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Bacillus megaterium BMJBN02 induces the resistance of grapevine against downy mildew

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Summary

Grape downy mildew caused by *Plasmopara viticola* is one of the most destructive diseases of grapes. All grape cultivars are susceptible to *P. viticola*. However, the resistance of grape plants could be induced in plant defense with some help of microbes. In this study, *Bacillus megaterium* BMJBN02 obtained from farmland soil was shown to regulate the resistance of grapevine against downy mildew. The salicylic acid (SA) content and the expression of pathogenesis-related (PR) genes of grapes under different treatments were examined using high-performance liquid chromatography-mass spectrometry (HPLC-MS) and reverse transcription-quantitative polymerase chain reaction (RT-qPCR), and it was found that SA content and the expression of PR genes could play a role in regulating the resistance of grapevine against downy mildew. The five-year plot experiment showed that the resistance effectiveness of isolate BMJBN02 was approximately equal to that of 0.1 % nicotinyl morpholine (commercial fungicide). Therefore, this study provides a valuable candidate method that uses *B. megaterium* BMJBN02 by regulating the resistance of grape against downy mildew for quality and yield of grape in commercial productivity.

K e y w o r d s : grape downy mildew; *Plasmopara viticola*; *Bacillus megaterium*; induced systemic resistance; salicylic acid; pathogenesis-related genes.

Introduction

Grape downy mildew (GDM), caused by *Plasmopara viticola* (*P. viticola*), leads to a reduction in the yield and quality of berries (Billet *et al*. 2019), and efficient control measures are not yet available. *Plasmopora viticola* is a obligate biotrophic oomycete (Wong *et al*. 2001). Hitherto, the common methods for controlling this disease have included the usage of pesticides and the breeding of resistant varieties (Zhang *et al*. 2017). Breeding resistant varieties is a most effective strategy. However, as the cycle of breeding resistant varieties may require time-consuming and costly research, an alternative highly efficient and bio-safe method of controlling grape downy mildew is required.

Rhizobacteria have previously been reported as commercial bioagents against pathogenic diseases (Mahanty *et al*. 2017). According to their resistance mechanisms, there are two strategies: one is to produce antibiotics and cell wall-degrading enzymes against pathogens (Guo *et al*. 2004, Ramesh *et al*. 2009); the other is to induce systemic resistance of host plant defences against pathogens (Deslandes *et al*. 2002). The diversity of rhizobacteria with multiple resistant mechanisms offers possibilities for the biological control of grape downy mildew disease.

In this study, we screened rhizobacterial isolates from samples of farmland soil. The varying resistance of leaf discs of grape plants were treated with these different rhizobacteria isolates to aquire the most efficient isolate against pathogen *P. viticola*. Under different treatments by this efficient isolate against *P. viticola*, the SA content and the expression of *PR* genes, which are most important in grapevine immunity, were detected. In order to determine the efficiency of this isolate, a plot experiment of this isolate was conducted in field application. This study provides an overview of underlying mechanism of resistance mediated by rhizobacteria isolate BMJBN02 against downy mildew, and it provides valuable insights into candidate methods for protecting the quality and production of grapes.

Material and Methods

G r a p e c u l t i v a r : Four-year-old *Vitis vinifera* L. 'Muscat' (grafted onto the rootstock of *V. riparia* × *V. labrusca* 'Beta'), a highly sensitive cultivar, was used in the plot experiment. Aseptic seedlings of 'Muscat' (on own roots) were used in detecting the SA content and the expression of *PR* genes.

P a t h o g e n : *Plasmopara viticola* (Berk et Curt.) Burl. & de Toni was cultivated on the highly sensitive grape cultivar 'Muscat' in a culture incubator, at room temperature of 25 ± 1 °C. This pathogen was obtained from the Plant Protection Institute, Shandong Academy of Agricultural Sciences. Soil samples were collected from the

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soil of leek or cucumber farmland near Shouguang City, Shandong Province, China.

Isolation of rhizobacterium: One gram of the soil sample was added to 100 mL of broth medium and incubated at 37 °C on a rotator shaker (200 rpm) for 2 d. The 0.1 mL suspension of 10^{-6} dilution in sterile 0.90 % NaCl solution was transferred to Luria-Bertani (LB) screening medium and incubated at 37 °C for 2 d. The colonies were isolated and purified by streaking on LB agar plates and stored at 4 °C for further analysis.

Screening of bio-control bacterial isolates: Diseased leaves with the symptoms of downy mildew were washed in flow water which has been sterilized. Centrifugation with 2000 g for 7 min was repeated 3 times. In this study, the sporangia of *P. viticola* were removed of hyphae by the filtration of skim cotton as filter and then diluted with sterile water to create a sporangial suspension with concentration of 1.0×10^5 mL^{-1} using a Neubauer chamber for subsequent tests. The concentration of bacterial isolate whose cells were centrifuged and diluted with sterile water to create a suspension was 1×10^8 CFU·mL^{-1} for subsequent detections.

