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INHIBITION OF POSTHARVEST GRAY MOULD DECAY AND INDUCTION OF DISEASE RESISTANCE BY *PSEUDOMONAS FLUORESCENS* IN GRAPES

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After suffering from mechanical injury and fungal infection, grapes are perishable. *Botrytis cinerea*, the causal agent of gray mould, is a critical pathogen for grapes. In this study, the inhibitory effect of *Pseudomonas fluorescens* on the formation of gray mould on grapes during the postharvest storage was investigated on "Kyoho" grape. The results suggest that a living cell suspension of *P. fluorescens* significantly inhibited spore germination of *B. cinerea*, and significantly reduced the incidence of grape gray mould. Moreover, compared with the control, the fruit inoculated with *P. fluorescens* had elevated activities of polyphenol oxidase (PPO), peroxidase (POD), catalase (CAT), phenylalanine ammonia-lyase (PAL), chitinase (CHI), and β-1,3-glucanase (GLU). Increase in enzyme activity correlated with enhanced host resistance. In addition, there was little difference in storage quality between the treated group and control group, indicating no adverse effects of the induced defence response on fruit quality.

Keywords: *Pseudomonas fluorescens*, grape, gray mould, induced resistance, storage quality

Grape is one of the most important fruits, which is widely cultivated and has a large production in the world. As a typical berry fruit, grapes are very perishable and susceptible to infection by fungal pathogens during transport and storage. The gray mould caused by *Botrytis cinerea* is one of the most common and most damaging diseases during the postharvest process of grapes, causing significant economic and resource losses in the world (Furuya et al., 2011).

Traditionally, the application of chemical fungicides, such as sulphides and pyrrole, are the main method to control gray mould. The emergence of fungicide resistance and environmental pollution along with the increasing consumer demands for reduction of residues in fruit emphasize the need for alternative disease control strategies. Biological control is a promising alternative posing lower risk to human health and the environment (Wallace et al., 2018).

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Pseudomonas fluorescens, a common and abundant Gram-negative bacterium that inhabits soil and plant surfaces, has the capacity to inhibit or suppress a variety of pathogenic fungi. However, most of the research on *P. fluorescens* has focused on the establishment of rhizosphere soil and its ability to produce antibiotics (Wallace et al., 2017). While there are a few reports on controlling postharvest diseases by *P. fluorescens* for the gray mould of grapes; little information is available about the induction of disease resistance.

Therefore, *P. fluorescens* was used as antagonist in this experiment, and the "Kyoho" grape was used as test material. The aim of this study was to investigate the effects of *P. fluorescens* on grapes.

1. Materials and methods

1.1. Fruit materials and microorganisms

1.1.1. Fruit. Grapes (*Vitis vinifera* L. × *V. labrusca* L. "Kyoho"), without chemical fungicide application, were hand-harvested randomly in Shijin Wine Plantation of Chongqing. They were immediately transported to our laboratory, then classified and selected to obtain fruit with identical maturity and size and no visible injures. The intact fruit were kept with their own stem (3–5 mm) to maintain fruit integrity.

1.1.2. Pathogen. The pathogen (*Botrytis cinerea*) was obtained from Dr. Wen, College of Plant Protection, Southwest University, and maintained on PDA (potato dextrose agar media) at 4 °C. After culturing the pathogens on PDA for 7 d at 25 °C, the conidial suspension of *B. cinerea* was prepared by rinsing the spores from the culture dishes with sterile distilled water (SDW) containing 0.05% (v/v) Tween-80. Finally, the concentration was adjusted to 1×10^5 spore ml^{-1} with sterile saline solution using a hemocytometer (Qiujing Biochemical reagent Instruments Co., Ltd. Shanghai, China).

1.1.3. Antagonist. P. fluorescens was kindly provided by Dr. Zsolt Zalán from National Agricultural Research and Innovation Centre Food Science Research Institute, Budapest Hungary. *P. fluorescens* was maintained at 4 °C on NA (nutrient broth agar medium). Liquid culture was inoculated with a loop of original culture into 50 ml of NB in 250-ml Erlenmeyer flasks, and incubated for 16 h on a rotary shaker at 200 r.p.m. After this, the bacterial concentration was approximately 1.5×10^{10} CFU ml^{-1}. From this bacterial cell suspension, different preparations of the antagonist were prepared. (A) Culture filtrate was prepared by filtering the bacterial cell suspension using a 0.22 μm polycarbonate membrane filter (Hefei Biosharp Co., Ltd. China). (B) 1×10^8 CFU ml^{-1} unwashed cell culture suspension was prepared by diluting with culture filtrate obtained in (A). (C) Autoclaved culture was prepared by autoclaving the suspension obtained in B) at 121 °C for 20 min. (D) Washed cell suspension was prepared by using SDW to wash the bacterial cell suspension twice to remove growth medium and adjusted to a concentration of 1×10^8 CFU ml^{-1}. SDW was used as control in our investigation. The concentration was adjusted to suit nephelometry (WZT-1M, Jinjia Scientific Instruments Co., Ltd. Shanghai, China).

1.2. In vitro antifungal assay

1.2.1. Effects of P. fluorescens *on the budding of* B. cinerea *spores.* According to the method of WANG and co-workers (2011), 4 ml of a 1.0×10^5 spore/ml suspension of *B. cinerea*, 2 ml of washed cell suspensions with different concentrations (0.25, 0.50, 0.75, and 1.0 MCF. MCF: Mai's turbidity unit), and SDW were added into 50 ml Erlenmeyer flasks containing 14 ml PDB. After 16 h incubation at 28 °C on a rotary shaker at 150 r.p.m., the spore germination and germ tube elongation were counted and measured. The spore was considered germinated when germ tube length was equal or greater than spore length.

1.2.2. Effect of P. fluorescens *on the mycelium growth of* B. cinerea. *P. fluorescens* was co-cultured with the pathogen on 1/2 PDA/NA (50 ml PDB, 50 ml NB, and 1.5 g agar) dishes to test antagonistic activity. One hundred microlitres of the four mentioned preparations (1.1.3 A, B, C, and D) and SDW were added to the whole dish, then a hole was made (6 mm in diameter, 2 mm in depth) in the centre of the dish by using a hole punch. Finally, a 6 mm mycelial disc was placed in the hole, which was obtained from the edges of a 7-d-old culture of the fungus. The dishes were incubated at 25 °C, and after 7 d, vertical diameters of each mould plaque were measured in two perpendicular directions by a Vernier calliper (Fengliang International Group Co., Ltd. Hong Kong, China).

1.3. Effect of P. fluorescens *on gray mould in vivo*

A wound (3 mm diameter ×3 mm deep) was made on each side of the pretreated grape berry using a sterile needle at the equatorial side. Then, the perforated grapes were randomly divided into 5 groups, and into each wound 10 µl *P. fluorescens* suspension (0.25, 0.50, 0.75, and 1.0 MCF) was inoculated. After 2 h, 10 µl of a 1.0×10^5 spore/ml suspension of *B. cinerea* was inoculated into each wound. Sterile distilled water was used as control. After natural air drying, the treated grapes were placed into turnover boxes, and all treated boxes were placed in a constant temperature and humidity incubator (LHS-150CLY, Qixin Scientific Instruments Co., Ltd. Shanghai, China) at 20 °C for 20 h (LHS-150CLY, Qixin Scienti). The treated fruit were incubated for 8 d. Each group contained 30 fruit in 3 replicates.

When the lesion diameter of gray mould on the surface was greater than 2 mm, it was recorded as diseased. The lesion diameters were determined by the mean of the horizontal and vertical diameters of each lesion, and the disease incidence (%) was calculated as disease incidence (%)=(number of diseased fruit/total number of fruit)×100. Any fruit wound with visible mould growth was considered to be infected.

1.4. Effect of P. fluorescens *on the defence enzymes of grapes*

Grapes were wounded as described above (1.3). The wounds were treated with 10 µl of 0.5 MCF bacterial suspensions using a micropipette. Wounds treated with SDW served as control. After the surfaces of the fruit were dry, all treated fruit were packed and placed in a constant temperature and humidity incubator at 20 °C under 90% RH. At various time intervals (0, 2, 4, 6, and 8 d), samples were taken from 10 independent fruit to analyse the activities of the defence-related enzymes.

Five grams of frozen grape sample was taken to extract crude enzyme solution by the method of Wang and Wang (2013). The supernatants were used to determine enzyme activity and protein content. Protein content was determined according to the Coomassie brilliant blue method (Bradford, 1976). The activity of PPO and POD was measured using the method of Srivastava and Dwivedi (2000). The activity of PAL was measured according to the method of Koukol and Conn (1961). The CAT was determined by the method of Cakmak and co-workers (1993). The chitinase activity was measured according to the method of Cao and Jiang (2005), and the β-1,3-glucanase activity was measured according to the method of Abeles and co-workers (1971). All results were expressed as U mg^{-1} protein.

1.5. Effect of P. fluorescens *suspension on storage quality of grapes*

The intact fruit were impregnated with sterile water and *P. fluorescens* suspension with a turbidity of 0.5 MCF for 2 min. After drying, the fruit were packed and placed in a constant temperature and humidity incubator at 20 °C under 90% RH. Each group contained 30 treated fruit replicated 3 times and cultured for 20 d. The disease incidence and quality of the fruit were determined at the end of the storage. The disease incidence was determined as described earlier. The total suspended solid (TSS) content of the fruit was measured directly using a hand-held sugar meter, and the results were expressed as percentage (Larrigaudiere et al., 2002); the titratable acid (TA) content was determined by titration, with phenolphthalein as indicator, and the results were expressed as the percentage of tartaric acid; the content of ascorbic acid in grape fruit was determined by 2,6-dichlorophenol indophenol titration method; the soluble sugar content was determined by phenol-sulphuric acid method; the results were expressed in mg g^{-1} glucose (Cao & Jiang, 2005); and the contents of glucose and fructose in fruit were determined by HPLC (Zhang & Hou, 2014).

1.6. Statistical analysis

Variance analysis was performed on the data using SPSS 20.0. Differences in significance were analysed using Duncan-type multiple comparisons, and the differences at $P<0.05$ were considered significant. Mapping was performed using Origin 9.0.

2. Results and discussion

2.1. The effect of P. fluorescens *on gray mould in vitro*

Most fungal pathogens infect fruit from wounds, stomata, and lenticels through spore germination to form germ tubes, causing postharvest diseases. Our study showed that *P. fluorescens* can significantly inhibit the spore germination and germ tube elongation of *B. cinerea*. In the control group, 80.39% of *B. cinerea* spores germinated, while the 0.75 MCF bacterial suspension treatments reduced spore germination to 8.67%. In addition, spore germination was completely inhibited by 1.0 MCF bacterial suspension. Similarly, the germ tube lengths of *B. cinerea* spores at different concentrations of *P. fluorescens* 0.25, 0.5, 0.75, and 1.0 MCF bacterial suspensions were 21.06, 13.65, 4.38, and 0 μm, respectively (Table 1).

Table 1. The effect of *P. fluorescens* on spore germination and germ tube elongation of *B. cinerea*

Treatments	Spore germination (%)	Germ tube length (μm)
Control	80.39±0.86a	50.50±4.69a
0.25 MCF bacterial suspension	57.96±1.12b	21.06±4.86b
0.5 MCF bacterial suspension	24.50±1.50c	13.65±1.44c
0.75 MCF bacterial suspension	8.67±0.53d	4.38±1.25d
1.0 MCF bacterial suspension	0.00±0.00e	0.00±0.00e

Control: SDW replaced the bacterial suspension. Bacterial suspension was prepared by using SDW to wash the bacterial precipitation twice to remove residual culture medium and adjusted to the design concentrations with the addition of sterile distilled water.
Values in a column followed by a different letter are significantly different according to Duncan's multiple range test at $P<0.05$ level.

