

*Original Contribution***BIOCONTROL OF GRAY MOLD ON *ROSA HYBRIDA* CV. BACCARA WITH *BACILLUS SUBTILIS*****E. S. Mousavi^{1*}, D. Naderi^{2*}, S. Kalateh Jari¹, V. Abdossi¹, H. Dehghanzadeh³**¹Department of Horticulture, Science and Research Branch, Islamic Azad University, Tehran, Iran²Department of Horticulture, Isfahan (Khorasgan) Branch, Islamic Azad University, Isfahan, Iran³Department of Agricultural Science, Payame Noor University (PNU), Iran**ABSTRACT**

The bacteria *Bacillus subtilis* was investigated for control of gray mold, postharvest quality and antioxidant enzymes of *Rosa hybrida* cv. Baccara. The results indicated that the treatment of *Bacillus subtilis* suspension of 1×10^8 cfu mL⁻¹ with resulted in a remarkably improved control of *Botrytis cinerea* infections. CAT activity in treated flower by antagonism were significantly more than those control ($P \leq 0.05$) at 25°C, RH 60-70%. POD activity cut flowers increased during the flower bud development with the lowest activity present at water-sprayed control. Enhanced by antagonism could be due to either induced resistance or direct effects of these chemicals on *Botrytis*. The proper concentration of *Bacillus subtilis* can thus provide an effective strategy to increase postharvest vase life of *Rosa*. Postharvest antagonism application prolonged vase-life in cut rose flowers by improving the reactive oxygen species (ROS) scavenging capacity related to CAT and POD activity.

Key words: *Bacillus subtilis*, *Rosa hybrida* cv. Baccara, CAT activity, POD activity

INTRODUCTION

Gray mold disease caused by the fungus *Botrytis cinerea* under cool, wet conditions; it can be a limiting factor in the production, marketing, and storage of flowers as roses, gerberas, and chrysanthemums. Currently ranking among the most serious disease in horticultural field crops, were reported in rose breeding centers Mahalat and Dezful in Iran and made many problems for growers (1, 2). Infections are not always visible at harvest, but they develop rapidly in transit to market under the conditions of high humidity maintained in shipping containers. The disease mainly infected rose in flower buds stage and cause rot before harvest, as well as necrotic lesions of the petals at postharvest (3). The infected petals, colored spots spread irregular that are surrounded by a red border. The current program for gray mold of rose is often unsuccessful. Control recommendations include frequent fungicide sprays in the greenhouse during the winter, with a postharvest dip of cut flowers in a fungicide solution year-round. This

program is prevented by unsightly residues on the flowers and by the development of populations of *B. cinerea* resistant to benomyl, vinclozolin and iprodione (4).

Fungicide resistance is widespread and can develop within one season (5). The importance of this disease is because of hidden and invisible contamination during the harvest rose. Indeed the flower could be without any typical disease symptom at the time of harvesting but appears between 6 to 12 hours after harvesting of infected flowers so, infected flowers cannot be sold in the market.

Natural defenses that protect plants are wide and varied. Plants can be resistant by management, production methods and environmental conditions before and postharvest (6). Recently microbial antagonists have been used to control postharvest diseases. One of the features of induced resistance is associated with different biochemical changes in plants including disease-related protein, peroxidase enzyme activity, total phenolic compounds and phenylalanine amonialiase (7). Consideration of these problems has resulted in interest in biological control of *B. cinerea*, which if available, could introduce needed

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flexibility into the management program. *B. cinerea*, have been reduced by preinoculation of the phylloplane with epiphytic bacteria or fungi. Monhamed and Caunter using the biological control agent *pseudomonas fluorescens* reduced the severity of gray mold of apple caused by *B. cinerea* (8). *Bacillus lichniformis* also was reported to control gray mold in apple (9). The role of volatile compounds of *Bacillus subtilis* was reported in inducing resistance against *B. cinerea* in Arabidopsis (10).

The present study we aimed to assess the efficacy of the integrated control of gray mold on *Rosa hybrida* cv. Baccara by combining the application of some bacterial strains in greenhouse conditions and whether this combination can increase the postharvest vase life roses?

METHODS AND MATERIALS

1. Pathogen inoculums

B. cinerea was isolated from a strawberry showing typical gray mold. *B. cinerea* was freshly cultured on PDA plates at 23°C spore suspensions were prepared by removing the spores from a 7-day old culture with a sterile inoculators and then suspending in sterile distilled water to the required concentration of 1×10^4 spores mL⁻¹, which was estimated using a hemacytometer.

2. Antagonist *Bacillus subtilis*

The bacteri *Bacillus subtilis* was cultured in 250 mL Erlenmeyer flasks with 100 mL (acid PDA, Difco, Detroit, MI, with 3 g/L oxgall and HCl added to acidify to pH 4.5-5.0) on a gyratory shaker at 180 r min⁻¹, 28°C for 24 h. The bacterial cells were acquired by centrifuging at 6000 × g for 15 min (at 4°C) and then re-suspended in sterile distilled water. The bacteria concentrations were adjusted as needed for different experiments using a hemacytometer.

3. Flower

Flowers (*Rosa hybrida* cv. Baccara) were harvested in the bud stage early in the morning from a greenhouse in Yazd, Iran. They were transferred in suitable covers at laboratory of horticulture, College of Agriculture and Natural Resources Ardakan. First, the size of 40 cm stem length was cut to prevent vascular obstruction and potential wilting in the water. They were surface-disinfected with 2% sodium hypochlorite for 2 min, rinsed with tap water and dried in air before use.

Rosebuds were sprayed with 10⁵ and 10⁸ colony-forming units (cfu/ ml) of the bacteria

as treatment. The experiment was conducted three times, with 12 roses per treatment.

The ability of *Bacillus subtilis* to control *B. cinerea* at several inoculum densities was determined by spraying of *Bacillus subtilis* with solutions containing 10⁵, and 10⁸ conidia per milliliter. In control treatments, *B. cinerea* was sprayed at these rates on roses that had not been treated with the bacteria. There were eight roses per treatment, and they were maintained in plastic boxes as in other experiments. Lesions caused by *B. cinerea* in the presence of *Bacillus subtilis* were counted after 1 days of incubation and compared with the numbers of lesions observed when *Bacillus subtilis* was not applied (in control treatment: water-sprayed and fungi).

For the CAT assay, frozen tissues (1 g) were ground in cold 50mM potassium phosphate buffer (pH 7.0). The supernatant obtained after centrifugation at 4 °C was used to determine enzyme activity. CAT was measured by monitoring the decrease in hydrogen peroxide (H₂O₂) at 240nm (11, 12).

For the determination of POD activity, petal (1.5 g) was mixed with 8 mL of ice-cold Ascorbate 10 μM, hydrogen peroxide 10 mM, and 10 ml of extract, ground thoroughly and centrifuged as described above. POD activity was evaluated following the method of Hemeda and Kelin (1990). POD was measured by monitoring the decrease in hydrogen peroxide (H₂O₂) at 240nm.

4. Statistical analysis

The experiments were done in factorial based on completely randomized design (CRD). Data were analyzed by using MSTATC. In order to determine significances, Duncan Multiple Test will be done (5%, 1% level)

RESULTS

1. The CAT activity

The CAT activity was significantly ($P \leq 0.01$) increased by treatment with different antagonist concentration, when compared to the control. In addition, the effect of time is significant on enzyme activity (**Table 1**).

Enzyme activity in treated Roses with antagonist showed a significant increase compared to control infected roses (fungi) and flowers water-sprayed cut flowers (control) on the third day, whereas there were not significant on the first day.

CAT activity was higher ($P \leq 0.01$) in petal of plants, which were sprayed, with different

concentrations of antagonism compared to water-sprayed plants for 5 and 7 days (**Table 1**). The highest CAT activity in petal was observed 7 days after antagonist spray (3.52 and 2.93 $\mu\text{mol H}_2\text{O}_2 \text{ red min}^{-1} \text{ mg}^{-1}$ protein with 10^5 and 10^8 (B₁, B₂), respectively). However, enzyme activity decreased with increasing time in water-sprayed cut flowers control (**Figure 1**). Enzyme activity in the first three days had no significant difference (**Table 1**). It was concluded Botrytis increased CAT activity in the Rose after the fifth day; but the antagonist increased it of the third day (**Figure 1**).

2. The POD activity

The POD activity was significantly ($P \leq 0.01$) increased by treatment with antagonist concentration, when compared to the control. In addition, the time has significant effect on enzyme activity (**Table 1**).

The highest relative POD activity was obtained in cut flowers sprayed in 10^5 - and 10^8 antagonism (0.97 and 0.87 $\mu\text{mol H}_2\text{O}_2 \text{ red min}^{-1} \text{ mg}^{-1}$ protein) with 10^5 and 10^8 (B₁, B₂) 5 and 7 days after spraying, respectively. Control flowers did not show significant increase in the enzyme activity at 7 days compared to the previous days (**Figure 2**). In the first three days, no significant difference has been observed in enzyme activity in infected control flowers (fungi) but there was a significant

decrease on the seventh day. The flowers treated with the antagonist rising seen after the 3 day (**Table 1**).

