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RESEARCH ARTICLE

Optimization of water dispersible granules containing biocontrol bacteria and stress alleviation on cotton

Shouyan Han[1], Chongdie Wu[1], Yongbin Fan[2], Jianwei Cao[3], and Aiying Wang[1*]

[1]Shihezi University, College of Life Sciences, Shihezi City, Xinjiang, China.
[2]Xinjiang Production and Construction Corps Key Laboratory of Oasis Town and Mountain-basin System Ecology, Shihezi City, Xinjiang, China.
[*]Corresponding author (way-sh@126.com).
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ABSTRACT

Cotton (*Gossypium hirsutum* L.) is economically important in Xinjiang, China, for many years; Verticillium wilt, arid climate, and soil salinization reduce cotton production, which has inhibited development of the cotton economy. Thus, creating a microbial agent specifically for cotton to resisting environmental stress is crucial. In this study, water dispersible granules strains (*Bacillus tequilensis* C-9 and *B. sphingosporium* A1) were screened for antagonistic bacteria of *Verticillium dahliae* from the rhizosphere soil of *Ferula sinkiangensis* K.M. Shen, and screened for carrier and auxiliary that could enhance the survival of strains A1+C-9. To test the efficacy and practicality of the water dispersible granules, their growth-promoting effect was examined. Meanwhile, bursts of reactive oxygen species and changes in the physiological indicators related to cotton growth were detected after the application of these granules to cotton that had been subjected to biotic and abiotic stresses to study the ability of the water dispersible granules to induce immunity in cotton. It was found that the treated group could increase the above-ground and below-ground fresh weight of cotton by 20.06% and 22.71% relative to the control group, and the biocontrol effect on cotton was increased by 46.55%. In salt and drought stress, catalase activity could be increased by 14.61% and 15.52%, respectively, relative to the control group. Comprehensive analyses showed that the application of water dispersible granules that contained A1+C-9 helped the cotton to grow and develop, inhibited *V. dahliae* from infecting cotton plants, reduced the damage from drought and salt stress, and increased the resilience of cotton to stress.

Key words: Abiotic stress, biotic stress, cotton, Verticillium wilt, water dispersible granules.

INTRODUCTION

Verticillium wilt is caused by *V. dahliae*, a soilborne bacteria, which is aggravated by years of continuous cropping (Liu et al., 2021b; Xi et al., 2021; Zhang et al., 2022a). There are no effective fungicides to control this pathogen, and it causes serious losses of crops (Song et al., 2020). Owing to the complexity of arid environments and climate change, water scarcity is one of the most serious constraints on global agricultural production (Li et al., 2019). Northwest China is an arid and semiarid region with high evaporation and a general shortage of freshwater resources (Sun et al., 2021). Drought has become one of the factors that seriously affects the production of cotton, so it is of great importance to develop water dispersible granules to help enhance the drought resistance of cotton (*Gossypium hirsutum* L.) Salt is an environmental factor that limits plant growth and reduces crop yields. The cultivation of cotton in Xinjiang, China, is dominated by drip irrigation technology, and the increasing prevalence of secondary salinization of the soil renders improvements in the tolerance of salt during the cultivation of cotton to be important for the sustainable development of agriculture. Currently there is no better solution for the control of Verticillium wilt that the improvement of resistance in cotton (Numan et al., 2018; Shen et al., 2021; Ahmad et al., 2022).

With the increasing concern over environmental pollution and food safety caused by the use of chemical pesticides in agricultural production, beneficial microorganisms are considered to be one of the most promising approaches (Olanrewaju et al., 2017; Chen et al., 2021). The biological control of plant parasitic nematodes and the control of Verticillium wilt in cotton with biocontrol agents are examples of the advantageous uses of biological control agents (Deketelaere et al., 2017; Subedi et al., 2020). In addition, microbial inoculants are a green and eco-friendly fertilizer and are an important way to achieve sustainable agricultural development. Thus, they have been widely used throughout the world.

Water dispersible granules are bacterial agents that consist of granules that disintegrate and disperse in water before being applied to the crop (Yanagisawa et al., 2017). The demand for water dispersible granules in the pesticide market has been increasing since the 1980s, primarily owing to their ability to flow well and the low risk of dust inhalation compared with traditional wettable powder formulations (Moiroux et al., 2018). Cotton cultivation in Xinjiang mostly utilizes drip irrigation, and the use of water dispersible granules with dripping water improves their applicability. The biocontrol bacteria used in the water dispersible granules in this study were *Bacillus tequilensis* C-9 and *Bacillus sphingosporium* A1. Our previous studies showed that both strains were antagonistic to *V. dahliae* and promoted growth. Strain C-9 is Gram-positive, while strain A1 is Gram-negative, and the two strains do not inhibit each other when mixed in culture (Guo et al., 2018).

In this study, the carrier and additives of the water dispersible granules and the method of application were optimized for both strains A1 and C-9. The extent of resistance of cotton to biotic stresses was also investigated when the water dispersible granules were applied. In addition, the effect of application of water dispersible granules was studied when the cotton was subjected to drought stress and salinity stress. The aim was to investigate the effect of the application of A1+C-9 in water dispersible granules on the induction of resistance and their ability to promote the growth of cotton under stress.

MATERIALS AND METHODS

Experimental strains, carriers and additives

Bacillus tequilensis C-9 and *B. sphingosporium* A1 were used as the prodrugs of the water dispersible granules, which were isolated from the inter-root soil of *Ferula sinkiangensis* K.M. Shen and were isolated and preserved by the Key Laboratory of Agricultural Biotechnology of Shihezi University, Shihezi, China. The activated and well-grown two strains of biocontrol bacteria were inoculated into 50 mL potato dextrose broth medium, and incubated at 160 r min^{-1} (Shaker incubator) and 25 °C for 16 h to make seed liquid. The seed liquid of the two strains of bacteria was inoculated into 50 mL potato dextrose broth medium for 72 h at 28 °C and 160 r min^{-1} (Shaker incubator) according to the inoculation ratio of 5%, and then the fermentation broth of a single strain was made at a concentration of 10^{10} cfu mL^{-1}. The seed solution of the two strains of bacteria prepared above was inoculated into 50 mL fresh potato dextrose broth medium according to the inoculation ratio of A1:C-9 = 1:9, and then double-mixed culture was carried out, and shaking flask culture was carried out at 160 r min^{-1} and 28 °C for 72 h, to obtain the raw bacterial fermentation solution (Guo et al., 2018).