The antifungal activity of *P. fluorescens* on *B. cinerea* mycelial growth was measured on 1/2 NA/PDA in various treatments. After 16 h of cultivation at 28 °C, all four treatments by *P. fluorescens* restrained the mycelial growth of *B. cinerea* significantly (Table 2). It was observed that unwashed cell culture mixture and washed cell suspension had more effective biocontrol effects in vitro (Table 2), compared with autoclaved cultures and culture filtrate. It indicated that competition for nutrient and space played an important role in the biocontrol capability of *P. fluorescens* against *B. cinerea*.

*Table 2***.** The effect of *P. fluorescens* on the mycelial growth of *B. cinerea*

Treatments	Mycelium growth (mm)	Inhibition rate (%)
SDW	89.34±0.93a	0.00±0.00d
Autoclaved cultures	74.01±0.11b	16.41±0.99c
Culture filtrate	47.59±0.37c	46.73±0.15b
Washed cell suspension	0.00±0.00d	100.00±0.00a
Unwashed cell culture mixture	0.00±0.00d	100.00±0.00a

Values in a column followed by a different letter are significantly different according to Duncan's multiple range test at $P<0.05$ level.

2.2. The effect of P. fluorescens *on grapes for the control of gray mould*

As illustrated in Figure 1, *P. fluorescens* had a significant inhibitory effect on postharvest gray moulding of grapes ($P<0.05$). The disease incidence and lesion diameter of grapes inoculated with *P. fluorescens* suspension was significantly lower than the control. The disease incidence was 70.77% and the lesion diameter was 4.57 mm in control fruit, while in fruit inoculated with 1.0 MCF *P. fluorescens* suspension these values were 30.37% and 2.23 mm, respectively, on the 8th day of storage. The concentration of *P. fluorescens* suspension had a significant effect on the disease incidence (Fig. 1A) and lesion diameter (Fig. 1B).

This study showed that *P. fluorescens* was effective in controlling postharvest decay of grape fruit caused by *B. cinerea*, and the higher the concentration, the better the antifungal effect (Table 1 and Fig. 1) was. When the concentration of the *P. fluorescens* suspension was higher than 0.5 MCF, increasing the concentration could effectively reduce the lesion diameter, but it had no significant effect on reducing the disease incidence of gray mould (Fig. 1A and Fig. 1B).

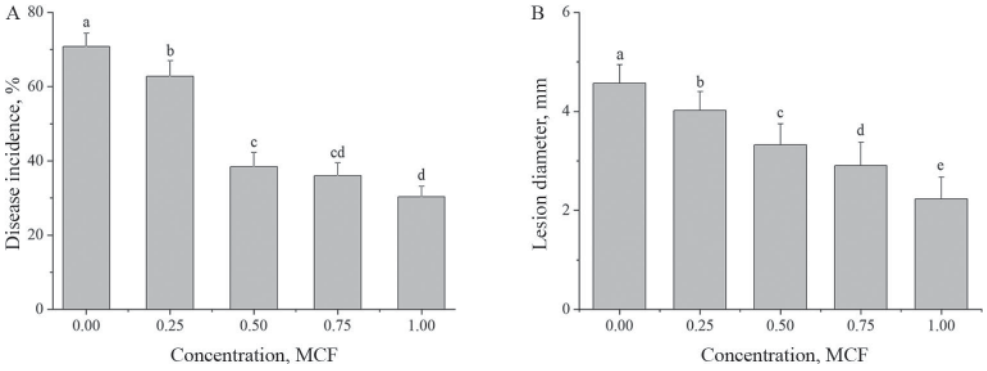

Fig. 1. Effects of different concentrations of *P. fluorescens* on disease incidence (A) and lesion diameter (B) of grapes during the incubation at 20 °C for 8 days. Each column represents the mean of triplicate assays from one experiment. Vertical bars represent the standard errors of the means. Different letters above the bars indicate statistically significant differences ($P<0.05$) within the same panel