3. Vase life

In this experiment, the lifetime days from spraying to aging was measured.

Vase life of cut Rosa determined by observing senescence symptoms, i.e., in-rolling of petals or wilting of one third of petals in each flower. The vase life was terminated on day 3, 5 and 7, when cut flowers were treated with 10^5 , 10^8 or respectively compared to same days in control (fungi and water sprayed) (**Figure 3**).

There were significant differences between treatment vase life ($P \leq 0.05$) (**Table 1**). The longest vase life observed when it was treated with 10^8 cfu mL^{-1} antagonist spray. Flower vase life of became short when it was treated with water. The vase life of rose cut flowers was extended by the different concentrations of antagonism used in and **Figure 4**. The vase life was longer in 10^8 , which resulted in 7.11 days compared to other concentrations.

Fungi treatment resulted in the lowest period to reach wilting percent. Thus, wilting occurred on the 3-rd day after treatment with fungi compared to 4 days in control (**Figure 3**).

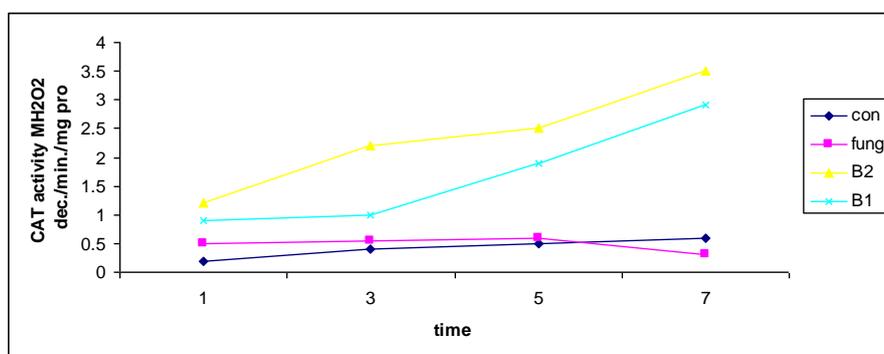


Figure 1. Changes in CAT activity in *Rosa hybrida* cv. Baccara

Con: control flower (only sprayed with distilled water); Fungi: infected control flower (only sprayed with *B.cinera*); B1: Infected flower sprayed with 10^5 -cfu mL^{-1} ; B2: Infected flower sprayed with 10^8 -cfu mL^{-1}

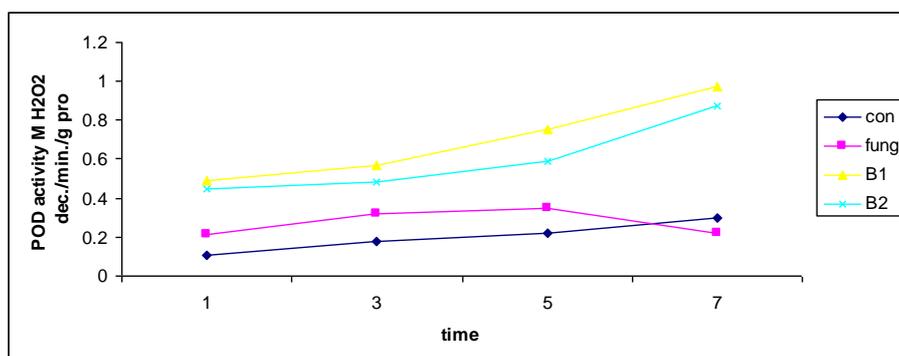


Figure 2. Changes in POD activity in *Rosa hybrida* cv. Baccara

Con: control flower (only sprayed with distilled water); Fungi: infected control flower (only sprayed with *B.cinera*); B1: Infected flower sprayed with 10^5 -cfu mL^{-1} ; B2: Infected flower sprayed with 10^8 -cfu mL^{-1}