The leaf disc method (He, 1999) was used to screen efficient bio-control bacterial isolates. Fresh leaves from 'Muscat' were sampled using a hole-puncher. One plate of leaf discs floated on the surface of 1000 µL distilled water were used as a negative control (NC); another plate of leaf discs floated on the surface of bacterial isolate (concentration of 1.0×10^7 CFU·mL^{-1} per bacterial isolate, 1000 µL) was served as test group; a third plate of leaf discs floated on the surface of 1000 µL Bordeaux mixture (2 % copper sulphate: 2 % calcium hydroxide = 9:1 (Volume/Volume) served as positive control (PC). Each treatment included three repetitions. All leaf discs were inoculated with the sporangial suspension of *P. viticola* at 40 µL on the other side of per leaf disc. After 7 d post-inoculation, each leaf disc was observed and classified by standards of disease condition and disease level according to He's method (HE 1999).

Identification of effective bio-control bacterial isolate: Cells grown on LB agar medium were examined for their morphological and cultural characteristics (HARRIGAN and MCCANCE 1966). Sequencing of the 16S rRNA gene was performed using primer 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and primer 1492R (5'-TACGGYTACCTTGTTACGACTT-3'). The PCR product was purified and sequenced as described previously (TANASUPAWAT *et al*. 2004). For sequence analysis of the 16S rDNA, sequence homologies were examined by comparing the sequences obtained with those of the GenBank database using BLAST-N software and was identified according to the closest relative. The isolates were also identified using phylogenetic tree analysis by Neighbor-joining (NJ) method for estimating evolutionary distances between all pairs of sequences simultaneously in software MEGA version 4.0 (Windows 7.0 version).

Extraction and analysis of Salicylic acid (SA): Aseptic seedlings of 'Muscat' (on own roots) were acquired by the method of grape tissue culture (TORREGROSA *et al*. 2002) for the extraction and analysis of SA. In this study, the aseptic seedlings have been prepared *in vitro* by sterilization of grapevine, and then been moved in pots with sterile soil for the research, which have been subsequently sprayed by the suspensions. The aseptic seedlings were used in four treatments the plant light incubator, 25 °C, relative humidity 60 %: (1) 'Control': aseptic seedlings were sprayed with sterile water; (2) '*P. viticola*': aseptic seedlings were sprayed with suspension of pathogen *P. viticola*; (3) 'BMJBN02': aseptic seedlings were sprayed with suspension of isolate BMJBN02; (4) 'preBMJBN02- *P. viticola*': aseptic seedlings were sprayed with suspension of isolate BMJBN02 and inoculated with suspension of pathogen *P. viticola* 4 h later. All treatments were run in triplicate. Extraction of SA from the treated seedlings was performed according to the method of PAN *et al*. (2010). Fifty microliters of the sample solution were injected into a reversephase C18 column for HPLC-ESI-MS/MS analysis. Separation and analysis of SA from the grape samples was performed according to the procedures (suppl. Tab. 1) described by the methods of Liu (LIU *et al*. 2016, LIU *et al*. 2013).

RT-qPCR and Pathogenesis-related (*PR*) gene analysis: Aseptic seedlings of 'Muscat' (on own roots) were acquired by the method of grape tissue culture (TORREGROSA *et al*. 2002) for the detection of *PR* genes. In this study, the aseptic seedlings have been prepared *in vitro* by sterilization of grapevine, and then been moved in pots with sterile soil for the research, which have been subsequently sprayed by the suspensions. The aseptic seedlings were used in four treatments in the plant light incubator, 25 °C, relative humidity 60 %: (1) 'Control': aseptic seedlings were sprayed with sterile water; (2) '*P. viticola*': aseptic seedlings were sprayed with suspension of *P. viticola*; (3) 'BMJBN02': aseptic seedlings were sprayed with suspension of isolate BMJBN02; (4) 'preBMJBN02-*P. viticola*': aseptic seedlings were sprayed with suspension of isolate BMJBN02 and inoculated with suspension of *P. viticola* 4 h later. All treatments were run in biological triplicate. Total RNA of tested seedlings was extracted with Trizol reagent (Invitrogen, Carlsbad, USA). The expression of *PR* genes and reference gene in the sample plants were examined by real-time quantitative PCR (RT-qPCR) (CHEN *et al.* 2005). Total RNA was converted to cDNA using a SYBR Prime-Script RT-qPCR reagent kit (TaKaRa, Otsu, Shiga, Japan) primed with a RT-qPCR primer with the *vvEF1-γ* gene as reference gene (CHEN *et al*. 2005; suppl. Tab. 2), and the gene expression was calculated by the $2^{-\Delta\Delta CT}$ method RT-qPCR was performed on a CFX96 real-time system (Bio-Rad, Shanghai, China). All samples were run in technical triplicate.

The incidence of disease investigated before and after treatments (four treatments in the analysis of SA and *PR* gene), and the disease index were conducted fourteen days after inoculation with *P. viticola*. The classification of disease levels, disease index and incidence rate were undertaken according to the method of HE (1999).

