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Construction of pH-responsive micro nano delivery system (Cysteine-Zinc@Boscalid） and antifungal activity against *Botrytis cinerea*

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A smart pH-responsive pesticide delivery system (Cysteine-Zinc@Boscalid, CZ@BOS) was constructed via a self-assembly method by Cysteine and $ZnCl_2$ to enhance inhibition effects on *Botrytis cinerea*. Results showed that CZ@BOS's properties were verified using XRD, FTIR, SEM and CZ@BOS displayed uniform morphology with an average particle size of 1395.82 nm. The cumulative release percentage of CZ@BOS reached 100% after 400 min of continuous release and exhibited responses to pH. Meanwhile, it can be seen from the release mechanism that the release behavior of Boscalid from CZ@BOS is highly consistent with the Frist-order model. Antifungal results and Inoculation experiment show CZ@BOS has a stronger antifungal effect than Boscalid and BOS-WG against *Botrytis cinerea* at a concentration of 175 mg/L, the inhibition rate of CZ@BOS against *Botrytis cinerea* can reach 86%, while the inhibition rate of BOS-WG is only 60%. Therefore, the intelligent pH-responsive CZ@BOS system developed in this study is a highly promising material for targeted pesticide delivery.

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Keywords: Cysteine-Zinc (CZ), Boscalid(BOS), Antifungal, release, *Botrytis cinerea*

1. Introduction

The excessive use of pesticides will inevitably have a negative impact on the agricultural economy and ecology, including high costs, serious environmental pollution and agricultural product safety. Boscalid is a nicotinamide fungicide with broad-spectrum antibacterial activity, which can prevent and treat almost all types of fungal diseases, and has good control effects on powdery mildew, gray mold, rot disease, and various rot diseases [1-2]. Due to its poor light stability and low water solubility, Boscalid often requires higher dosages in practical applications to maintain effectiveness, which, in turn, leading to numerous environmental problems [3-6]. In order to reduce or eliminate the various problems caused by the repeated use of pesticides and achieve the goal of delivering drugs to specific parts for targeted treatment [7], the most effective way is to design pesticide controlled-release formulation that can release in response to various stimuli (pH, temperature, humidity, light, etc.) [8-9]. Micro nano-delivery systems can provide a feasible method for delivering pesticides to specific parts of plants without changing the activity of

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pesticides [10-11]. Recently, application of nanotechnology in the field of agriculture has provided new opportunities for the development of sustainable agriculture relying on the high efficiency, environmentally friendly, good water dispersibility, high biological activity [12-17]. This technology is mainly used for targeted delivery of drugs, and the constructed nanocarriers exhibit response behavior to external stimuli (including light, temperature, pH, etc.), which has broad prospects in pharmaceutical and therapeutic delivery systems [18]. Owing to their biocompatibility, biodegradability [19], functionalizability [20], and ease of design/synthesis, amino acids and their derivatives can assemble with other components into ordered nanostructures via non-covalent interactions. Thus, many modified amino acids demonstrate significant potential across various application fields [21-24]. As described in reference [25], Cystine can coordinate with equimolar cadmium ions to form three-dimensional (3D) Cys/Cd nanorods for enhanced overall structural stability. Currently, modified amino acids are widely used in the medical field, but have not been applied in the prevention and control of agricultural fungi, and the combination of amino acids and zinc for the prevention and control of *Botrytis cinerea* has not been seen.

Therefore, in this work, zinc Cysteine-encapsulated Boscalid was prepared via self-assembly, aiming to prevent and control *Botrytis cinerea* through the slow release of Boscalid. The synthetic route of Cysteine-Zinc@Boscalid(CZ@BOS) is showed in Figure 1. The successful synthesis of CZ and CZ@BOS was verified by a series of characterizations, and the system demonstrated high antifungal activity. Therefore, this work presents a straightforward method for fabricating CZ@BOS achieving remarkable prevention and control of *Botrytis cinerea*.