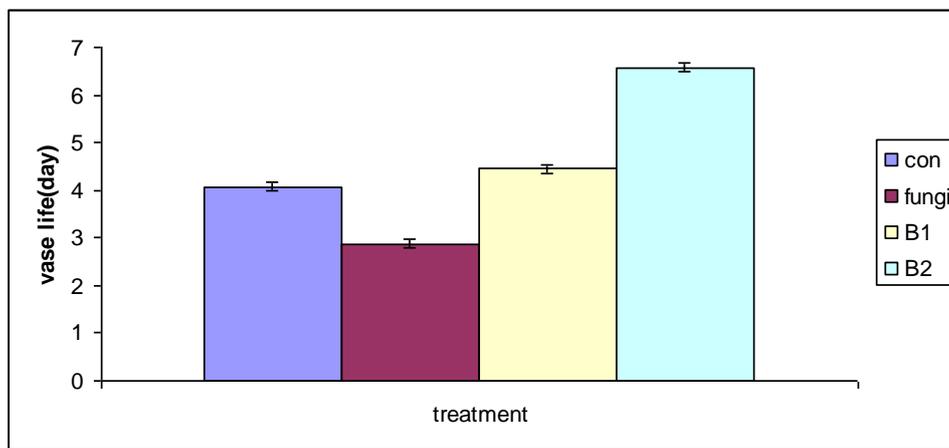


Figure 3. Vase life after spraying

Con: control flower (only sprayed with distilled water); Fungi: infected control flower (only sprayed with *B.cinera*); B1: Infected flower sprayed with 10^5 -cfu mL^{-1} ; B2: Infected flower sprayed with 10^8 -cfu mL^{-1}

Table 1. Analysis of Variance for Vase life, CAT & POD activity

Source	DF	MS	MS	MS
		CAT	POD	vase life
con	3	1.38991*	0.54654**	24.7774*
time	3	0.10528**	0.34297*	0.3053*
con*time	9	0.55935**	0.01321**	0.1349**
Error	32	0.0294	0.01829	0.0219
Total	47			



Pictutre 4. Effects of treatment on vase-life and development of cut Rose flowers, letter inside present treatment: B₁, B₂, control, fungi

DISCUSSION

Some plants show a hypersensitivity reaction to prevent the development of bacteria as too much ROS in infected areas causing cell death (13). Following, an acquired resistance occurs in infected cells areas. As a result, the cell walls can be firm and phytoalexins accumulate. Various proteins are active; including antioxidant enzymes and their pathogenesis-related protein (14).

Antioxidative enzymes such as SOD and CAT are one of the most efficient systems, which protect the cells against the risk of ROS (15).

With developing senescence, much more H_2O_2 accumulates in petals (Hossain et al., 2006). Although, CAT control H_2O_2 levels due to its scavenging and detoxifying properties (16).

This enzyme has a dual role and is able to react with oxygen and produce phosphoglycolate

instead of phosphoglycerate, which ultimately leads to the release of CO_2 . Therefore, increasing the levels of catalase (remove H_2O_2), reduce photorespiration and CO_2 compensation. In this experiment, CAT activities were

measured in petals and increased sharply with time and concentration (17).

An increase in CAT activity with time and concentration was also reported in petals of Edrisi *et al* (18).

Peroxides are involved in the building process a wall of a cell, such as phenol oxidation, suberization and lignified plant cells, which leads to the defense against disease (19). Increase in peroxidase activity associated with the induction of resistance (20). Too late plants

defending result in easily colonized plant tissues by disease (21).

De Gara *et al* indicated that peroxidase reactions reduce the infiltration rate of the disease in lignin polymerization complex structure of the cell wall and cell wall hardening (22). Nevertheless during pathogenesis Active Oxygen Species at high concentrations may cause dissociative reactions, lipid peroxidation and cell membrane damage, degradation of proteins and turned off chlorophyll. Thus, antioxidant activity is necessary to maintain AOS relatively at low levels (23).

Reduction POD activities of infected plants may due to the accumulation ROS, which in turn decline the ability of the antioxidant defense system, resulting in enzyme activity decreased. Farahan and Etebarian found that peroxidase activity be able to be a reason for induction of antifungal-like substances that inhibit fungal development in tissue and defense-related enzymes (24).

Plant defense systems against this active oxygen contain enzymes that are able to shift, scavenging and neutralize free radicals and oxygenated intermediaries. Enzymes such as (POD), (SOD) and CAT, which appear various isoforms in the cells have different features and can, improve scavenging reactive oxygen species (ROS) (25).

Plant peroxidases are catalyst reduction of H_2O_2 in their daily activity cycle (26).

Antagonism is able to increase the vase-life of cut rose flowers and delay senescence by increasing the scavenging capacity of cells.

Reactive oxygen species also are other important factors that speed up the aging process (27). ROS damage the cell membrane and cause various interactions which leads to cells destruction. Vase life of cut flowers can be influenced by compounds that neutralize ROS or cause defects in its production mechanisms. It has been found that the pattern of activity of antioxidant enzymes and improving antioxidant capacity of cells is a very important factor in controlling the aging process (28).

CONCLUSION

In order to establish the most suitable condition for biological control of gray mold we tried some concentrations of *Bacillus subtilis*. CAT, POD activity and vase life of cut Rosa determined. All traits increased by

spraying *B. subtilis*. The results concluded from the investigation will be useful for the users including greenhouse managers to improve the health of their roses.

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