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IDENTIFICATION OF HE-NE LASER BASED MUTATION INDUCED BACTERIA AGAINST GRAPE DISEASE

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Abstract

In the present study six bacterial strains from the soil of grape orchard were isolated and identified. Their antagonistic ability against *Uncinula necator* and *Plasmopara viticola* which cause downy mildew and powdery mildew of grapes, respectively were studied. Through phenotypic, physiological and biochemical studies, SF6 strain was identified as *Bacillus subtilis* which can effectively inhibit the growth of *Uncinula necator* and *Plasmopara viticola* of grapes. In order to enhance the antagonistic ability of the SF6 strain, mutation was induced by He-Ne laser radiation and, six mutant strains were obtained. Among the six mutant strains, SF66 showed the strongest antagonistic activity to *Uncinula necator* and *Plasmopara viticola* of grapes with its genetic stability. The finding of this research provides important impetus for the development of beneficial antagonistic microorganisms through He-Ne laser radiation breeding.

Introduction

Owing to the unique flavor, rich nutritional and healthcare value, grapes have become one of the fruits industries that all the countries around the world are now competing to develop (Chen *et al.* 2018). In recent years, with the continuous expansion of cultivated grape area, the harm of grape diseases has been aggravated year by year, some of diseases have caused disasters, which seriously affects grape quality and hinders further development of grade industry. Main pathogenic bacteria causing grape diseases are *Plasmopara viticola* and *Uncinula necator*. The former infects the green parts of grape vines, especially the young tissues, while the later could cause severe grape defoliation, causing serious harm to grape vines (Cristobal *et al.* 2018).

Biocontrol agents are of strategic significance for its contribution in establishment of green organic grape production system in a safe, non-toxic and high-efficient and economical way (Deng *et al.* 2020). In the past twenty years, a great variety of control strategies have sprung up in biocontrol of plant pathogens. And some antagonistic bacteria, such as *Agrobacterium*, *Bacillus* and *Pseudomonas*, have been regarded as beneficial in crop protection. Isolation and screening of antagonistic microorganism play an essential role in application of biological control. *Pseudomonas* has been using for a long time, and in recent years, the application of *Bacillus* has been given a lot of attention, especially, application of *Bacillus subtilis* has aroused great interest (Raj *et al.* 2019, Thankaraj *et al*. 2019).

In the meantime, breeding of antagonist has attracted great attention of many scientists. Modern genetic engineering and metabolic engineering techniques have been regarded as the effective approaches to microbial breeding, however, application of such modern techniques always run into various obstacles, may be because of the limited knowledge in physiological genomics and biological chemistry (Boso *et al.* 2019). As a result, traditional breeding methods are still the effective approaches to obtain improved strains. Low-power laser radiation technology

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has drawn much attention in the microbial mutation breeding sector, and has been used in screening of phenol-degrading bacteria (Pons *et al*. 2018). Nevertheless, there is a few reports on breeding of antagonist to grape diseases and insect pests by low-power He-Ne laser mutation.

This research was undertaken with an aim to provide important impetus for the development of beneficial antagonistic microorganisms through He-Ne laser radiation breeding.

Materials and Methods

The *Uncinula necator* and *Plasmopara viticola* of grapes are offered by Microbiology Institute of Shaanxi, which were grown on PDA plate stored at 30°C.

The T3 He-Ne laser generator (wavelength of 632nm, power of 10mW) with a light spot of 1.5mm is provided by Northwestern University Photoelectric Equipment Plant.

The strain was isolated from infected grape orchard soil in Weinan, Shaanxi province. Single collarium colony selected from all the monoclonal plates were inoculated to other fresh plates to check its purity. When the monoclonal bodies, the antagonism to pathogens was tested *in vitro*.

The inhibition zone method was used for preliminary screening. With indicator bacteria of *Uncinula necator* and *Plasmopara viticola* of grapes, respectively, the antagonist ability was tested by measurement of the antimicrobial spectrum formed by all strains. The specific operations were as follows: pour plate method was used to prepare pathogen plates, and the bacteria to be tested was inoculated by spot inoculation method onto the cooled and solidified pathogen plates, and after cultivation for 48 hrs at constant temperature of 28 °C, inhibition condition and diameter of inhibition zone was observed. Such experiment was repeated three times for each group. Finally, according to diameter of the inhibition zone, the strains with the greatest inhibitory activity were selected and kept for later use.

The Oxford Cup method was used in secondary screening to test the antagonistic ability of bacterial fermentation liquor. The antagonist ability was measured based on the record. Gram staining, spore staining and bacteria sizing were carried out with reference to microbiological experiment, Bergey's manual of bacterial identification and common bacterial systematic identification manual.

Physiological and biochemical identification with reference to microbiological experiment standards (Fu *et al*. 2015), catalase reaction, sugar or alcohol fermentation experiment, VP experiment, amylolysis experiment, citrate utilization test, casein hydrolysis, nitrate reduction test, salt tolerance test, growth in NA culture medium with pH 5.8, cellulose decomposition experiment, hydrogen sulfide production experiment and indole production experiment were conducted, respectively.

Extraction of total bacterial DNA, recovery of 16S rDNA fragment and extraction of *E. coli* plasmid was performed with corresponding kits (provided by Tiangen Biotech Co., Ltd.). , The 16S rDNA sequence was amplified, sequenced and analyzed following Boso *et al.* (2019) with a pair of universal primers 27F (5'—AGAGTTG TCATGGCTC—3') and 1492R (5'—TACGGYTACCTTGTTACGACTT—3'). The primers were synthesized by Shanghai Biotech Co., Ltd. Nucleotide samples were synthesized into 1500bp templates of 16S rDNA and PCR mixture reaction system (30 μl), 1 μl primer, 15 μl pfu PCR Master Mix, 13 μl double steam water. The PCR conditions were 95℃ for 3 mins, 95℃ for 50 sec., 52℃ for 50 sec, 72℃ for 2 mins, 30 cycles, 72℃ for another 10 minutes. 10μl of the amplified PCR products was taken and added to the agarose gel with 1.5% mass fraction for electrophoretic analysis, and then ethidium bromide with a final concentration of 0.5μg/ml was added into the gel. After electrophoretic mobility at 70 voltage for 30 min, the gel was placed in the gel imaging system for digital scanning. The Gel containing the target fragment was then cut off with a surgical blade under UV

lamp, purified and extracted with the purification kit (UNIQ-10 Column DNA Gel Extraction Kit) of Shanghai Biotech Co., Ltd. PGM-T carrier was used to connect and introduced into the *E. coli* DH5α. After culture, white clones were selected and tested by electrophoresis and amplification primer, and then, the target fragment was submitted to the automatic sequencer of Shanghai Biotech Co., Ltd. for sequencing.

The strains slants cultured for 30h were taken, and then 5ml sterile normal saline was used to clean the bacterial lawns respectively and poured into a conical bottle containing glass beads. After shaking the bottle for 30 mins on a cyclotron oscillator, the OD600 value was adjusted to 0.986 by photoelectric turbidimetric counting method, so that the concentration of bacterial suspension became 10^8 CFU/ml.

Under aseptic conditions, 2ml SF6 bacterial suspension was taken and placed in aseptic test tubes respectively, and the output power of He-Ne laser was adjusted to 9mW. The test tubes were then exposed to He-Ne laser 25cm away for 5, 10, 15, 20, and 25 mins respectively. The original bacterial suspension was used as the experimental control. the above experiment was repeated 3 times.

The new colonies were inoculated onto the original bacterial plate, and the inhibition zone method was used to test the antagonist ability. The survival rate and positive mutation rate were calculated following equations:

(i) Survival rate % = Viable bacteria count after mutation/ viable bacteria count before mutation *100%

(ii) Positive mutation rate% = Number of positively mutated strains/ viable bacterial count after mutation *100%

After the mutation, six mutant strains (SF1, SF2, SF3, SF6, SF7, SF9) were obtained and transferred onto NA medium for 1 day, the output bacteria were regarded as the first generation. Thereafter, 50 generations were cultured in the same way. Thus, the genetic stability of mutant strains was determined according to change of the antagonistic ability of the strains.