The cultivar of cotton (*Gossypium hirsutum* L.) was Xinlu Early 7, which originated from the same source as the bacteria. *Verticillium dahliae* strain Vd278 was applied with a spore concentration of 1.0×10^7 cfu mL^{-1} and an inoculum volume of 25 mL per pot. The auxiliary candidates are shown in Table 1.

Optimization of water dispersible granules with each additive for strains A1 and C-9

Preparation of aqueous dispersion of biocontrol bacteria and optimization of the formulation scheme with reference to the method of Ming et al. (2015). The most suitable carriers, dispersants, disintegrant agents, binders and protectors for the biocontrol bacteria were screen on the basis of biocompatibility. The optimal ratio of wetting agent was selected from 2%, 3%, 4% and 5% sodium dodecylbenzenesulfonate based on its wettability. The selection of the most suitable dispersants from 3%, 4%, 5% and 6% were based on their dispersibility, suspension and disintegration time. The optimal ratio of disintegrants was selected from 3%, 4%, 5% and 6% based on their disintegration time. The optimal ratio of binder agents was selected from

1%, 2%, 3%, 4% and 5% based on their disintegration time and rate of granulation. The optimal ratio was selected from the 0.1%, 0.5%, 1.0% and 1.5% protectants based on the number of viable bacteria after 15 d of storage. Finally, the wettability, disintegration time, suspension and dispersibility and rate of granulation of the A1+C-9 water dispersible granules were measured, and a high temperature stability test was conducted.

Table 1. Drugs selected for various Auxiliary type.

Auxiliary type	Drug names
Carrier	Kaolin
	Diatomite1
	Calcium carbonate
Wetting agent	Sodium dodecylbenzenesulfonate
Dispersants	Sodium lignin sulfonate
	Sodium tripolyphosphate
	Carboxymethylcellulose sodium
Protector	Humic acid
	Carboxymethyl cellulose
	Xanthan gum
Disintegrating agent	Calcium chloride
	Ammonium sulfate
	Sodium chloride
Binder	Polyvinyl alcohol, vinyl alcohol polymer
	Polyethylene glycol
	Soluble starch

Selection of the most suitable treatment for A1+C-9 water dispersible granules
Cotton seeds were surface sterilized and planted in a 15 cm diameter nutrient bowl with autoclaved soil. The water dispersible granules were applied as clay and root irrigation at 0.5%, 1%, 1.5% and 2% in 100 mL per pot of cotton. The control consisted of the application of 100 mL distilled water. After the cotton seedlings had grown three true leaves, they were inoculated with *V. dahliae* using the wounded root infusion method. A total of 2 to 5 g leaves from each treatment were collected before and 12, 24, 36 and 48 h after inoculation with *V. dahliae* to determine the activities of peroxidase (POD), superoxide dismutase (SOD), phenylalanine ammonia lyase (PAL) and polyphenol oxidase (PPO), and the method and concentration of the applications were explored by comparing the four types of enzymes. Enzyme activity was determined with reference to the method of Tian et al. (2015).

Cotton seedlings and application of bacterial agents
We also investigated the effect of A1+C-9 water dispersant granules on cotton growth promotion, disease resistance, and resistance to drought, salinity and alkalinity. The cotton seeds were surface sterilized and planted in a 15 cm diameter nutrient bowl. At 5 d after emergence, 100 mL A1+C-9 water dispersant granules were added to each pot. The method of application and concentration of the granules were selected based on the results of the previous section, and the control group was treated with an equal amount of distilled water. The following experiments were repeated three times for each treatment.

Growth promoting effects of A1+C-9 water dispersible granules on cotton
After the cotton seedlings had grown for 30 d, their height, leaf area, fresh weight of the above- and belowground parts and the root lengths were measured, and the development of their roots was monitored.

Induction of disease resistance in cotton by water dispersible granules

Take cotton seedlings that have grown 3-4 true leaves after applying water dispersible granules were inoculated with a suspension of *V. dahliae* spores by root wounding and perfusion. The cotton leaves were collected 12 h after inoculation with the pathogen to detect the burst of reactive oxygen species (ROS) and the hypersensitive response (HR) reaction. The ROS burst was detected by staining with 3,3'-diaminobezidine (DAB) (Zhang et al., 2016), and the HR was detected by trypan blue staining. The cotton leaves were collected at 0, 12, 24, 36 and 48 h after inoculation with *V. dahliae*, and the activities of POD, SOD, PAL and PPO were assayed. We investigated the disease of cotton 15 d after inoculation with *V. dahliae*.

The criteria for grading the Verticillium wilt were as follows: Grade 0, no disease symptoms; Grade 1, there was disease on the cotyledons; Grade 2, there was one true leaf that was diseased; Grade 3, two true leaves were diseased; Grade 4, three or more true leaves were diseased, and the plant tips died, or the total plant died back.

Incidence disease (%) = (Total number of plants with disease/Total number of plants surveyed) × 100% (1)

Disease index = 100 × Σ (Disease level × Number of plants of the disease class)/(Highest level of disease × Total number of plants) (2)

Biocontrol efficiency (%) = (Disease index in the control group - Disease index in the treatment groups/Disease index in the treatment groups × 100% (3)

Effect of water dispersible granules on induced resistance in cotton under drought and salt stress

Take cotton seedlings that have grown for 30 d and have been treated with bacterial agents for drought and salt stress. The drought treatment group was watered with 100 mL 250 mmol L^{-1} mannitol every 3 d, and the salt treatment group was watered with 100 mL 200 mmol L^{-1} NaCl every 3 d. The leaves were collected at 12 h after treatment for the ROS burst assay, and the cotton plant leaves were sampled at 24 h after treatment to determine their activities of SOD and CAT and their content of malondialdehyde (MDA) (Li and Li, 2000; Tian, 2015). After 15 d of stress treatment, the cotton phenotypes were observed and photographed.