Plot experiment: The experiment was carried out during 2017-2021 growing seasons in the vineyard of Caishi town in Jinan City, Shandong Province, China, using the susceptible cultivar: 'Muscat' (with the rootstock of

V. riparia × *V. labrusca* 'Beta'). In the test of which BMJBN02 concentration is efficient for plot experiment, three treatments were set according to the concentrations of isolate BMJBN02 in 2017 with negative control and positive control, and the grape variety was four-year-old 'Muscat' (with the rootstock *V. riparia* × *V. labrusca* 'Beta'). In the treatments, the experimental isolate BMJBN02 at concentrations of 2.0 × 10^7 CFU·mL^{-1} (Treatment 1), 1.0 × 10^7 CFU·mL^{-1} (Treatment 2) and 2.0 × 10^6 CFU·mL^{-1} (Treatment 3), were sprayed onto grapevines before spraying with *P. viticola* (concentrations of 1.0 × 10^5 mL^{-1}) 24 h. The plot treated with clean water was set as 'Negative Control', and the other one treated with 0.1 % nicotinyl morpholine was set as 'Positive Control'. Three plots were set in the field for each one treatment. Plot size measured 3 rows which were 7.5 m long and 1.8 m apart. 7 repeats with intervals of 3 d, or 3 repeats with intervals of 7 d after inoculation with *P. viticola* during the field trial were done. The incidence of disease was investigated before and after experiment, and field investigations were conducted on the twenty-eight day after inoculation with *P. viticola*. The classification of disease levels, disease index and incidence rate were undertaken according to the method of He (1999).

The field experiments to test the biological control efficiency of isolate BMJBN02 were undertaken across the 2018-2021 growing seasons, with three treatments, negative control and positive control in each growing season. In the treatments, the experimental isolate BMJBN02 at concentrations of 2.0×10^7 CFU·mL^{-1} (Treatment 1), 1.0 × 10^7 CFU·mL^{-1} (Treatment 2) and 2.0×10^6 CFU·mL^{-1} (Treatment 3), was sprayed primarily onto grapevine before inoculation with *P. viticola* 24 h. The above treatments were repeated three times with 7 d intervals in one growing season. The plot treated with clean water was set as negative control, and the other one treated with 0.1 % nicotinyl morpholine was set as positive control. Each treatment or control included 5 plots in a randomized block arrangement, 3 rows with 15 m long and 1.8 m apart for each plot, and was applied three times with intervals of 7 d during the field trial. Disease severity (DS %) (Yu *et al*. 2017) was measured three times during 7 day intervals after inoculation of *P. viticola*, during each growing season, and expressed as percent coverage of leaf area with typical symptoms of grape downy mildew according to He (He 1999). The obtained data used in the estimation of the final disease severity (FDS %) (Yu *et al*. 2017).

Results

Isolation and Screening of bio-control bacteria: bacterial isolates: Sample bacterial isolates were isolated from leek or cucumber farmland of Shouguang City. A total of 32 isolates were collected and denoted as isolate BMJBN02, (SL1), isolates SL2-SL19 and isolates SC1-SC13, respectively. The isolate BMJBN02 and isolates SL2-SL19 were isolated from the soil of leek farmland near Shouguang City, and the isolates SC1-SC13 were isolated from the soil of cucumber farmland near Shouguang City.

As shown in Tab. 1, 32 isolates, independently, were tested by the method of leaf disc for screening the highest efficient isolate. Seven days after inoculation, white mold layers with germinating sporangia, forming hyphae and sporangia were observed in the negative control; however,

Table 1

The effectiveness of isolates from soil by the leaf disc method test

Sample		Incidence rate (%) ± SE	Sample		Incidence rate (%) ± SE
NC	distilled water	89.27 ± 1.33a	16	SL16	35.85 ± 1.78c
PC	Bordeaux mixture	16.46 ± 1.05a	17	SL17	33.96 ± 1.99c
1	BMJBN02	15.49 ± 1.03a	18	SL18	37.74 ± 2.32d
2	SL 2	19.82 ± 1.32a	19	SL19	47.17 ± 2.22d
3	SL 3	21.87 ± 1.67b	20	SC1	51.56 ± 1.54b
4	SL 4	24.64 ± 1.77c	21	SC2	35.14 ± 1.73c
5	SL 5	17.57 ± 1.54b	22	SC3	26.09 ± 1.09a
6	SL6	33.33 ± 1.78c	23	SC4	56.67 ± 1.89c
7	SL7	23.08 ± 1.99c	24	SC5	19.88 ± 1.77c
8	SL8	30.99 ± 1.31a	25	SC6	55.56 ± 1.35a
9	SL9	29.58 ± 1.97c	26	SC7	37.50 ± 1.89c
10	SL10	28.99 ± 2.02c	27	SC8	57.89 ± 2.01c
11	SL11	38.10 ± 2.32d	28	SC9	38.96 ± 1.77c
12	SL12	17.28 ± 1.11a	29	SC10	57.69 ± 1.54b
13	SL13	26.09 ± 1.78c	30	SC11	18.79 ± 1.33a
14	SL14	39.68 ± 1.45b	31	SC12	42.67 ± 1.98c
15	SL15	38.64 ± 1.57b	32	SC13	37.25 ± 1.65b

Note: Analysis with LSD method ($P \leq 0.05$), different letters represent a statistically significant difference in vertical column; the same as below. Scott-Knott test should be done when many treatments were experienced. Incidence rate (%) = (the number of discs showing downy mildew infection / total number of discs in test) × 100 %.

mold was not always observed on leaf discs treated with isolate BMJBN02, where anaphylaxis necrosis occurred at the inoculation site. The effectiveness of isolate BMJBN02 was the highest of the 32 isolates, exhibiting the lowest incidence rate at 15.49 %, and the regions inoculated with *P. viticola* became necrotic lesions with the rest of the same leaf disc remaining healthy.