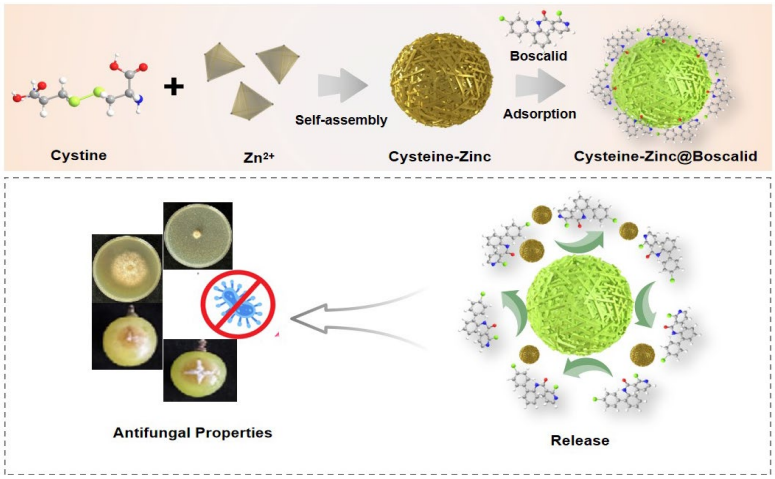

Fig. 1. Schematic illustration of CZ@BOS synthesis and its release properties and antifungal activity.

2. Experimental

2.1. Materials

Cysteine(AR), was purchased from Shanghai Lanji Technology Development Co., Ltd; tris(hydroxymethyl)aminomethane(99.5%) was purchased from solarbio; Boscalid(97%) was purchased from Hailir Pesticides and Chemicals Group Co.Ltd; Commercial Boscalid

formulation(BOS-WG, 50%) was purchased from BASF; Potato Glucose Agar(AR), were purchased from Qingdao Haibo Biotechnology Co., Ltd; *Botrytis cinerea* obtained from the Institute of New Pesticide Innovation&Research; Ultra-pure water was used in the experiment; Zinc Chloride(AR), Anhydrous ethanol(AR), Acetonitrile(AR), Acetone(AR), Methanol(AR), were purchased from China National Pharmaceutical Group Chemical Reagent Co., Ltd; Filtration membrane, were purchased from Tianjin Keyilong Experimental Equipment Co., Ltd.

2.2. Preparation of Cysteine-Zinc (CZ)

Firstly, 0.24 g of Cystine was dissolved in 50 mL of distilled water. The resulting solution was then slowly added to the Tris-HCl buffer solution (pH 8.0), and stir thoroughly at room temperature. Then, add the resulting mixture to a $ZnCl_2$ aqueous solution (0.292 g $ZnCl_2$, 100 mL distilled water). Allow the mixture to stand, followed by centrifugation (11,000 rpm, 20 min), and drying (60 °C, 6 h) to obtain a white powder of Cysteine-Zinc (CZ).

2.3. Preparation of Cysteine-Zinc@Boscalid (CZ@BOS)

0.1 g of Boscalid was dissolved in absolute ethanol under ultrasonication. Subsequently, 0.4 g of Cysteine-Zinc (CZ) was added to the solution，sealed and light-shielded at room temperature, stirred for 24 h, centrifuged (10000 r/min, 10 min), washed with anhydrous ethanol three times, and freeze-dried at low temperature for 24 h to obtain Cysteine-Zinc@Boscalid(CZ@BOS).

2.4. Characterization

The morphology of samples was observed by TEM (Hitachi H-7650, Japan) and SEM (SU8010, Hitachi Company, Japan). The structure of samples was observed by FTIR (Nicolet，IR200, USA). XRD images were obtained by powder X-ray diffractometer (Germany Brooke Co., Ltd.). Mastersizer 3000 Zetasizer laser particle size analyzer was used for analyzing the particle size of samples. