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POTENTIAL OF COMPOUND BACTERIAL AGENT IN THE BIOCONTROL OF MUSKMELON PATHOGENS

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Abstract

The bacterial strain TG6 was screened from the soil of a muskmelon greenhouse, which could effectively inhibit the growth of *Sphaerotheca fuliginea*, *Fusarium oxysporum, Phytophthora melonis* and *Pseudoperonospora cubensis* of muskmelons. TG6 was identified as *Bacillus subtilis* through morphological, physiological, biochemical and molecular studies. Taking TG6 as the original strain, TG67 and TG69 were obtained by He-Ne laser mutagenesis. The microbial compound antagonist strain TG67 and TG69 was prepared in the ratio of 1:1 and applied to the biological control of greenhouse melon. Muskmelon field experiments showed that the bactericide could effectively control the occurrence of various diseases of muskmelon in greenhouse.

Introduction

In our rapidly evolving society, the prevalence of plant diseases poses significant threats to agricultural yields and, consequently, to the income of farmers. Plant diseases are caused by biological or abiotic factors that induce pathological changes within the plant's physiology and morphology. This disrupts normal growth and results in various abnormal manifestations. Currently, the primary strategies for managing crop diseases include agricultural, physical, chemical, and biological control methods (Rivas-Garcia *et al.* 2019).

Muskmelons were basically cultivated in greenhouse. However, greenhouses are susceptible to various diseases because of the high temperature, high humidity and other features. Greenhouse muskmelons are prone to be infected by some pathogens and develop fusarium wilt, downy mildew, powdery mildew and phytophthora blight and so on (Cao *et al.* 2020, Lv *et al.* 2018). Melon wilt disease is most prevalent during the flowering, fruit setting, and fruit expansion phases. Early symptoms include the wilting of one or two branches, progressing to root decay and browning, and ultimately leading to the wilting and death of the entire plant. Such afflictions can drastically reduce the quality and yield of melons, and in severe instances, result in total crop failure (Liu *et al.* 2023). *Fusarium oxysporum* has been identified as the primary pathogen behind melon fusarium wilt, a soil-borne fungal disease (Du *et al.* 2022). Its mycelium, sclerotium, and chlamydospore can survive in the soil over winter and accumulate with each passing year, serving as the primary infection source for subsequent seasons.

The selection of disease-resistant varieties, traditional agricultural control, chemical control and biological control measures were adopted to control muskmelon wilt. In general, the diseases are controlled by spraying of pesticides on a regular basis. Nevertheless, long-term and massive use of chemical manures and pesticides would lead to serious deterioration of the ecological environment for crops and seriously affect the growth of crops. On this basis, greater attention has

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been paid to green and safe protective biological products and the application of such products in crop protection in greenhouse muskmelons (Padda *et al*. 2017, Pal *et al.* 2017). Biological control has emerged as a promising area of research in plant disease management due to its environmentally friendly attributes, such as being non-toxic, leaving no harmful residues, not affecting beneficial microorganisms, and providing long-lasting disease prevention. Commonly utilized biocontrol agents include *Pichia anomala Kurtzman*, *Pseudomonas fluorescens,* and various Bacillus species, with Bacillus being the most frequently used for plant disease prevention (Zhang *et al.* 2023).

To address the high incidence and reduced yields of greenhouse muskmelon diseases traditionally associated with chemical pesticides and fertilizers, this study utilized advanced microbial breeding technology. The aim was to select and cultivate antagonistic bacteria capable of effectively suppressing muskmelon diseases and to formulate these into a microbial consortium for disease management in greenhouse muskmelons. This approach is designed to enhance the growth and disease resilience of the muskmelon crops.

Materials and Methods

Four fungal pathogens ie *Sphaerotheca fuliginea, Fusarium oxysporum, Phytophthora melonis* and *Pseudoperonospora cubensis* of muskmelons were obtained from Shaanxi Institute of Microbiology.They were cultured on PDA medium and stored in a 4℃ refrigerator. Following the dilution plate method described by Ali *et al.* (2022), the bacteria against the pathogens were screened out by plate confrontation method (Bu *et al.* 2021). The morphological identification of the bacterial strain with the strongest antagonistic ability was carried out according to Al-Daghari (2020), and its physiological and biochemical identification was made with reference to microbiological experimental standard (Yang *et al.* 2022).