Identification of the effective biocontrol bacterial strain: The effective biocontrol bacterial isolate BMJBN02 was a gram-positive, facultatively aerobic, spore-forming rod-shaped bacterium. The 16S rDNA sequence of isolate BMJBN02 was related to *Bacillus megaterium*, based on its phenotypic characteristics and the sequence analysis of the phylogenetic tree of 16S rDNA (Fig. 1). The sequence was 1393 bp and was submitted to GenBank (Accession No. MN945400.1).

Analysis of SA content: The microorganism-free grape seedlings, which were grown from grape tissue culture, were used for the analysis of SA content and *PR* genes which were associated in SAR with the typical symptom of anaphylaxis necrosis. In Fig. 2, it is shown that after inoculation with *P. viticola*, the SA content did not vary significantly between the 'Control' and '*P. viticola*' treatments across the period of the experiment, whereas treatment 'preBMJBN02-*P. viticola*' caused an increase in SA in the pathogen-treated groups when compared to the control (with an increase at 4 hours after treatment (hpt), reaching a peak at 8 hpt, followed by a decrease at 24 hpt); and that treatment 'BMJBN02' exhibited obvious variation with peaks and maintained the maximum increase in SA levels. The SA contents of leaves subjected to treatment 'preBMJBN02-*P. viticola*' and treatment 'BMJBN02' increased rapidly after 4 hpt, and relatively high levels were maintained up to 24 h (with a small decrease from the peak level; Fig. 2).

Expression of *PR* genes: Not only SA exhibited a predominant role in disease resistance, but also some *PR* genes, examined by RT-qPCR, in SAR were taking the important role (Tab. 2 and Fig. 3). The four important and highly conserved genes (*PR1, PR2, PR5* and *PR10.2*) were generally at much higher levels in grape plants treated with isolate BMJBN02 (including treatment 'BMJBN02' and treatment 'preBMJBN02- *P. viticola*') than those treated only with *P. viticola* (treatment '*P. viti-*