Statistical analysis

Significance analysis were carried out using SPSS 25.0 in the table (IBM SPSS Software Inc., Armonk, New York, USA). Bar graphs were plotted and significance analysis calculated using Paired Comparison Plot package in OriginPro 2022-UNTITLED (OriginLab Corporation, Northampton, Massachusetts, USA). Line graphs were plotted and significance calculated using Microsoft Excel 2021. Furthermore, the letters in the bar graphs, line graphs, and tables represent the degree of difference, when the letters are the same it means that there is nonsignificant difference between the two groups, and when the letters are different it means that there is a significant difference between the two groups, i.e., $p < 0.05$.

RESULTS

Optimized choice of carrier and additives

The results showed that diatomaceous earth had the least effect on the rate of survival of A1 and C-9. The rates of viability are shown in Table 2. Therefore, diatomaceous earth was chosen as the carrier for the development of the bacteria. As shown in Table 3, in terms of the effect on the survival rate of A1 and C-9, the five substances, sodium dodecylbenzenesulfonate as the wetting agent, sodium lignin sulfonate as the dispersant, ammonium sulfate as the disintegrating agent, polyvinyl alcohol and soluble starch as the binders, and humic acid as the protector, were more effective with an effective survival rate of more than 85% for A1 and more than 70% for C-9. Thus, it was selected as an additive formulation for further optimization.

Table 2. Rate of viable bacteria using different carriers. CK: Control.

Carrier	Number of colonies		Effective viable rate	
	A1	C-9	A1	C-9
	——— 10^{10} cfu mL^{-1} ———		——— % ———	
Kaolin	13.67 ± 2.52	6.33 ± 2.08	73.22	31.65
Diatomite1	16.33 ± 3.06	14.33 ± 1.53	87.47	71.65
Calcium carbonate	10.67 ± 2.89	6.67 ± 3.21	57.15	33.35
CK	18.67 ± 3.21	20.00 ± 2.65	–	–

Table 3. Rate of viable bacteria using different auxiliary type. CK: Control.

Auxiliary type	Auxiliary names	Number of colonies		Effective viable rate	
		A1	C-9	A1	C-9
		——— 10^{10} cfu mL^{-1} ———		——— % ———	
Wetting agent	Sodium dodecylbenzenesulfonate	18.67 ± 3.21	15.00 ± 2.00	96.59	71.43
Dispersants	Sodium lignin sulfonate	18.33 ± 2.08	17.67 ± 0.58	94.83	84.14
	Sodium tripolyphosphate	16.00 ± 1.00	14.00 ± 1.00	82.78	66.67
	Carboxymethylcellulose sodium	15.33 ± 0.58	13.33 ± 1.15	79.31	63.48
Disintegrating agent	Calcium chloride	14.33 ± 1.15	12.00 ± 2.00	74.13	57.14
	Ammonium sulfate	17.33 ± 0.58	18.00 ± 1.00	89.65	85.71
	Sodium chloride	15.67 ± 1.53	11.33 ± 2.52	81.07	53.95
Binder	Polyvinyl alcohol, vinyl alcohol polymer	16.67 ± 1.15	17.33 ± 1.53	86.24	82.52
	Polyethylene glycol	14.33 ± 2.52	15.67 ± 0.58	74.13	74.62
	Soluble starch	16.67 ± 1.15	18.33 ± 2.08	86.24	87.29
Protector	Humic acid	18.67 ± 2.52	19.33 ± 0.58	96.59	92.05
	Carboxymethyl cellulose	16.33 ± 3.06	14.67 ± 1.53	84.48	69.86
	Xanthan gum	13.00 ± 1.00	14.33 ± 1.53	67.25	68.24
CK		19.33 ± 2.08	21.00 ± 3.00	–	–

Optimization of additives

By measuring the wettability of sodium dodecylbenzenesulfonate water dispersible granules with different concentrations (Figure1a), the shortest wettability time was 50.13 s when the dosage was 3%, and the wettability time was basically stable when the dosage was 3% ~ 4%. Thus, 3% was selected as the concentration of water dispersible granules. When the binder was polyvinyl alcohol or soluble starch, there was little difference in the granulation effect of the water dispersible granules (Figure 1b), but there was a significant difference in their disintegration time (Figure 1c). The disintegration time of soluble starch was greater than 75 s for all the dosages, but the disintegration time of polyvinyl alcohol at 1% and 2% was less than 65 s. This indicates that the product disintegrates better when 1% or 2% polyvinyl alcohol is used as the binder. Therefore, polyvinyl alcohol was chosen as the protective agent. Low amounts of humic acid still protected the activity of the strains after 15 d storage (Figure 1d), but with increasing amounts of humic acid, high concentrations inhibited the number of living bacteria of the strains. In this study, the best protection was achieved when the humic acid concentration was 0.1%.

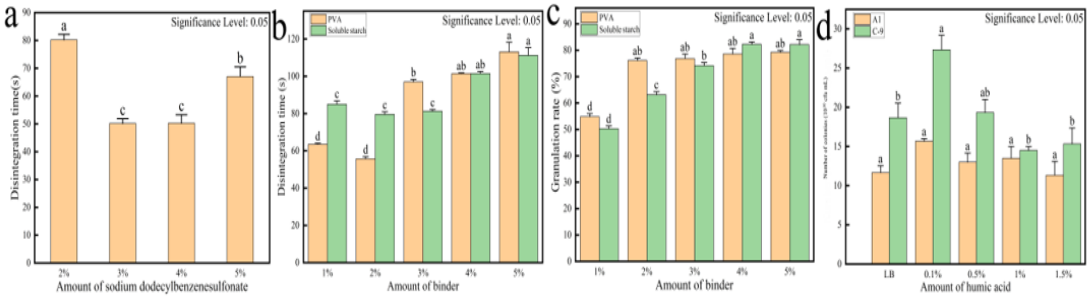

Figure 1. Histogram of optimization results for amount of wetting agent, binder and protectant. a: Wettability results using different concentrations of sodium dodecylbenzenesulfonate. b: Respective disintegration times using different concentrations of polyvinyl alcohol and soluble starch as binders. c: Results of respective granulation properties using different concentrations of polyvinyl alcohol and soluble starch as binders. d: Number of viable bacteria using different concentrations of humic acid as a protectant A1 *vs*. C-9 strains.

The dispersants sodium lignosulfonate dispersed and suspended better (Table 4). However, the disintegration time tended to decrease and then increase as the dosage increased, with the shortest disintegration time occurring at 4% of sodium lignosulfonate. Therefore, this concentration was chosen as the optimal dosage. The disintegrant ammonium sulfate also had excellent dispersibility and suspension (Table 5), but the disintegration time decreased followed by an increase with the increasing dosage. The disintegration time was the lowest when the dosage was 2%. Therefore, 2% ammonium sulfate was the most effective.

Table 4. Screening results for dispersants. Distinction (++): Thickness below 5 mm; credit (+): 5-6 mm; fail (-): Below 6 mm.

Amount	Dispersibility	Sodium lignosulfonate disintegration time (s)	Suspension
3%	Qualification	50.53 ± 2.00^{b}	++
4%	Qualification	45.73 ± 2.55^{b}	++
5%	Qualification	65.50 ± 1.91^{a}	++
6%	Qualification	68.60 ± 1.81^{a}	++

Table 5. Screening results for disintegrating agent. Distinction (++): Thickness below 5 mm; credit (+): 5-6 mm; fail (-): Below 6 mm.

Amount	Dispersibility	$(NH_4)_2SO_4$ disintegration time (s)	Suspension
1%	Qualification	65.00 ± 5.21^{a}	++
2%	Qualification	38.03 ± 6.63^{b}	++
3%	Qualification	50.47 ± 5.30^{ab}	++
4%	Qualification	62.30 ± 4.56^{a}	++
5%	Qualification	67.90 ± 9.74^{a}	++

Based on the experimental results shown above, the final optimized formulation of the A1+C-9 water dispersible granules was determined as shown in Table 6. The finished product was prepared from the optimized formulation and tested for performance as follows: Wetting time was 42.39 ± 1.15 s; disintegration time was 39.43 ± 5.95 s, and there was excellent suspension and dispersibility and 82% rate of granulation. According to the State Standard of the People's Republic of China, the bacteriological agent used in this study had excellent performance.

In addition, the high temperature stability test showed that the number of bacteria in this study was in the same order of magnitude before and after hot storage (Table 7), and the hot storage results were stable, which indicated that the formulation of these bacteria was stable.

Table 6. Optimized formulation of biocontrol bacteria water dispersible granules. A1: *Bacillus sphingosporium*; C-9: *B. tequilensis*.

Inoculant components	Name	Dosage
Raw bacterium	Raw bacterium of A1 and C-9	Number of active bacteria > 8.0×10^{10} cfu mL^{-1}
Wetting agent	Sodium dodecylbenzenesulfonate	3%
Dispersants	Sodium lignin sulfonate	4%
Disintegrating agent	Ammonium sulfate	2%
Binder	Polyvinyl alcohol, vinyl alcohol polymer	2%
Protector	Humic acid	0.1%
Carrier	diatomite1	Complementary to 100%

Table 7. High temperature stability of biocontrol bacteria water dispersible granules. CK: Control.

Number	Number of bacteria before heat storage	Number of bacteria after heat storage
	$\times 10^{10}$ cfu g^{-1}	$\times 10^{10}$ cfu g^{-1}
1	8.2	5.5
2	8.7	5.0
3	8.9	5.7
CK	9.9	4.0

Suitable methods to apply water dispersible granules

When inoculated with *V. dahliae* for 0 h after application of water dispersible granules, the activities of PAL and POD were found to be maximal when dosage was as high as 1.5% (Figure 2ac) and were significantly different from those of the control ($P < 0.05$). The enzyme activity also showed an increase and then a decrease as the concentration of water dispersible granules increased. In the case of PPO (Figure 2b), there was nonsignificant difference between control and water dispersible granules applied at various concentrations. The SOD activity was significantly higher when water dispersible granules were applied compared with control (Figure 2d), while there was nonsignificant difference between the different concentrations.

When inoculated with *V. dahliae* (Figure 2), the activity of the four enzymes tended to increase and then decrease with time and reached their maximal levels of activity at 24 h. The maximum activity was achieved at a concentration of 1.5% of water dispersible granules. In addition, different methods of application of water dispersible granules also affected activity of cotton defense enzymes to some extent.

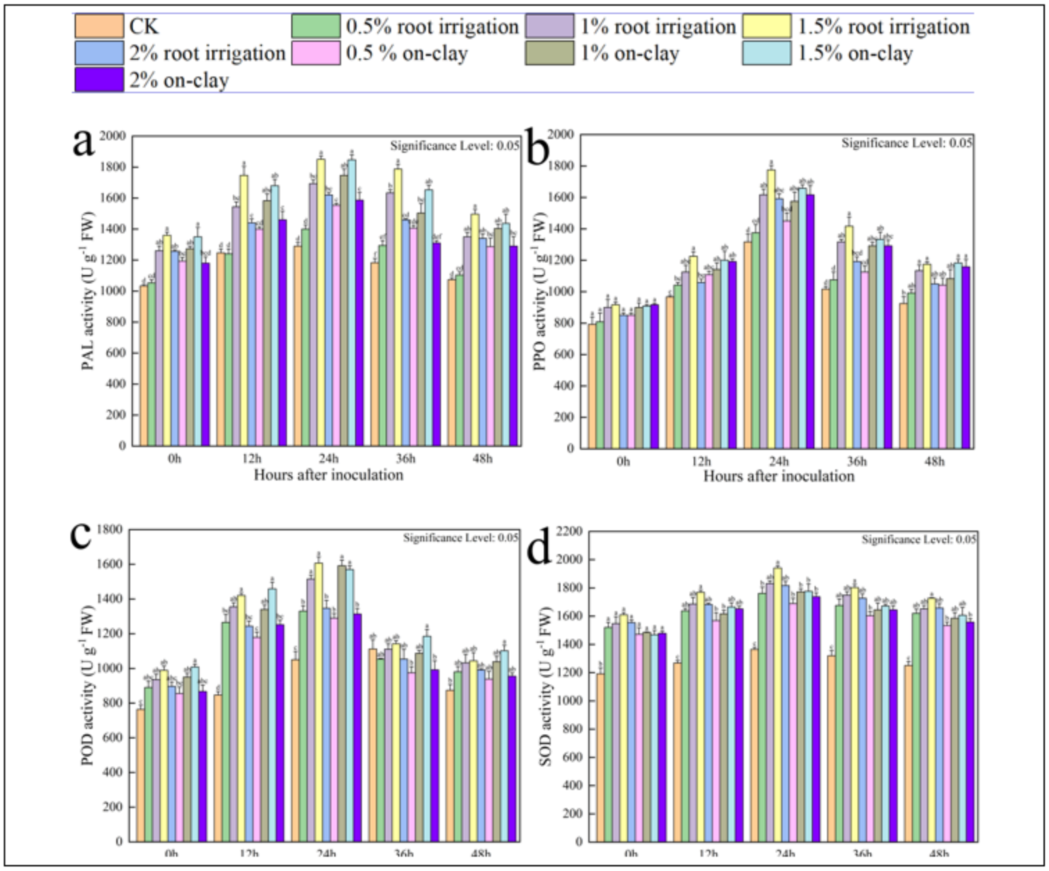

Figure 2. Effect of application of different concentrations of water dispersible granule on defensive enzyme activity in cotton. CK: Control group; PAL: phenylalanine ammonia lyase; PPO: polyphenol oxidase; POD: peroxidase; SOD: superoxide dismutase.

Promotional effect of water dispersible granules

After 30 d of growth, all the biological traits of cotton plants that had been treated with water dispersible granules improved compared with control plants (Figure 3). This was particularly significant in terms of plant height, leaf area and fresh weight. Compared with control, plant height, leaf area of the first to fifth true leaves, fresh weight of the above- and belowground parts increased by 15.94%, 29.26%, 9.16%, 21.47%, 7.24%, 72.13%, 20.06% and 22.71%, respectively, in treatment group. Although there was nonsignificant difference between control and treatment groups in terms of root length, a more developed root system was observed in the treatment group (Figure 3b).

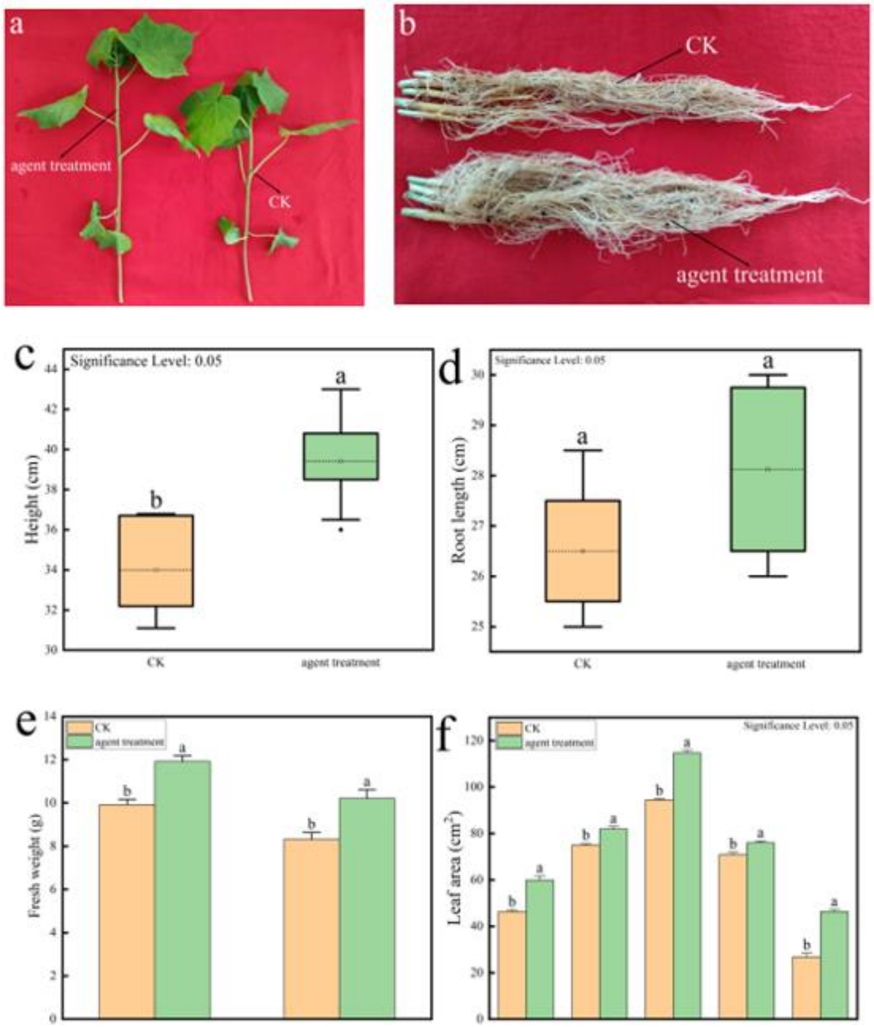

Figure 3. Growth indicators of plants after application of water dispersible granules. The a and b diagrams show growth state of cotton after the application of bacterial agents, CK is the control, The c, d, e graphs are histograms of plant height, root length and fresh weight of cotton respectively, f is the leaf area, where 1, 2, 3, 4 and 5 represents the first to fifth true leaves of the plant.