Referring to previous methods (Hernandez *et al,* 2021), the kits provided by Shanghai Biotech Co., Ltd. were used to extract DNA of the strain screened out and recover its 16SrDNA fragment (Hernandez Montiel *et al.* 2021). Universal primers synthesized by Shanghai Biotech Co., Ltd. (67F:5'-AGAGTTGTCATGGCTC-3' and 1492R: 5'-TACGGYTACCTTGTTACGAC TT-3') were used for PCR amplification of 16SrDNA. PCR reaction conditions are (95℃ for 3 min, 95℃ for 50 sec, 52℃ for 50 see, 72℃ for 2 min, 30 cycles, 72℃ for another 10 min). After purification of the PCR products obtained, the target fragment was submitted to Shanghai Biotech Co., Ltd. for gene sequencing. The sequence obtained was also provided with a phylogenetic tree constructed according to the 16SrDNA sequence of similar species on NCBI via MEGA 5.0 (Sui *et al*. 2020).

After slant culture of the selected strain for 24 hours, 5mL sterile normal saline was used to wash the slant and then poured into the conical bottle containing glass beads, which was shaken vortically for 30 minutes. The photoelectric turbidimetric counting method (Phoka *et al.* 2020) was used to adjust the OD value of the bacterial suspension to 0.986 so that the concentration reached 10^8 cfu/mL.

Under aseptic conditions, 2 mL of the above bacterial suspension was taken and added to a sterilized test tube. After that, the output power of the He-Ne laser was adjusted to the sterile mode (Veloso *et al*. 2020). And then, 2 mLTG6 bacterial suspension was taken and poured into sterilized test tube before adjusting the output power of the He-Ne laser to 9 mW. At this time, both the two test tubes were placed 25 cm away from the laser and exposed to it for 5 minutes, 10 minutes, 15 minutes, 20 minutes and 25 minutes respectively. With the original bacterial suspension as the control, the experiment was repeated for three times.

Under aseptic conditions, 1 mL of laser-irradiated bacterial suspension was taken and placed in a test tube containing 9mL of aseptic water as 10^{-1}. After shaking and mixing, it was diluted in

the same way to make it 10^{-5}, 10^{-6}, 10^{-7}. And then 0.2 mL of diluents at the three gradients were applied on a flat plate, with three parallel diluents for each. After culture at 37℃ for 20 hours, colony morphology was observed and colony count was carried out with the original bacterial suspension as the control. The previously selected mutant strains were activated and transferred to NA medium. And medium confrontation experiments were performed on the strains every 10 generations to analyze their antagonistic ability against pathogens and test their genetic stability. If there was no significant change in resistance to pathogens after passage, it indicated that the strain had good genetic stability

At 35℃, the selected mutants were added into the liquid medium with 2% inoculation quantity, and the high-concentration bacterial suspension was obtained after shake culture for 72 hours on a shaking table (150 rpm). The high-concentration bacterial suspensions of all mutant strains after expanded culture were evenly mixed in a certain proportion to prepare the compound bacterial antagonist. Then, all kinds of bacteria that can efficiently transform apple residues to produce biochemical fulvic acid bacterial manure bred through selection by the laboratory in the early stage, and the liquid biochemical fulvic acid was produced by fermentation (Sajeena *et al.* 2020, Ruangwong *et al*. 2021). The complex microbial inoculants obtained in the former steps were composited with fluvic acid, thus, the biochemical fulvic acid microbial compound bacterial manure for greenhouse muskmelons was developed, which was diluted 100-500 times before application.

In this experiment, muskmelons from adjacent greenhouses with normal use of chemical manures and pesticides were used as the control. During the planting period, the relative content of chlorophyll in leaves of muskmelons were measured by chlorophyll meter in every 7 days. The effective antagonistic bacteria on leaf surface of muskmelons and the active number of beneficial microorganisms in the rhizosphere soil of muskmelons were detected by dilution-plate method (Gilardi *et al*, 2020, El-Mougy and Abdel Kader 2019). And then the yield per mu was counted and the production cost was calculated. After that, morphology of leaves and fruits of the experimental group and the control group were observed, and the ripe muskmelons were submitted for inspection to detect their sugar content (Shi *et al*. 2022).

Results and Discussion

Six strains of *Bacillus subtilis* with good morphology were obtained through dilation plate isolation, and the antagonistic ability of each strain against the four pathogens of muskmelons was tested by plate confrontation method (Janga *et al*. 2017).