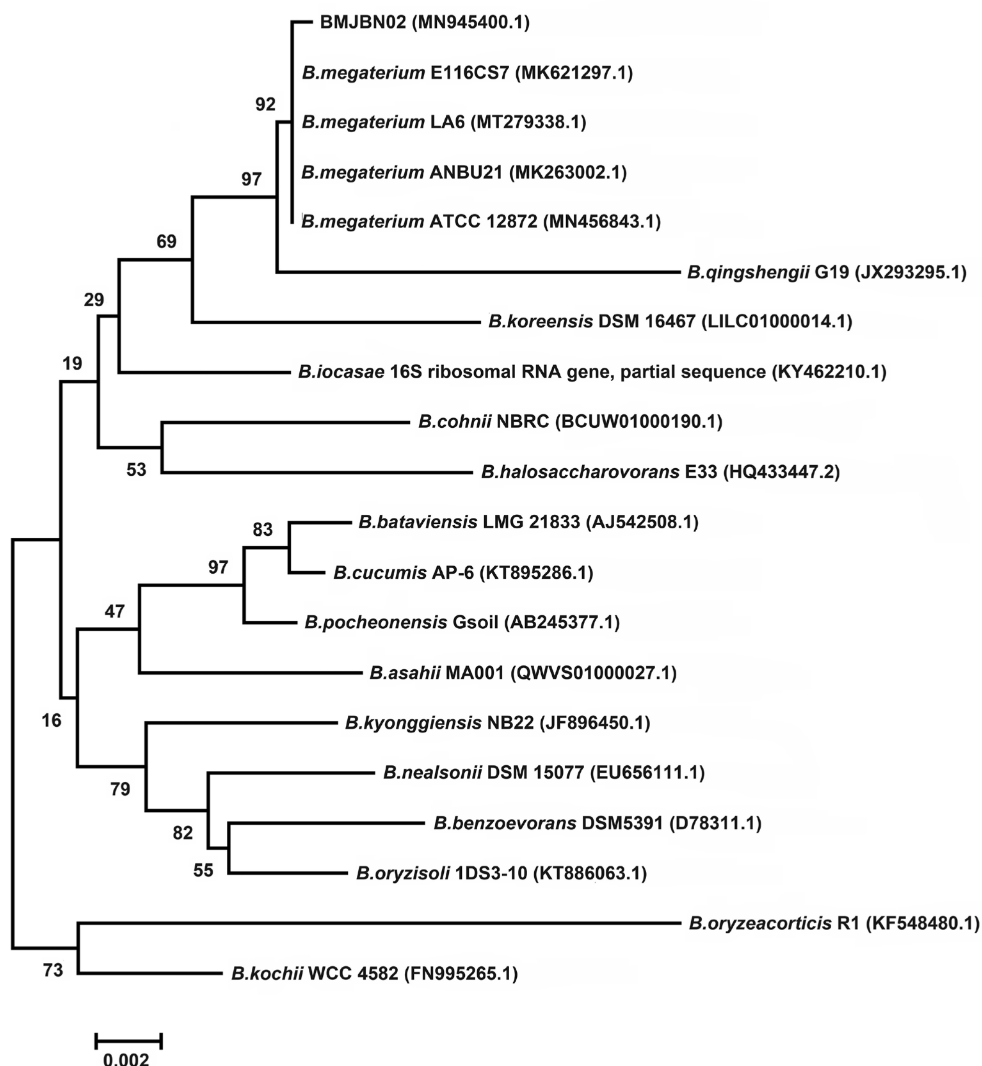

Fig. 1: Phylogenetic trees of isolate BMJBN02 by MEGA 4.0, phylogenetic tree of isolate BMJBN02 based on 16S rDNA gene sequence.

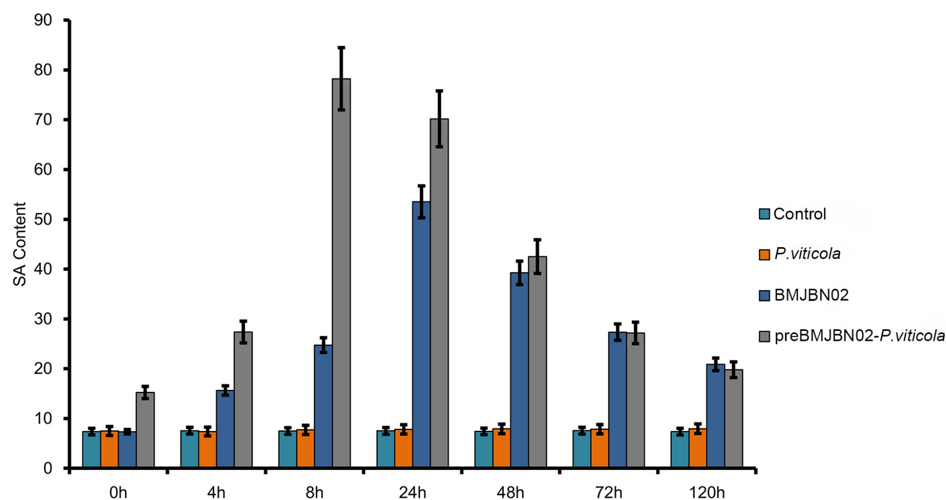

Fig. 2: Variation of SA content in grapes in different treatments. Abscissae represent time since inoculation and ordinates represent SA content ($ng \cdot g^{-1}$, fw). Error bars represent standard error (± SE) based on the number of collected samples in three biological replicates. Different letters represent a statistically significant difference ($P \leq 0.05$) among treatments as determined by Fisher's least significant difference test.

Table 2

The efficience of isolate BMJBN02 against downy mildew in the analysis of SA content and PR genes

Treatment	0 dpi		14 dpi	
	Disease index	Incidence rate(%) ± SE	Disease index	Incidence rate(%) ± SE
Control	0.00	0.00	0.00 ± 0.06a	0.00 ± 0.11b
P. viticola	0.00	0.00	11.75 ± 0.95c	20.67 ± 0.88d
BMJBN02	0.00	0.00	0.00 ± 0.05a	0.00 ± 0.08a
preBMJBN02-*P. viticola*	0.00	0.00	1.56 ± 0.13b	4.33 ± 0.25c

Notes: The classification of disease level is as follows: Grade 0 - The healthy leaf; Grade 1 - The lesion area no more than 5% of the whole leaf; Grade 2 - The lesion area from 6 % ~ 25 % of the whole leaf; Grade 3 - The lesion area from 26 % ~ 50 % of the whole leaf; Grade 4 - The lesion area from 51 % ~ 75 % of the whole leaf; Grade 5 - The lesion area from 76 % ~ 100 % of the whole leaf. Disease index = [Σ (diseased leaves at all levels × relative level) / (total number of leaves under investigation × the highest level)] × 100; Incidence rate (%) = (the number of discs showing downy mildew infection / total number of discs in test) × 100 %.

