

Effect of *Bacillus Subtilis SY1* on antifungal activity and plant growth

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Abstract: Agriculture soil in some areas of China is seriously damaged due to years of irrational farming practices. Soil-borne disease is a major problem of soil pollution, which affects yield and quality of agricultural products. Ecological remediation of soil is an effective way to solve this problem. In this study, *Bacillus subtilis SY1* was successfully used to antagonize several normal fungal pathogens in eggplant. The growth and pathogenic tolerance of the host plant were improved after inoculation. In the seedling test, sprout tendency, accumulative germination percentage, sprout index, and vigour index of seeds increased 24%, 24%, 35%, and 64%, respectively. Inoculation also made the seedlings stronger and improved their plant-morphologic characters significantly. When infected by fungal pathogen, the activity of protective enzymes in inoculated seedlings improved, which helped lessening membrane damage by superoxide anion.

Key words: fungal pathogen, antagonism, growth promotion, protective enzymes

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1 Introduction

The excessive use of chemical pesticides and chemical fertilizers in modern agriculture has resulted in deterioration of soil fertility and emergence of

pesticide-resistant mutants of insects and plant pathogens worldwide^[1,2]. To cope with these problems, an environmentally friendly way-biological control using antagonistic microorganisms is becoming more and more attentive, and much research has been carried out in recent years^[3,4]. The inhibitory effect of *Bacillus subtilis* on plant pathogenic fungi has been frequently reported in laboratory, greenhouse, and field studies^[5-7]. *B. subtilis* is able to synthesize more than 60 different types of antibiotics, mainly in polypeptides, many of which possess antifungal effects and belong to the iturin family^[8]. Besides the anti-fungal effects, some compounds produced by *B. subtilis* may also act as plant growth promoters^[9]. *Bacillus subtilis SY1* was used in the experiments to determine its biocontrol effect to some pathogenic fungi in vegetable soil. The eggplant planting experiments indicated that *B. subtilis SY1* not only antagonized several pathogenic fungi but also promoted the seedling growth and increased its stress tolerance.

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2 Materials and methods

2.1 Materials

1) Microbial agents

B. subtilis SY1 was maintained on plate count agar (PCA) at 4°C before the experiments.

The eight vegetable plant pathogens were provided by the Vegetable Research Institute of the Academy of Agriculture Sciences in Tianjin, China. The fungal pathogens were maintained on potato dextrose agar (PDA) medium at room temperature in the darkness.

2) Soil

The soil used in this study was taken from a field in Tianjin. After being sieved through an 8-mesh (about 2-mm pore size) screen and air-dried, the soil and vermiculite were mixed in the ratio 4:1 (wt/wt). The prepared soil was kept in plastic bags at room temperature.

2.2 Experimental method

2.2.1 Antagonistic test

In vitro antagonistic examination, the antifungal activity of *B. subtilis SY1* was tested against eight typical pathogen diseases on the PDA media. Spore suspensions of different fungi (five-day-old cultures with a concentration of more than 10^6 cfu/mL) were prepared in 0.85% sterilized saline. Ten-mL melted solid water-agar medium was poured into the plates, and then the sterile stainless steel columns were put on the cooled solid agar. When another 10-mL melted solid PDA medium was cooled to 40-50°C, 1-mL spore suspension of each fungus was added and well-mixed with the PDA medium. The mixture was then put onto the water-agar medium plates using transfer pipet. When the agar had cooled to become solid, the stainless steel columns were taken away and 50 μ L *B. subtilis SY1* suspensions were put into the hole except for the controls that had 50 μ L sterilized saline instead. Each treatment was repeated three times. The plates were then incubated at (28 \pm 1)°C for seven days and the diameters of fungal inhibition ring were detected. After that, the mycelia on the edge of the inhibition ring were inoculated on the sterile PDA agar medium and incubated at (28 \pm 1)°C for three days. Through microscopic examination, if the mycelia grow

that means the antagonistic effect between *B. subtilis SY1* and the fungi is inhibition; if not, it means the effect is sterilization.