Induction of disease resistance in cotton by application of water dispersible granules

More brown material was observed in leaves of cotton plants treated with water dispersible granules after DAB staining, while less precipitated brown material was observed in the control group. This showed that application of water dispersible granules enhances the burst of ROS in cotton plants (Figure 4), thus enhancing the disease resistance of cotton. Less blue precipitation was observed in leaves of control cotton plants after trypan blue staining, whereas blue precipitation in leaves of cotton plants treated with water dispersible granules increased significantly compared with the control group. This indicates that water dispersible granules enhance the HR response in cotton. Therefore, promoting programmed cell death of cotton cells infected with *V. dahliae* inhibited proliferation of this pathogen (Figure 4).

These findings were consistent with previous results that showed that basal activity of the four defense enzymes was significantly higher in cotton treated with water dispersible granules than that in control (CK) (Figure 5). All four defense enzyme activities in treatment group reached their maximum at 24 h, while all defense enzyme activities in control group reached their maximum at 36 h except for PPO, which reached its maximum at 24 h, indicating that treatment group responded more intensively and rapidly to infection by *V. dahliae* (Figure 5).

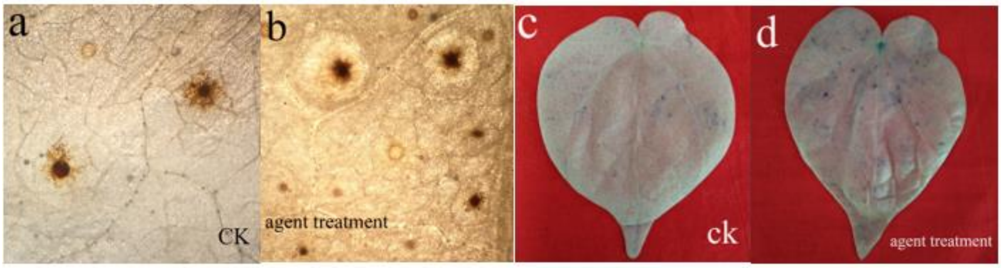

Figure 4. Detection of reactive oxygen species and cell death in cotton leaves. CK: Control.

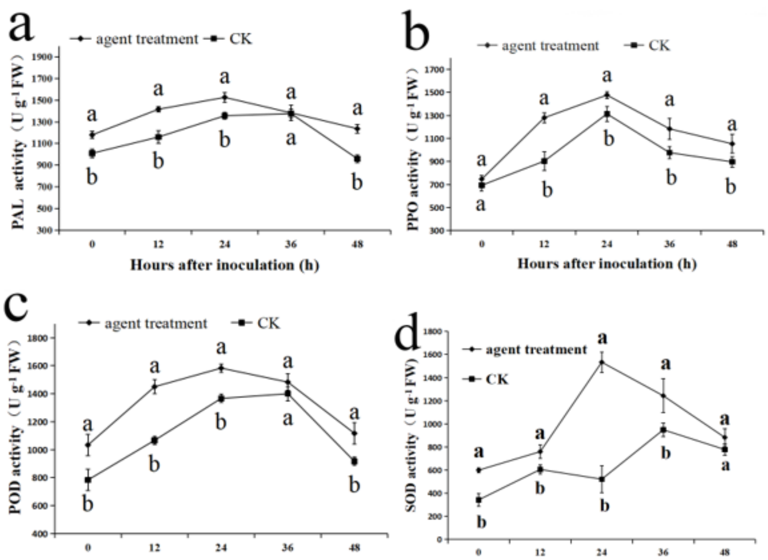

Figure 5. Effects of biocontrol water dispersing agent on the defense reaction system of cotton. CK: Control group; PAL: phenylalanine ammonia lyase; PPO: polyphenol oxidase; POD: peroxidase; SOD: superoxide dismutase.

After 15 d of infection of cotton with *V. dahliae*, the degree of disease in cotton was significantly reduced after treatment with the bacterial agents (Figure 6). The rate of incidence of disease of cotton plants in control group that were not treated with water dispersible granules was 63.33%, and the disease index was 48.33. In contrast, the rate of incidence of cotton plants in treated group was 33.33%, and disease index was 25.83. Thus, there was a biocontrol effect of 46.55% against Verticillium wilt of cotton. In addition, the number of plants that reached grade 3 and 4 in control group was higher than that in group treated with water dispersible granules.

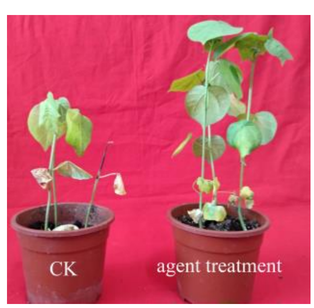

Figure 6. Effect of potted experiment of biocontrol water dispersing agent. CK: Control.

To investigate association between water dispersible granules and disease index, a correlation analysis between defense enzymes and disease index was conducted using SPSS 25.0, which showed a negative correlation between the disease index and enzyme activity. This indicates a negative correlation between disease index and application of water dispersible granules (Table 4).

Effect of water dispersible granules on induced resistance in cotton under drought and salt stress

The cotton was stained after 12 h of stress to observe the burst of ROS in leaves, and more brown material was observed in both groups treated with drought and NaCl in cotton leaves that were treated with water dispersible granules (Figures 7b-7d). In contrast, there was less brown precipitated material in the control group (Figures 7a-7c). This indicates that the application of water dispersible granules enhances burst of ROS in leaves of cotton plants, thus improving the resistance of cotton when subjected to adversity.

Observing the phenotypic differences in cotton revealed that all the cotton plants grew normally before the stress treatment, but cotton plants in treatment group grew and developed better than those in the control group (Figure 8a). After the mannitol and NaCl stress treatments, cotton plants showed some degree of wilting, but wilting was more severe in plants that had not been treated with bacterial agents.