icola'). Correspondingly, grape plants treated with isolate BMJBN02 (including treatment 'BMJBN02' and treatment 'preBMJBN02- *P. viticola*') and those treated with only *P. viticola* (treatment '*P. viticola*') resulted in much higher and earlier expression of *PR* genes in comparison to the control. Although the *PR1* gene and the *PR2* gene were also greatly induced (over 3-fold) in treatment 'BMJBN02' and treatment 'preBMJBN02- *P. viticola*' at 4 hpt, treatment '*P. viticola*' showed an induction of these genes at 24 hpt, which was relatively late compared to treatment 'BMJBN02' and treatment 'preBMJBN02-*P. viticola*'. Specifically, expression of *PR5* gene showed an immediate increase at 4 hpt in all treatments, but that of *PR5* gene in treatment '*P. viticola*' was not as strongly induced. Furthermore, though the expression of *PR10.2* gene was inhibited at first (4 hpt) and then increased slightly with the peak at 72 hpt in treatment '*P. viticola*', those of *PR10.2* gene in treatment 'BMJBN02' and treatment 'preBMJBN02 - *P. viticola*' started to increase from 4 hpt and maintained elevated levels of expression until 24 hpt. From these results, we can infer that the levels and the reaction time of expression of *PR* genes are closely connected to the resistance of grape plants against downy mildew; the higher the level and the earlier the expression of *PR* genes, the stronger the resistance of grape plants.

Plot experiment: In 2017, according to the test of which concentration is efficient, examination after treatment application showed that the isolate BMJBN02 concentration of 2.0×10^7 $CFU \cdot mL^{-1}$ (Treatment 1) was the most effective concentration against grape downy mildew, with an incidence rate and disease index approximately equal to values resulting from treatment with 0.1% nicotinyl morpholine (positive control) (Tab. 3). Besides, there is no much difference of the disease index and incidence rate (%) between spraying with an interval of 7 d and spraying with an interval of 3 d. Considering the convenience of application, 7-d has been chosen as the interval used in the 2018-2021 plot experiment. In Tab. 4, data indicate that there are large differences in final disease severity (FDS%) among three treatments. Moreover, all treatments reduced

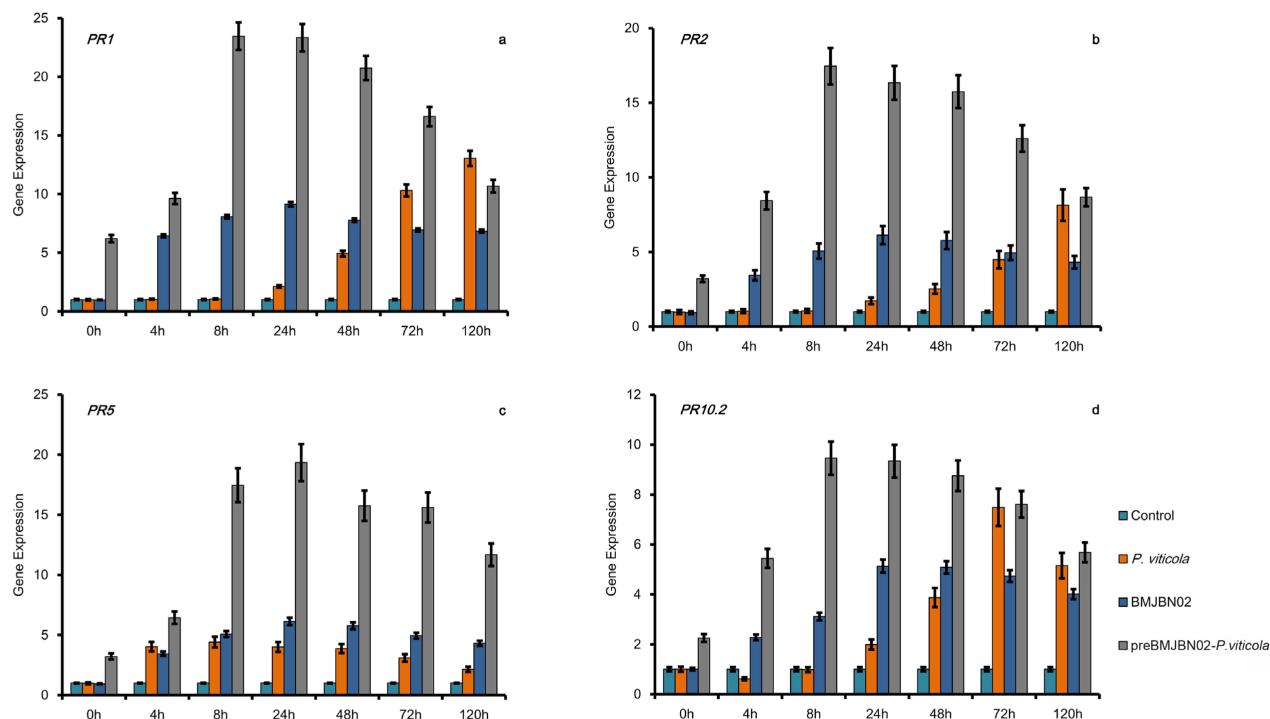

Fig. 3: Relative expression of *PR* genes in different treatments. All the values were normalized to the expression level of *vv EF1-γ*. Data represent the change in gene expression in leaf samples. Error bars represent standard error (± SE) based on the number of collected samples in three biological replicates. Different letters represent a statistically significant difference ($P \leq 0.05$) among treatments as determined by Fisher's least significant difference test.

Table 3

The effect of plot experiment with isolate BMJBN02 in 2017

Treatment	0 dpi	28 dpi with intervals of 3 days,		28 dpi with intervals of 7 days,	
	Incidence rate(%)±SE	Disease index	Incidence rate(%)±SE	Disease index	Incidence rate(%)±SE
Negative control	0.00	44.81 ± 2.88d	53.12 ± 2.73d	60.00 ± 3.17e	68.71 ± 2.97d
Positive control	0.00	8.44 ± 1.43a	9.30 ± 1.89a	9.00 ± 2.13a	10.70 ± 2.11a
1	0.00	8.17 ± 1.21a	9.12 ± 1.33a	8.46 ± 1.87ab	10.32 ± 1.76a
2	0.00	14.19 ± 2.02b	11.99 ± 1.87b	16.88 ± 2.56c	13.07 ± 2.33b
3	0.00	21.97 ± 2.08c	22.89 ± 2.10c	29.09 ± 2.81d	33.21 ± 2.67c

Note: dpi: days after inoculation with *P. viticola*. The sample grape: highly sensitive variety 'Muscat'. Treatment 1: experimental isolate BMJBN02 concentrations of 2.0×10^7 CFU·mL^{-1}, Treatment 2: experimental isolate BMJBN02 concentrations of 1.0×10^7 CFU·mL^{-1}, and Treatment 3: experimental isolate BMJBN02 concentrations of 2.0×10^6 CFU·mL^{-1}; Positive control: treatment of 0.1 % nicotinyl morpholine; Negative control: treatment of clean water. Analysis with $P \leq 0.05$, different letters represent a statistically significant difference in vertical column; the same as below. Scott-Knott test should be done when many treatments were experienced.

FDS% compared to the negative control. The experimental isolate BMJBN02 concentrations of 2.0×10^7 CFU·mL^{-1} (Treatment 1) reduced FDS% to 28.81 % in 2018 to 26.13 % in 2021; the experimental isolate BMJBN02 concentrations of 1.0×10^7 CFU·mL^{-1} (Treatment 2) reduced FDS% to 31.97 % in 2018 and 31.20 % in 2021; the experimental isolate BMJBN02 concentrations of 2.0×10^6 CFU·mL^{-1} (Treatment 3) reduced FDS% to 41.13 % in 2018 to 38.77 % in 2021. In other words, the FDS values under different treatment conditions showed a downward trend over the four growing seasons.

Discussion

The induction of plant disease resistance by rhizobacteria could help enhance host resistance against pathogens. Our results indicate that *Bacillus megaterium* BMJBN02 screened from soil samples regulates the resistance of grape plants against downy mildew. It was found that *Bacillus megaterium* BMJBN02 regulates the resistance of grape plants by playing a role in affecting SA content and the expression of related *PR* genes in SAR by the detection of HPLC-MS and RT-qPCR. The plot experiment showed

Table 4

Effects of isolate BMJBN02 on final disease severity (FDS %) of grape downy mildew, from 2018 to 2021 growing seasons

Treatment	FDS (%)±SE			
	2018	2019	2020	2021
Negative Control	71.37 ± 4.89b	77.85 ± 5.47d	75.21 ± 5.31d	80.24 ± 4.72d
1	28.81 ± 4.05a	28.34 ± 4.47a	27.65 ± 4.06a	26.13 ± 3.85a
2	31.97 ± 5.05c	31.78 ± 5.23c	30.33 ± 5.01c	31.20 ± 4.53c
3	41.13 ± 5.47d	41.33 ± 5.73e	39.37 ± 5.68e	38.77 ± 5.18e
Positive Control	28.77 ± 4.09a	31.49 ± 4.72b	30.67 ± 4.65b	33.23 ± 4.33b

Note: The sample grape: highly sensitive variety 'Muscat'. Treatment 1: experimental isolate BMJBN02 concentrations of 2.0×10^7 CFU·mL^{-1}, Treatment 2: experimental isolate BMJBN02 concentrations of 1.0×10^7 CFU·mL^{-1}, and Treatment 3: experimental isolate BMJBN02 concentrations of 2.0×10^6 CFU·mL^{-1}; Positive control: treatment of 0.1 % nicotinyl morpholine; Negative control: treatment of clean water. Analysis with $P \leq 0.05$, different letters represent a statistically significant difference in vertical column; the same as below. Scott-Knott test should be done when many treatments were experienced.

that the resistance imparted by *Bacillus megaterium* BMJBN02 was approximately equal to that of 0.1 % nicotinyl morpholine, an efficient fungicide against grape downy mildew.

Research on bacteria as a strategy against grape diseases, especially downy mildew on grape plants, is in its infancy. Most of these bio-control isolates were isolated from soil of plants. For example, *B. amyloliquefaciens* S13-3 isolated from soils was demonstrated to act against *C. gloeosporioides* (Mochizuki *et al*. 2012) and has demonstrated biocontrol potential against downy mildew (Zhang *et al*. 2017). Although *Bacilli* from soil have been reported to inhibit the growth of pathogens (Mochizuki *et al*. 2012, Liang *et al*. 2016), their actions of adjusting the resistance of grape plants have rarely been studied. This study was first to isolate *Bacillus* from farmland soils and to demonstrate that it adjusts host resistance against the disease of grape downy mildew.