2.2.2 Seed germination test

The seeds used in this test were first sorted to ensure that they were uniform in size and breed. They were then sterilized with 70% ethanol for 7 min and rinsed 3 times with sterilized water. After that, batches of 100 seeds prepared to be inoculated were soaked for 8 h in a *B. Subtilis SY1* bacterial suspension at a concentration of approximately 3×10^8 cfu/mL in 0.85% sterilized saline. Control seeds were soaked in sterile water for the same period of time. Then seeds were placed evenly on water-soaked filter paper in petri dishes and incubated in a chamber at (28 \pm 1)°C and 80% relative humidity for a photoperiod of 16 hours. Experiments were repeated for three times. The standard of germination based on the embryo length of each seed was longer than 2 mm. The germination of seeds were detected every 24 h and the sprout tendency were measured on the third day. The germination percentage, sprout index, vigor index and the length of radicle were determined after seven days. The parameters of germination can be expressed as accumulative germination percentage using

$$G = Ga/Gn \times 100\% \quad (1)$$

where *Ga* is the amount of the germinated seeds on the seventh day and *Gn* is the amount of the total seeds.

Sprout index

$$GI = \sum(Gt/Dt) \quad (2)$$

where *Gt* is the amount of the seedlings on the *t*th day, while *Dt* is the amount of incubated time.

Vigour index

$$VI = S \times \sum Gt/Dt \quad (3)$$

where *S* is the length of radicle after seven days.

The sprout tendency is the amount of germinated seedlings on the third day.

2.2.3 Plant test

Uniform germinated control and germinated inoculated seeds were selected and sown into the pots respectively. Each pot contained 20 imbibed seeds, planted at a depth of approximately 1 cm below soil surface. Twenty-five mL of water or *B. Subtilis SY1* bacteria suspensions were added to each microcosm

before they were placed in a random design into a growth cabinet set to maintain the temperature at 28°C, a 95%-relative humidity and a photoperiod of 16 h. The plants were irrigated with water every other day. At 6 and 12 days after sowing, 20-mL *B. Subtilis SY1* bacterial suspension at 10^8 cfu/mL was added as a drench to the roots of each plant except for the controls where water was added. After 20 days some of the seedlings of each treatment were dug out carefully, seedlings of inoculated and controlled were selected randomly, and then the plant-morphologic characters in the leaves were measured. Each experiment was repeated for three times. After that 50 mL of the pathogen cell suspension at 10^3 cfu/mL (*Fusariumoxysporum fsp melongenae Matuo etSchl.*) was irrigated into the left pots. After 5 days, wilting and some disease spots turned up to the seedlings. The enzymes and concentration of MDA, chlorophyll, and carotenoid in the leaves were determined. The plant-morphologic characters and the enzymes were determined according to the methods reported by Hesheng Li^[10] and Qi Zou^[11].

3 Results and discussion

3.1 Effect of *B. subtilis SY1* on fungi

B. subtilis SY1 had a certain degree of antagonism to all of these eight fungal pathogens. The antifungal activity of *B. subtilis SY1* is shown in Table 1 and Figure 1. The radius size indicates the antagonistic activity. The bigger the size, the stronger the antifungal activity is. Under

the same condition, the antagonism of *B. subtilis SY1* to the B (*Alternaria solani*(*Ell.et Mart.*)), F (*Fusariumoxysporum f.sp melongenae Matuo etSchl.*) and H (*Fusarium oxysporum (Schl.) f. sp. Lycopersici (Sacc.) Snyder et Hansen*) were significant and the average inhibition radius were more than 15 mm. Next were C (*Pythium aphanidermatum*) and G (*Botrytis cinerea Pers.*), and followed by A (*Phytophthora parasitica Dast.*), D (*C. gloeosporioides (Penz.) Sacc.*) and E (*Verticillium dahliae Klebahn*). Meanwhile, it can be found that the antagonistic effect maintained for a long time without decrease. After 10 days of incubation the pathogenic mycelia did not cover the surface of the inhibition ring, which indicates the antagonism was very strong^[12].

Table 1 Antagonistic activity of *B. subtilis SY1* to 8 common plant pathogenic fungi

Pathogenic Fungi	Antifungal activity	Antifungal effect
A: <i>Phytophthora parasitica Dast.</i>	++	sterilization
B: <i>Alternaria solani</i> (<i>Ell.et Mart.</i>)	+++	Inhibition
C: <i>Pythium aphanidermatum</i>	+++	sterilization
D: <i>C. gloeosporioides (Penz.) Sacc.</i> ,	++	Inhibition
E: <i>Verticillium dahliae Klebahn</i>	+	Inhibition
F: <i>Fusariumoxysporum f.sp melongenae Matuo etSchl.</i>	+++	Inhibition
G: <i>Botrytis cinerea Pers.</i>	+++	sterilization
H: <i>Fusarium oxysporum (Schl.) f. sp. Lycopersici (Sacc.) Snyder et Hansen</i>	+++	sterilization

Note: "+", "++", "+++", and "++++" represent the average inhibition radius are less than 5 mm, between 5 and 10 mm, between 10 and 15 mm, and more than 15 mm, respectively.