Based on the antagonistic ability of the selected bacteria on muskmelon pathogens, the TG6 antagonistic bacteria with the strongest inhibitory effect (Table 1), was selected for follow-up studies.

Table 1. Inhibition activity of antagonistic bacteria against test pathogens.

Strains of *B. subslitis*	*Sphaerotheca fuliginea*	*Fusarium oxysporum*	*Pytophthora melonis Katsura*	*Pseudoperonospora cubensis*
TG61	8.2 ± 0.36	9.4 ± 0.28	8.9 ± 0.25	8.3 ± 0.18
TG63	8.1 ± 0.42	7.6 ± 0.41	7.2 ± 0.24	8.5 ± 0.32
TG65	6.7 ± 0.41	6.3 ± 0.40	6.6 ± 0.31	5.8 ± 0.86
TG66	7.6 ± 0.23	6.4 ± 0.31	6.7 ± 0.32	6.8 ± 0.16
TG67	12.9 ± 0.56	13.8 ± 0.12	11.2 ± 0.36	11.9 ± 0.42
TG69	11.5 ± 0.32	11.1 ± 0.68	12.2 ± 0.79	10.8 ± 0.68

TG6 bacterium was rod-shaped, gram-positive with spore in oval shape with an enlarged sac. The colony was dry, non-transparent and pleated, with irregular diffusion at the edges. TG6 was a strictly aerobic bacterium, resistant to sodium chloride at 2, 5, 7 and 10%, and can produce acid by glucose fermentation. IMViC test amylolysis and casein hydrolysis test results were positive.

Based on the phenotypic traits and physiological and biochemical characteristics, TG6 was consistent with the standard *Bacillus subtilis* strain. Consequently, the TG6 strain was preliminarily identified as a *Bacillus* sp.

The PCR amplification results of 16S rDNA fragment of TG6 strain were about 1, 500 bp. The PCR products were submitted to the company for sequencing, and sequence about 1,471 bp was obtained. Then, the sequence was compared to the 16SrDNA sequence closely relative bacteria in the database of NCBI. Bioedit7.0 was used for multiple comparisons to analyze its homology and construct the phylogenetic tree (Fig. 1). The obtained sequence showed 99.99% homology with B. *subtilis*. According to its phylogenetic characteristics, the TG6 strain was identified as *Bacillus subtilis*.

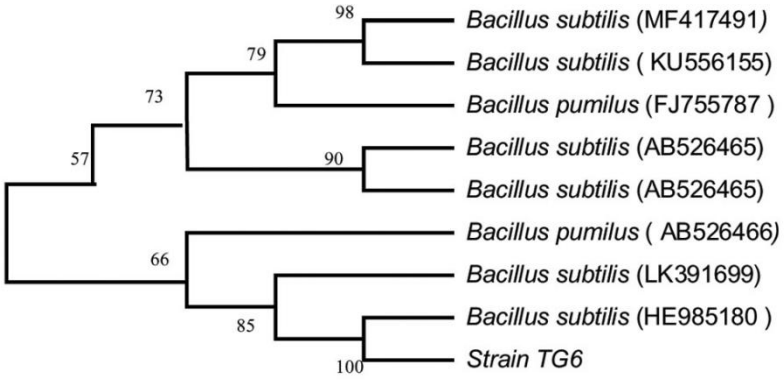

Fig. 1. Phylogenetic tree of strain TG6.

Six mutant strains TG61, TG63, TG65, TG66, TG67 and TG69 with larger colony and faster growth rate were selected for antagonism against *Sphaerotheca fuliginea*, *Fusarium oxysporum*, *Pytophthora melonis* and *Pseudoperonospora cubensis*. StrainTG67 and TG69 showed remarkable antagonistic effect against the pathogens (Table 1), Therefore, TG67 and TG69 mutant was screened for the following experiments.

Subculturing of TG67 and TG 69 mutant was conducted for 50 generations, and confrontation antagonism tests were conducted on the strains every 10 generations. The results showed that all the generations of strains have similar antagonistic effect, indicating a good genetic stability of TG67 and TG69 mutant strains (Table 2).

After the fermentation, the fermentation products of the two mutant strains TG67 and TG69 were mixed in the proportion of 1:1. The biochemical fulvic acid which prepared in our laboratory before (Fu *et al.* 2015) were mixed with the mixture of mutant TG67 and TG69, so as to prepare the microbial antagonistic fertilizer. The microbial antagonistic fertilizer can not only effectively inhibit the growth of pathogens, but also significantly improve soil fertility as well as disease resistance of muskmelon.