2.3. Induction of grape resistance by P. fluorescens

Plant disease resistance is a stress response, which affects the normal physiological metabolism of plant tissues or cells (Hammerschmidt, 2005). The improvement of fruit disease resistance is accompanied by an increase in defence-related enzyme activity. The induction of defence response in fruit has been considered as another major mechanism of antagonists to suppress infection with pathogens, and growing evidence have supported this point of view (Droby et al., 2009; Liu et al., 2016).

PAL is indirectly involved in the synthesis of various phenolic compounds. PPO can catalyze the formation of lignin and other phenolic oxides, constitute a protective barrier, and resist the invasion of pathogens (Mojtaba & Homayoon, 2002). The ability of antagonistic bacteria to adapt to oxidative stress at the wound of the fruit is one of the key factors that determine the ability to colonize. Catalase (CAT) and peroxidase (POD) are the key enzymes for active oxygen metabolism. β-1,3-Glucanase can degrade the fungal cell wall causing the protoplasmic membrane to rupture and directly kill pathogens to protect the host. Chitinase can degrade chitin, the main component of many fungal cell walls, thereby directly inhibiting the growth of pathogenic bacteria (Hariyadi & Parkin, 1991).

The experimental results (Fig. 2) show that after treatment with *P. fluorescens*, the activity of defence-related enzymes (PAL, PPO, CAT, POD, β-1,3-glucanase, and chitinase) in grapes increased to some extent, indicating that the induced disease resistance is one of the important mechanisms of *P. fluorescens* to effectively control grape gray mould.