Results and Discussion

Six bacterial strains were isolated from grape orchard soil. With the target of finding out antagonist of *Uncinula necator* and *Plasmopara viticola* of grapes, inhibition zone method was applied for primary screening of the six strains and measurement of the inhibition zone size (Table 1). Results indicate that antagonistic effects of the six strains against the two pathogens show obvious differences. For example, SF6 and SF3 strains have obvious antagonistic effect on the two pathogens, while the antagonistic effect of the other four strains varies greatly. For instance, SF1 strain showed strong inhibitory effect on the pathogen of grape powdery mildew, while its antagonistic effect on the pathogen of grape downy mildew was relatively poor.

Table 1. The antagonistic effect of the six strains' cell.

	Diameter of Inhibition zone (mm)	
Antagonistic bacteria	*Uncinula necator*	*Plasmopara viticola*
SF1	3.7 ± 0.22	1.4 ± 0.43
SF2	2.7 ± 0.40	2.4 ± 0.66
SF3	2.4 ± 0.36	2.1 ± 0.15
SF4	1.8 ± 0.35	1.7 ± 0.34
SF5	4.3 ± 0.29	4.1 ± 0.23
SF6	4.5 ± 0.18	4.3 ± 0.12

After preparation of sterile fermentation liquor for the six strains, and with the target of *Uncinula necator* and *Plasmopara viticola* of grapes, the Oxford Cup method was used for a second screening, with the emphasis on verification of the antagonistic ability of SF5 and SF6 strains which showed strongest inhibition effect in the preliminary screening (Table 2). Results indicate that antagonistic ability of the fermentation filtrate of the six strains decline to different extents, compared with bacterial cells, but SF6 is still with the strongest antagonistic ability. The diameter of the inhibition zone in SF6 fermentation filtrate only decreases slightly compared to that in the preliminary screening, which means its sufficient extracellular inhibition substances and stable antagonistic ability. On this basis, the SF6 strain with the strongest antagonistic ability was selected for follow-up studies of biocontrol agents.

Table 2. The antagonistic effect of the six strains' fermentation broth.

	Diameter of Inhibition zone(mm)	
Antagonistic bacteria	*Uncinula necator*	*Plasmopara viticola*
SF1	2.9 ± 0.26	0.9 ± 0.12
SF2	1.9 ± 0.23	1.9 ± 0.12
SF3	1.6 ± 0.13	1.3 ± 0.26
SF4	1.9 ± 0.28	2.1 ± 0.12
SF5	2.6 ± 0.36	2.5 ± 0.31
SF6	4.3 ± 0.30	3.1 ± 0.12

According to individual and colony morphological observations of SF6 antagonist bacteria, the young culture was rod-shaped, gram-positive, and could produce spores in oval shape and with enlarged spore sac (1.1×0.9μm). The colony was dry and pleated, non-transparent with irregular diffusion at edges. Judging from these morphological characteristics, SF6 might belong to *Bacillus* (Calvo *et al*. 2019).

Based on the physiological and biochemical indexes of SF6, it falls to the category of strictly aerobic bacteria, and can grow in broth medium at pH 5.7 with sodium chloride resistance of 3, 6, 9 and 12%. In addition, it can ferment a variety of carbohydrates including glucose (which only produces acid) and mannitol, but not indoles and hydrogen sulfide. It also showed following positive reactions: starch and casein hydrolysis, gelatin liquefaction, VP experiment (pH 7.79), citrate utilization, catalase and oxidase production, physiological and biochemical characteristics were the same as those of a standard strain of *Bacillus subtilis*. According to the morphological and physiological and biochemical characteristics, the antagonist SF6 could be basically identified as *Bacillus* sp. (Wang *et al*. 2021).