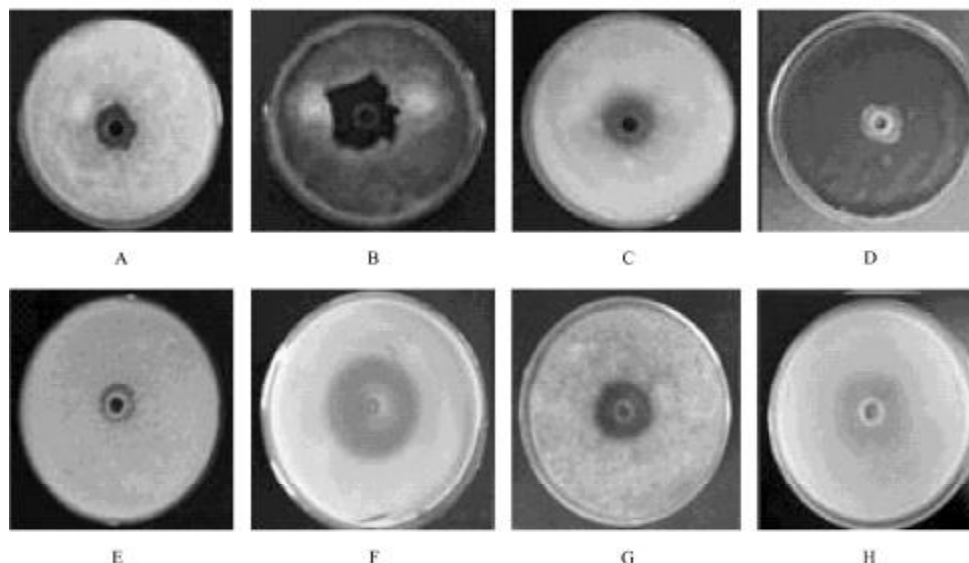


Figure 1 Antagonistic activity of *B. subtilis SY1* to 8 common plant pathogenic fungi

The results of further antifungal test showed that *B. subtilis SY1* had the sterilization effect on A (*Phytophthora parasitica* Dast.), C (*Pythium aphanidermatum*), G (*Botrytis cinerea* Pers.) and H (*Fusarium oxysporum* (Schl.) f. sp. *Lycopersici* (Sacc.) Snyder et Hansen) whose mycelia did not grow any more after inoculating onto the fresh PDA medium, while on another four fungi the antagonistic effect were inhibition, abnormal morphology of the mycelia was found. Through microscope it was clear to see that, compared with normal mycelia, expanding bubble was formed at the acme of inhibited mycelia and then breached for the antagonized mycelia. Most of the abnormal mycelia swollen and broken, and the material of the cell let out. Although what antifungal material *B. subtilis SY1* produced was not known, it antagonized all of these eight normal pathogenic fungi that always happen on Solanaceous plant, which means its potential good application in the farming land.

3.2 Seed germination characteristics

The accumulative germination percentage means the number of the total germinated seeds after incubation. As can be seen from the data in Table 2, this index was 80 for the inoculated seeds and 68 for the control. The sprout tendency and sprout index are important indices of seed germination. In this test they were 62 and 31.13 after inoculated, 24% and 35% higher than that of the control, respectively. Vigour index is another important parameter, which is the synthetical evaluation of the seed germination. The higher this value is, the stronger the seed is and the higher seed germination percentage is. After inoculation, the vigour index of the seeds increased from 127.5 to 209.53, 64% higher than that of the control. From the results above it can be seen clearly that inoculating *B. subtilis SY1* promoted the germination and early development of eggplant seeds, which would lay a good foundation for the seedling growth. The results obtained are consistent with those of some earlier researches^[13,14].