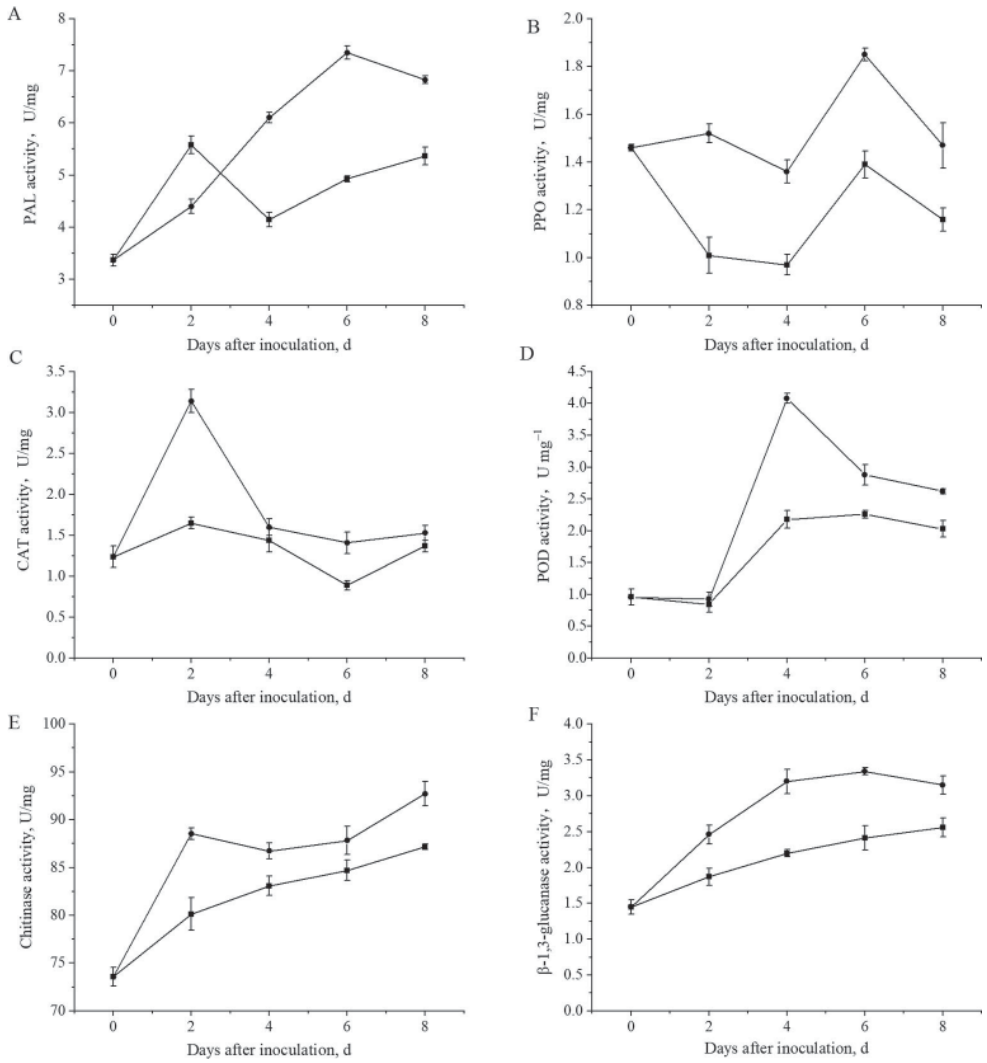

Fig. 2. The effect of *P. fluorescens* on activities of PAL (A), PPO (B), CAT (C), POD (D), chitinase (E), and β-1, 3-glucanase (F) in grapes. Each value is the mean of three experiments. Bars represent standard errors
—■—: Control; —●—: *P. fluorescens*

2.4. Effect of P. fluorescens *on storage quality of grapes*

In the process of growth and development (ripening and aging), physical properties of fruits and vegetables undergo a series of changes, including colour, shape, quality, hardness, etc. These changes directly reflect the quality of fruits and vegetables. In the postharvest storage process, the defence reaction due to pathogen infection can cause a certain degree of quality decline of fruits and vegetables. In this study, Table 3 shows that the soaking treatment with *P. fluorescens* suspension with 0.5 MCF could significantly reduce the incidence of disease during natural spoilage of grapes, and had a positive effect on the storage quality of grapes,

indicating good potential for commercial application. The incidence of grapes treated with *P. fluorescens* after storage at 20 °C for 20 d was only 40.40%, which was meaningfully lower than that of the control group (64.76%). In addition, after 20 d storage, the fruits soaked in *P. fluorescens* solution were higher in ascorbic acid, sugar, and TSS contents than control grapes.

Table 3. Effect of *P. fluorescens* on storage quality in grapes

Quality index		Incidence (%)	TSS (%)	TA (% tartaric acid)	Ascorbic acid (mg/100 g)	Fructose (mg g^{-1})	Glucose (mg g^{-1})
Days	Treatment						
0	——	——	14.50±0.14b	0.3930±0.0085a	6.51±0.05a	53.08±1.62c	71.35±6.48b
20	control	64.76±4.36a	14.70±0.14b	0.3667±0.0085b	61.38±3.01b	61.38±3.01b	79.46±4.60ab
20	*P. fluorescens*	40.40±0.86b	15.15±0.07a	0.3778±0.0014ab	68.16±1.64a	68.16±1.64a	94.59±10.00a

Note: the same column letter means that the difference is not significant, and different letters mean that the difference is significant ($P<0.05$)

3. Conclusions

In conclusion, the results of this study showed that *P. fluorescens* was an effective agent for the control of *B. cinerea* on "Kyoho" grapes stored at 20 °C, indicating that *P. fluorescens* may have the ability to control gray mould during commercial grape storage. In addition, potential mechanisms of inhibition may include competition for nutrients and space, targeting conidial germination and mycelial growth, and inducing disease resistance. These data suggest that the soaking treatment in *P. fluorescens* bacterium suspension significantly reduced the occurrence of grape diseases but did not affect the quality of the grapes. Therefore, *P. fluorescens* could potentially be used as a biocontrol agent against *B. cinerea* in the postharvest storage of grapes.

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