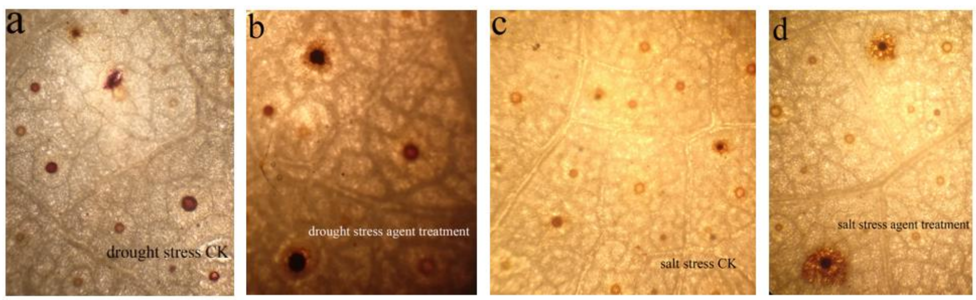

Figure 7. Reactive oxygen species assay in the leaf of cotton. CK: Control.

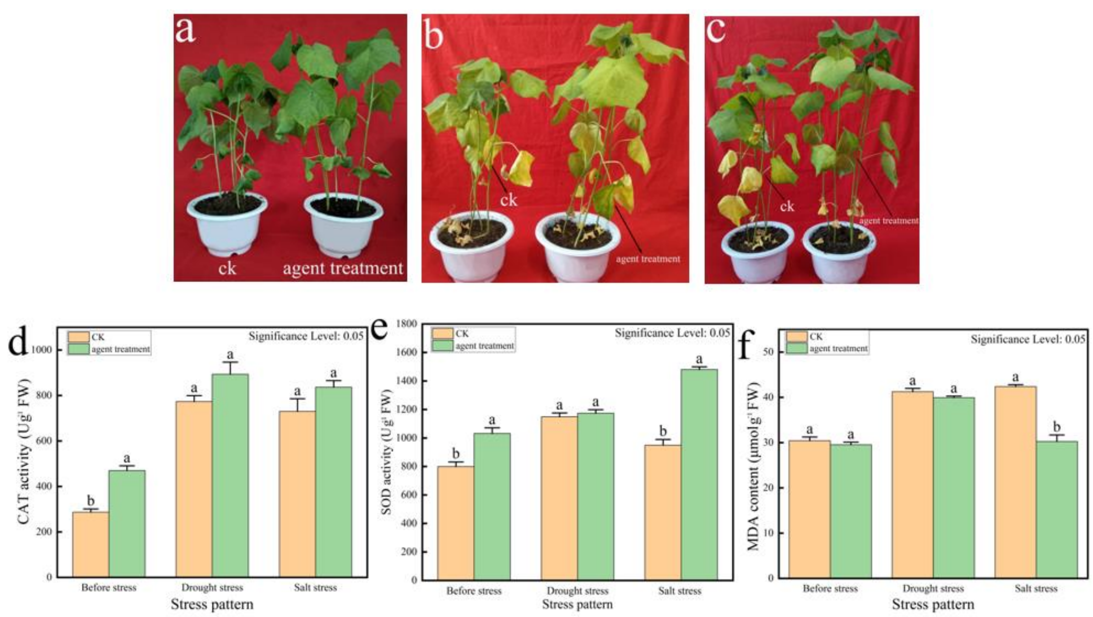

Figure 8. Changes in growth and enzyme activity of cotton under stress. The a picture shows before stress, b picture shows drought stress, c picture shows salt stress, d, e, f pictures show changes in cotton enzyme activity under stress. CK: Control group; CAT: catalase; SOD: superoxide dismutase; MDA: malondialdehyde.

The CAT activity of cotton plants treated with water dispersible granules increased by 63.95% compared with untreated CK group before the stress treatment. The SOD activity of cotton plants treated with water dispersible granules increased by 29.02% compared with that of the untreated CK group (Figure 8). After 24 h of stress treatment, there was nonsignificant difference in CAT activity between control and treatment groups for drought stress and salt stress (Figure 8d), while there was nonsignificant difference in SOD activity, as well as MDA content, between drought treatments and control groups (Figures 8e-8f). In salt stress, there was nonsignificant difference between the CAT activity in the group treated with water dispersible granules and control group (Figure 8d), while the SOD activity in the group treated with water dispersible granules increased by 56.02% compared with that of control group (Figure 8e). The MDA activity increased significantly by 28.64% in the control group compared with group treated with water dispersible granules.

DISCUSSION

Water dispersible granules are cheaper to produce, easy to use, can be stored for a long time, and are highly stable. The water dispersible granules in this study were a live preparation of bacteria, so there were stringent requirements for carrier and additives. This study was based on biocompatibility screening, and a suitable carrier for water dispersible granules, diatomaceous earth, was screened. The primary component of diatomite (Shanghai Macklin Biochemical Company, Shanghai, China) is SiO_2, which has a microporous structure, good absorption, a large specific surface area, good thermal stability, strong adhesion and a protective effect on microorganisms. Thus, it is one of the common carriers for the production of bacteriological agents. The storage stability of water dispersible granules directly affects the quality of their products and their application. The selection of suitable additives can improve the storage stability of microbial agents. Therefore, we screened the additives by biocompatibility to determine the most suitable additives and the degree of use to ensure the optimal performance of water dispersible granules. After optimization of dosage of each additive, performance of the aqueous dispersant was determined after a high temperature stability test and performance measurements.

The results of the experiments showed that water dispersible granules promoted activity of the defense enzymes of cotton and that the effect of water dispersible granules varied according to the method of application. As shown in Figure 2d, SOD activity of root irrigation method was significantly higher than that of clay method at a 24 h dosage of 1.5%, while a comparison of the other three enzyme activities revealed nonsignificant difference between the two methods at a dosage of 1.5% ($P < 0.05$). Thus, after a comprehensive comparison, we used a 1.5% aqueous dispersant in the later experiments using the root irrigation method.

