot 7H_2O$. The initial pH was kept at 5.0. A predictive matrix was produced through computational modeling in Design-Expert software. This revealed an optimal formulation consisting of 20.88 g/L glucose, 13.66

g/L ammonium chloride, and 10.74 g/L $ZnSO_4 \cdot 7H_2O$. This formulation has a maximum theoretical inhibition efficacy of 82.53%. Empirical verification at this precise formulation resulted in an inhibition rate of 85.42%.

It is noteworthy that low levels of the X-104 fermentation broth are found to stimulate eggplant germination and vegetative growth. Although auxin-like compounds play a role in this mechanism, the exact mechanism of this stimulation remains to be elucidated. In order to translate the promising results from the pot trials into practice field studies are needed. The study is essential in evaluating the biocontrol activity of the strain towards eggplant root rot disease and developing the biocontrol product.

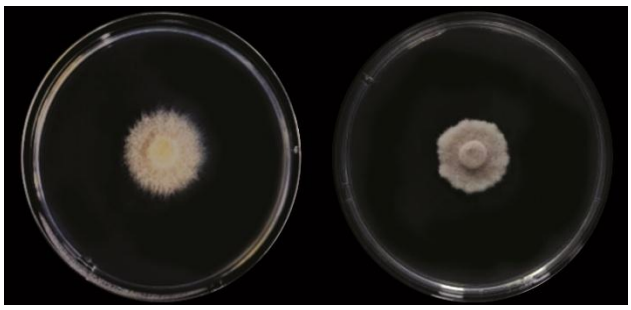

Fig. 9. Comparison of antibacterial effects between initial conditions (left) and optimized conditions (right).

Fig. 11. Influence of X-104 fermentation broth on eggplant seedlings Fermentation broth: Water = A: 1:100; B:1:75; C: 1:50; D: 1:25; E: 1:10; F: 1:5; G: CK

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