The composited compound biochemical fulvic acid microbial manure was applied on greenhouse muskmelons. The results showed that the average chlorophyll content of the leaves of muskmelons rose from 45 to 62. The number of viable beneficial bacteria in the soil increased

from 10^7/g to 10^{12}/g, the leaves were obviously thicker, larger and greener, and the fruit grew vigorously with nearly the same size, the surface was smooth and spot-free, the flesh was in golden color and with high sweetness.

Table 2. Hereditary stability of bacteria mutant TG67 and TG69.

Strain NO.	Generation	Inhibition zone diameter (mm)			
		Sphaerotheca fuliginea	*Fusarium oxysporum*	*Phytophthora melonis Katsura*	*Pseudoperonospora cubensis*
TG67	1	12.9 ± 0.42	13.8 ± 0.16	11.2 ± 0.38	11.9 ± 0.52
	10	12.9 ± 0.36	13.8 ± 0.22	11.2 ± 0.29	11.9 ± 0.50
	20	12.9 ± 0.31	13.8 ± 0.36	11.2 ± 0.32	11.9 ± 0.47
	30	12.9 ± 0.29	13.8 ± 0.35	11.2 ± 0.41	11.9 ± 0.42
	40	12.9 ± 0.30	13.8 ± 0.37	11.2 ± 0.46	11.9 ± 0.40
	50	12.9 ± 0.43	13.8 ± 0.46	11.2 ± 0.53	11.9 ± 0.36
TG69	1	11.5 ± 0.28	11.1 ± 0.60	12.2 ± 0.81	10.8 ± 0.56
	10	11.5 ± 0.30	11.1 ± 0.52	12.2 ± 0.73	10.8 ± 0.49
	20	11.5 ± 0.24	11.1 ± 0.46	12.2 ± 0.66	10.8 ± 0.38
	30	11.5 ± 0.19	11.1 ± 0.42	12.2 ± 0.52	10.8 ± 0.29
	40	11.5 ± 0.31	11.1 ± 0.36	12.2 ± 0.47	10.8 ± 0.26
	50	11.5 ± 0.28	11.1 ± 0.29	12.2 ± 0.36	10.8 ± 0.31

In this study, strain TG6 was isolated from the soil of a muskmelon greenhouse. It demonstrated effective inhibition of *Sphaerotheca fuliginea*, *Fusarium oxysporum, Pytophthora melonis Katsura* and *Pseudoperonospora cubensis* in muskmelons. Through morphological, physiological, biochemical, and molecular analysis, TG6 was identified as *Bacillus subtilis*. Derivative strains TG67 and TG69 were then developed from TG6 using He-Ne laser treatment.

The obtained mutant strains 67 and 68 were combined with biochemical fulvic acid to prepare antagonistic microbial fertilizer. The obtained microbial antagonistic fertilizer was applied to the field experiment of biological control of muskmelon in greenhouse.The results of field experiments showed that the microbial antagonistic fertilizer could effectively control the occurrence of various diseases of muskmelon in greenhouse.

Studies have shown that different biocontrol agent have significant differences in promoting plant growth and preventing fungal diseases. *Bacillus* can be induced by the release of antifungal metabolites (Compant *et al.* 2005, Esmaeel *et al.* 2018), volatile organic compounds, parasitism or inhibition of the growth of pathogenic bacteria through lysozyme. Some studies have shown that cellulase secreted by antagonistic bacteria can promote the cracking of fungal mycelium body wall, thus achieving the prevention and control of plant diseases (Richter *et al.* 2011, Liu *et al.* 2019).

In conclusion, the antagonistic strains of muskmelon were selected by laser mutagenesis technology and prepared into microbial compound antagonistic agents. On this basis, the compound bacterial agent and the biochemical fulvic acid compound bacterial fertilizer developed in the laboratory were prepared to prepare the compound bacterial fertilizer. Field experiment results showed that the compound bacterial fertilizer could not only effectively prevent various

diseases of greenhouse muskmelon, but also promote the growth of muskmelon and increase the sugar content of muskmelon. This study can significantly prevent the fungal disease in melon and reduce the latent capacity of pathogens in the field before harvest, which shows high social and economic benefits

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