Table 2 Germination of eggplant seeds under different treatments

	1 d	2 d	3 d	4 d	5 d	6 d	sprout tendency	Accumulative germination percentage	sprout index	Vigour index
control	0	26	24	8	0	0	50	68	23	127.5
inoculated	0	46	16	8	4	0	62	80	31.13	209.52

3.3 Seedling test

The impact of *B. Subtilis SY1* inoculants on seedling growth promotion was determined in this study. After inoculation, the plant morphology and antioxidant enzyme in the seedlings were better. It is shown in Figure 2, compared with control, inoculated seedlings showed enhanced shoot and root development, enhanced greening and lateral root formation. Whether it produced plant hormones is not known, but promotion of lateral root formation is a typical auxin effect^[15]. Obviously, enhanced lateral root formation increases the capacity to take up nutrients. The mean root length, stem length, stem thickness, leaf area, root total absorption area, root active absorption area, lateral roots quantity and plant dry weight were enhanced by 114.29%, 33.33%, 18.18%, 56.5%, 0.76%, 1.33%, 33.33% and 34.15%, respectively, as compared to the un-inoculated control (Table 3). Inoculation of *B. Subtilis SY1* promoted the growth of seedlings, improved the ratio of

stem to root, and increased the biomass of seedlings. The larger leaf area played an important role in better photosynthesis and respiration.

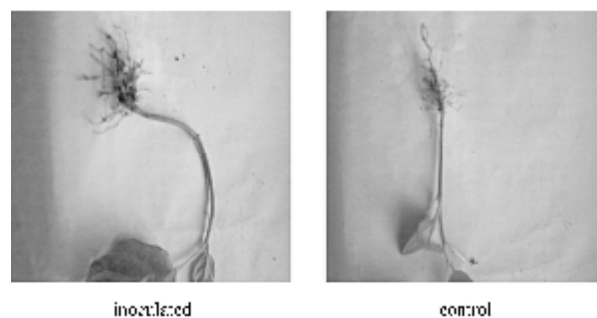


Figure 2 Root morphology of the seedlings after 20-day growth

Antioxidant protective enzyme activities have been used to document the ecological effect of agent *B. Subtilis SY1* applied to soil cultivation and plant growth. They have been proposed as a tool to monitor changes in stress tolerance of the host plant after inoculation^[16]. In these experiments, the authors detected activity and

concentration changes of MDA, PAL, PPO, SOD, CAT, POD, chlorophyll and carotenoid in leaves of eggplant

seedlings before and after infection by *Fusariumoxysporum f.sp melongenae* Matuo etschl.

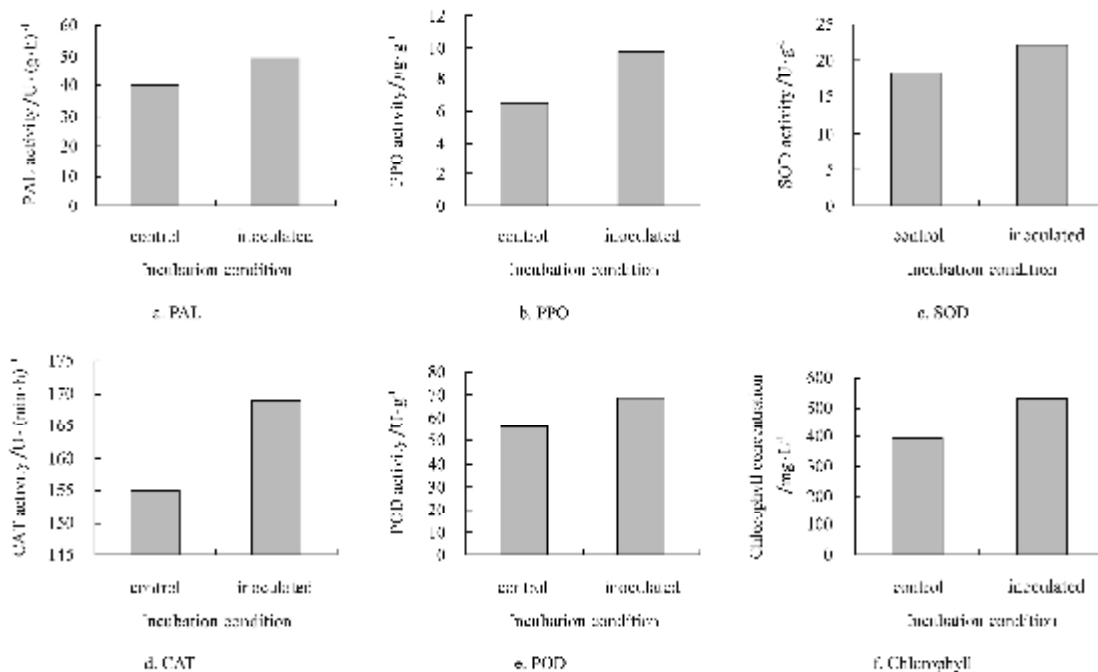
Table 3 Growth of eggplant seedlings under different treatments