The amplified PCR product of 16S rDNA fragments of SF6 showed about 1499 bp (Fig. 1). PCR products were purified and transferred into *Escherichia coli*. LB medium containing 100 μg/ml ampicillin was used to selected white colonies, the target fragment was then submitted to DNA sequencing analysis through electrophoresis and amplification with primers. The 1489 base sequence of 16SrDNA in SF6 could be obtained from GeneBank. A BLAST sequence similarity analysis was performed on 16S rDNA sequence with that of similar species in the GeneBank, and the results showed that SF6 strain share 100% homology with *Bacillus subtilis* NRRL NRS-744.

The phylogenetic tree was constructed by N-J method. According to the phylogenetic characteristics, the antagonist SF6 can be identified as *Bacillus subtilis* (Nartey *et al*. 2021).

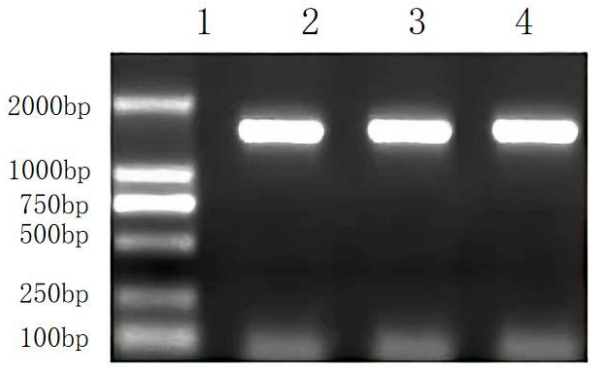

Fig. 1. Gel electrophoresis of PCR amplification for 16SrDNA of strain SF6.

Results of antagonist bacteria SF6 exposed to laser for different time durations. With the increase of exposure duration, the death rate of SF6 strain increased, and the rate of positive mutation also went up, however, when it rises to a certain extent, the rate of positive mutation starts to decline (Table 3). It also showed that the dosage of exposure of laser radiation for 15 minutes was the optimal.

Table 3. The effect of different exposure time of laser radiation on SF6.

Time (min)	5	10	15	20	**25**
Survival rate (%)	58.6	43	29.9	16.1	7.9
Variable ratio (%)	11.2	18.8	24	17.9	14.9

By the inhibition zone method, the six SF6 mutant strains which were bigger and grew faster, and with the antagonistic ability stronger than that of the parent SF6 strain were selected, and respectively numbered SF61, SF62, SF63, SF66, SF67 and SF69. Then, confrontation growth method was used to test their ability against grape pathogen of downy mildew and powdery mildew. The antagonism distance was regarded as the measurement standard, and the degree of change in which was detected by T test. SPSS13.0 software was used for data analysis. The parent SF6 was used as the control group. The specific statistics and analysis results are given in Tables 4 and 5. As is shown, compared to the control group, the six mutants showed different significance against the same pathogen. Just as indicated by Table 4, for grape pathogen of powdery mildew, there was a significant difference in the antagonistic ability of mutant SF62, SF66 and SF69 compared to the parent SF6, and according to the average value, SF66 had longer inhibitory belt. For grape pathogen of downy mildew, there was a significant difference in the antagonistic ability of mutant SF66 and SF69, compared to the parent SF6, and based on the average value, mutant SF66 is still with the widest inhibitory belt (Table 5). On these grounds, SF66 was selected for follow-up studies after taking integrated consideration of the antagonistic activity.

Table 4. The antagonistic distance against *Uncinula necator* and descriptive statistics using One-sample t-test.

Strains	Test Value (control parent)= 4.4(mm)						
	Width of Inhibition belt (mm)			Mean value	Std. Deviation	t	Sig.(2-tailed)
SF61	4.39	4.79	4.58	4.59	0.2001	0.877	0.493
SF62	4.89	5.09	4.79	4.92	0.1531	4.899	0.041
SF63	4.59	4.49	4.79	4.62	0.1499	1.499	0.269
SF66	5.09	4.99	5.19	5.09	0.0008	10.401	0.008
SF67	4.69	4.89	4.59	4.72	0.1598	2.639	0.109
SF69	5.19	5.59	5.39	5.39	0.2000	7.801	0.019

Table 5. The antagonistic distance against *Plasmopara viticola* and descriptive statistics using One-sample t-test.

Strains	Test Value (control parent)= 4.2(mm)						
	Width of Inhibition belt (mm)			Mean value	Std. Deviation	t	Sig.(2-tailed)
SF61	4.39	4.69	4.09	4.39	0.2999	0.499	0.587
SF62	4.39	4.49	4.69	4.52	0.1519	2.598	0.099
SF63	4.39	4.69	4.29	4.46	0.2079	1.379	0.300
SF66	4.89	4.99	5.29	5.06	0.2078	6.368	0.019
SF67	4.49	4.79	4.59	4.62	0.1519	3.779	0.058
SF69	4.79	4.70	4.89	4.82	0.0568	15.999	0.005

By the Oxford Cup method, the antagonistic ability of SF66 fermentation liquor showed significantly greater antagonistic ability against grape pathogen of downy mildew compared to the parent SF6 (Table 6).

Table 6. The antagonistic effect of the fermentation broth of the mutant and parent strain against the two pathogens *in vitro.*

Strain	Pathogen	
	Uncinula necator	*Plasmopara viticola*
SF6	+++	++
SF66	+++	+++

Note: + indicates that the diameter of inhibition zone is 1.5mm; ++ indicates that the diameter of inhibition zone is 3mm; +++ indicates that the diameter of inhibition zone is 4.5mm.

After culturing 50 generations of mutant SF66 with the significantly improved antagonist ability, confrontation antagonism experiment was performed on each generation of strains. Results showed that the antagonistic ability of all generations of mutants were basically the same, with slight change in the number of colonies, indicating a good genetic stability of SF66 mutant in antagonistic ability (de Azevedo *et al*. 2021, Beerahassan *et al*. 2021).

This study focuses on the common pathogens in grape production. Firstly, six strains resistant to grape diseases were isolated from grape orchard soil. After a preliminary and second screening, SF6 strain with the strongest antagonistic ability against powdery mildew and downy mildew of grapes was obtained. Through a series of identification experiments, including colonial and cell characteristics observation, 16S rDNA sequence analysis and comparison, SF6 was confirmed to be *Bacillus subtilis* based on the comprehensive evaluation of the above experiments. Owing to its strong antagonistic ability, continuous secondary metabolite production system and produce secondary metabolite with different structures; *Bacillus* sp. has been widely applied in biological control of fruit and vegetable diseases (Di *et al*. 2021). Moreover, it could produce endospores to enhance heat resistance and drought resistance (Naseri and Younesi 2021).

In order to enhance the antagonistic ability of the biological control strain, a mutation breeding method was applied on the SF6 strain. Mutation by He-Ne laser is a high-efficient technology in mutation breeding, which is characterized with energy density, small targets, good monochromaticity, and directionality. After being exposed to He-Ne laser, SF6 strain changes in its colonial morphology but also decrease in the number of colonies, which indicates laser exposure has the lethal and mutagenic effects on SF6 strain. The strain itself and fermentation liquor of the mutant strain SF66, which was screened out from the mutants with significantly improved antagonistic ability. Based on a comparative analysis of the antagonistic effects of the 50 generations, it was found that mutant strain SF66 has good genetic stability in its antagonistic ability, which is worthy of further studies.

The parent strain is with strong ability against pathogen of grape downy mildew but weak ability against grape powdery mildew, but after mutation, both the ability against grape downy mildew and antagonistic activity of the bacterial fermentation liquor were significantly improved. It is speculated that the mutagenesis may lead to the gene mutation of regulating enzymes in a metabolic process, which improves antibiotic yield thereby. Of course, further experiments are needed for verification.

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