SA accumulation, as well as the coordinated expression of *PR* genes with antimicrobial activity, is also necessary for the onset of SAR in plants (Dong 2004); therefore, the SA content and the expression of *PR* genes were detected in the present study. The grape plants in treatment '*P. viticola*' showed a mild change in SA content, and SA content clearly increased in grape plants of treatment 'BMJBN02' and treatment 'preBMJBN02-*P. viticola*'; in particular, grape plants in treatment 'preBMJBN02-*P. viticola*'exhibited an acute upregulation of SA content (Fig. 2). Combined with the results shown in Tab. 2, these results are consistent with the findings of previous investigations that SA content and its occurrence time are positively correlated with disease resistance of the host (Yu *et al*. 2012, Liu *et al*. 2016). It is notable that in treatment 'BMJBN02', SA content of tested grape plants increased rapidly and kept at higher level than that in treatment 'control', and that in treatment 'preBMJBN02-*P. viticola*', SA content of tested grape plants was significantly elevated and reached its peak at 8 hpt, maintaining the higher concentration during the subsequent infection processes (Fig. 2). In treatment 'BMJBN02', the levels of SA content were significantly elevated starting at 4 hpt (Fig. 2). The content of SA accumulation and the response time may have triggered a more effective process within the plant immune system to resist invasion by the pathogen (Vlot *et al*. 2009, Loake and Grant 2007).

The initiation of SAR is dependent on the level of SA content after systemic induction (Mauch-Mani and Metraux 1998), and also depends on the activation of a series of PR proteins with antibacterial activity (Van Loon 1997). To investigate this further, particularly in light of the contributions made by SA-mediated SAR, we analyzed the expression of four pivotal *PR* genes in SAR (Fig. 3). Some studies have shown that the expression of *PR* genes not only can produce proteins to impede the invasion of pathogens, but may also help the host plant block pathogen invasion, as shown by systemic detection of elevated *PR* gene expression among infected tissues, surrounding tissues, and remote uninfected parts (Mehrotra and Aggarwal 2003, Nandi 2016). Other investigations have revealed correlations of grape downy mildew resistance with enhanced expression of *PR* genes (Malacarne *et al*. 2011, Figueiredo *et al*. 2012). The previous research indicated that several defense-related genes are preferentially induced in the early phases of invasion of pathogens (Figueiredo *et al*. 2012). After *P. viticola* inoculation, high expression levels and speed of expression for *PR*5 of grape are consistent with the conclusion of Dufour *et al*. (2013). In this study, the result of expression of *PR10.2* is consistent with the previous research that the increased expression of *PR10* family gene enhanced the disease resistance of the host (Xie *et al*. 2010). The previous study was refered that the expression of *PR1*, *PR2* and *PR5* genes was positively correlated with host disease resistance mediated by salicylic acid (Delessert *et al*. 2005). Experimental results of *PR* genes were consistent with previous transcriptomic analysis which indicates that downy mildew resistance is mainly associated with the expression of *PR* genes of SAR (Polesani *et al*. 2010). Transcriptional changes in *PR* genes of grape have been related to a defense response (Polesani *et al*. 2010) and to the establishment of compatible inter-

action (HAYES *et al*. 2010, GAMM *et al*. 2011). Moreover, in treatment 'BMJBN02', a significant increase in SA content has also been shown to occur after treatment with isolate BMJBN02, coordinated with an increase in expression of *PR* genes. These results are consistent with the previous investigation that SA content and expression of *PR* genes are positively correlated with disease resistance of the grape (LI *et al*. 2015).

Isolate BMJBN02, its effective action confirmed by the leaf disc assays, SA content and expression of *PR* genes, was also tested in a field experiment. The fact that the isolate exhibited remarkable inhibitory effects on downy mildew during the 2017-2021 growing seasons suggests a prospect for practical application in large-scale vineyards. The same effect has been observed for other soil-isolated microbe isolates, such as *Streptomyces atratus* PY-1, *B. subtilis* KS1 and *Trichoderma atroviride* TRS25 on *P. viticola* in vineyards (LIANG *et al*. 2016, FURUYA *et al*. 2011, SZCZECH *et al*. 2017). Especially, isolate BMJBN02 also showed strong resistance effectiveness against grape downy mildew approximately equal to the effectiveness of 0.1 % nicotinyl morpholine, suggesting that application of the isolate may substitute for chemical pesticides. On the other hand, the FDS value per treatment showed a slowly downward trend during the past tested growing seasons. It could be concluded that the vineyard was kept away from grape downy mildew by isolate BMJBN02.

In conclusion, *B. megaterium* BMJBN02 isolated from farmland soil was shown to adjust host resistance of grape against downy mildew. Therefore, the application of this isolate could be useful for helping grapes resist downy mildew in vineyards.

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