Index	Mean root length /mm	Stem length /mm	Stem thickness /mm	Leaf area /mm ²	Root total absorption area /mm ²	Root active absorption area /mm ²	Lateral roots quantity	Plant dry Weight /g
Control	14	24	1.1	1.0055	0.3672	0.1875	3	0.0082
Inoculated	30	32	1.3	1.5736	0.37	0.19	4	0.011
Increase ratio /%	114.29	33.33	18.18	56.50	0.76	1.33	33.33	34.15

The results showed that the activity of PAL, which has a close relationship with plant's resistance to pathogen^[17], increased 22% after inoculation (Figure 3a). The higher the PAL activity is, the faster the metabolism of the p-hydroxyphenylpropane unit is, which leads to the synthesis of disease-resistance secondary metabolites such as lignin and phenolic compounds. In Figure 3b it can be seen that the activity of PPO was 6.54 $\mu\text{g/g}$ of the control, 33% lower than that of the inoculated. PPO is the main enzyme that can oxidize the phenolic substance to quinoid substance, which gives effect to sterilize or inhibit the reproduction of pathogen^[18,19].

As all know, the active oxygen in the plant would be accumulated after being infected by the pathogen, while the SOD has the ability to promote the active oxygen metabolism, which can clear the free radicals and protect the plant against injury^[20]. Figure 3c shows that the

activity of SOD increased 21% compared with the un-inoculated control. CAT and POD are another two enzymes, which have effects on oxygen free radical-scavenging. The activity of CAT in the plant after inoculation was 169 U/(min·g) (Figure 3d) which was 9% higher than that of the un-inoculated. The stronger the activity of CAT is, the higher ability of clearing the H₂O₂ and MDA. Besides, the POD is also involved in synthesis of lignin and oxidation of IAA, both of which contribute to the disease defense of plant^[21]. The activity of POD increased 23% after inoculation in the experiment. It can be seen from Figure 3h, due to the protective effect of the antioxidant enzyme, the accumulation of MDA was weakened after inoculation when infected with pathogen. The concentration of MDA was 1.71 $\mu\text{mol/g}$, which decreased by 26%.



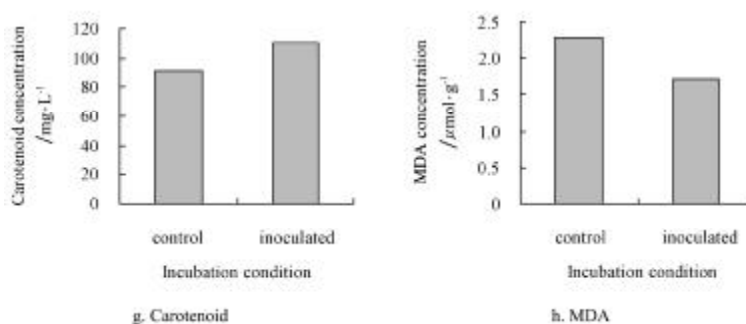


Figure 3 Changes of activity of MDA, PAL, PPO, SOD, CAT, POD, chlorophyll and carotenoid in leaves of eggplant seedlings after infected by *Fusariumoxysporum* f.sp *melongenae* Matuo etschl.

Chlorophyll and carotenoid play important roles in photosynthesis processing. The radiant energy absorbed by carotenoid was transferred to chloroplast and finally converted into steady chemical energy for plant via photochemical processes. In this test, the concentrations of chlorophyll and carotenoid increased 34% and 22% after inoculation, respectively. The above results showed that the inoculation of *B. subtilis* SY1 strengthened the pathogen resistance of the plant, which was expressed in terms of increase in antioxidant enzyme activity and decrease in MDA concentration. It can therefore be concluded that inoculation *B. subtilis* SY1 kept the seedlings in a defensive state when infected by the pathogen.

4 Conclusions

Bacillus subtilis SY1 showed antifungal effects on the eight normal pathogens to some extent in vitro; some of the effects were sterilization and some were inhibition. The reproduction of the pathogen fungi were inhibited when confronted by *B. subtilis* SY1.

In the seedling test, inoculating *B. subtilis* SY1 had a great effect on shortening germination time, raising sprout index, and promoting radical and embryo growths. The vigour index of the seeds increased 64% after inoculation, and the morphology of the seedlings improved significantly.

Besides growth promotion effect, *B. subtilis* SY1 also played an important role in enhancing stress tolerance of the host plant. The activity of protective antioxidant enzymes increased, and the concentration of MDA decreased after inoculation, which helped to keep the

seedlings away from injury.

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