Some studies have shown that either the treatment with probiotics or application of microbial fertilizers prepared with probiotics as active ingredients that can promote the growth of plants. For example, Giassi et al. (2016) found that bacteria have different abilities to promote the growth of different plant genotypes. Myresiotis et al. (2012) found that *B. pumilus* SE34 was highly effective at promoting the growth of tomato (*Solanum lycopersicum*). The mechanisms of biological control by which inter-root bacteria can indirectly contribute to plant growth include reducing the levels of disease by various methods, including antimicrobial resistance, induction of systemic resistance and competition for nutrients and ecological niches (Lugtenberg and Kamilova, 2009). Strain C-9 and A1 were isolated from cotton and ferruginous inter-rhizosphere soils, and strain C-9 has a high capacity for protease production, which plays a key role in the soil N cycle (Liu et al., 2021a). Strain A1 has ACC deaminase activity, an enzyme that plays an important role in the relief from biotic and abiotic stressors in plants, thereby enhancing their growth under adverse environmental conditions (Singh et al., 2022). In this study, the water dispersible granules of strains A1 and C-9 promoted height, fresh weight, leaf area and root system of cotton. This may be owing to the fact that rapid multiplication of a large number of functional bacteria increased activity of soil proteases, accelerated soil N cycle and improved growth conditions of cotton. This could be the reason why cotton grew much better.

Successful biocontrol agents colonize roots and suppress pathogens through mechanisms, such as ecological niche exclusion and competition, direct antagonism of the pathogens using antibiotics, parasitism or predation, and by triggering systemic host plant defense responses (Viterbo et al., 2010). When a pathogen invades a plant, the plant undergoes an HR response, which causes the cells around the point of infection to die to prevent the pathogen from infecting other parts of the plant, and ROS accumulate rapidly before the HR response occurs (Heath, 2000). Figures 4a-4b illustrate the short burst ROS in cotton and the more intense HR response when water dispersible granules were applied. We also found that water dispersible granules reduced incidence of Verticillium wilt and disease index in cotton, and the correlation analysis showed a negative correlation between defense enzyme activity and disease index (Table 8). Cotton defense enzymes synthesize compounds related to defense, strengthen cell walls and produce substances that are toxic to pathogens (Wang et al., 2006; Huang et al., 2010; Nilthong et al., 2012; Bu et al., 2014). After infection with *V. dahliae*, the defense enzymes in the cotton plants reacted quickly to defend against the pathogen. Within 48 h of *V. dahliae* infection, the defense enzymes in the treatment group were always higher than those in the control group, except for PAL. This indicated that water dispersible granule treatment significantly increased the stress and defense enzyme activity of cotton plants, thus, providing better protection against *V. dahliae* infection.

Table 8. Disease index and defensive enzyme Pearson correlation coefficient. PAL: Phenylalanine ammonia lyase; PPO: polyphenol oxidase; POD: peroxidase; SOD: superoxide dismutase.

Hours after inoculation	PAL	PPO	POD	SOD
12	−0.96**	−0.96**	−0.99**	−0.89*
24	−0.94**	−0.89*	−0.98**	−0.99**
36	−0.05	−0.86*	−0.69	−0.85*
48	−0.97**	−0.83*	−0.91*	−0.71

Microbial communities have been reported to influence plant traits by mitigating the effects of abiotic stresses on plant populations, and the saline/salt-tolerant bacteria that live in saline and arid ecosystems can promote growth of plants that grow in saline and arid conditions (Vurukonda et al., 2016). When faced with biotic and abiotic stresses, plants rapidly accumulate ROS as a first layer of defense (Qi et al., 2018). Figure 7 shows that when the water dispersant was applied, it enhanced stress response of cotton plants and improved their resistance to drought and salt stresses.

Under stress, the production of excessive ROS can also damage plants (Rohman et al., 2019), and the CAT enzymes are essential for scavenging ROS produced in plants and can protect them from damage (Raza et al., 2021). There was a significant difference between control and treated groups before stress, while after stress, there was nonsignificant difference between control and treated groups for either drought or salt treatment. This suggests that control treatment enhanced CAT activity to withstand the stress. However, in pre-treatment plants, there was significantly more CAT activity in the treated group than in control group, which also indicates that when water dispersible granules were applied, CAT activity increased, and thus, plants were protected. The SOD enzymes are part of the enzymatic antioxidant system, and SOD activity increases when plants are subjected to salt stress (Shankar et al., 2016; Woith et al., 2017). In this study, salt stress produced a significant difference in SOD activity between treated and control groups, which showed that the effect of A1+C-9 water dispersible granules differed under different stresses. The concentrations of MDA varied with biotic and abiotic stresses and also varied depending on the stress conditions. The accumulation of MDA can also produce cellular damage. Studies have shown that drought stress and salt stress cause the levels of MDA to accumulate in plants (Li et al., 2018; Chen et al., 2020; Xiong et al., 2020; Zhang et al., 2022b). In this study, application of water dispersible granules significantly reduced the content of MDA in presence of salt stress, but there was nonsignificant change in drought stress. These data clearly show that A1+C-9 water dispersible granules can reduce the degree of salt stress on cotton.

CONCLUSIONS

In this study, *Bacillus sphingosporium* A1 + *B. tequilensis* C-9 mixed bacterial water dispersible granules were optimized, and the most suitable formulation of these granules was finally determined after the determination of biocompatibility and the evaluation of wettability, dispersibility, suspension, disintegration and thermal stability. The optimal treatment of water dispersible granules was found to be 1.5% by root irrigation by detecting changes in activities of defense enzymes in cotton infected with *Verticillium dahliae* by different treatments of water dispersible granules. The application of water dispersible granule treatments promoted growth and induced disease resistance, drought resistance and salt resistance in cotton. The water dispersible granules not only enhanced the stress of cotton to external stresses and increased activity of cotton defense enzymes but also enhanced the resistance of cotton plants to cotton wilt, drought and salt stress, and promoted growth and development of cotton plants.

Author contribution

Methodology: C.W. Formal analysis: Y.F. Investigation: Y.F., J.C. Data curation: S.H. Writing-original draft: S.H. Writing-review & editing: S.H. Supervision: A.W. Funding acquisition: A.W. All co-authors reviewed the final version and approved the manuscript before submission.

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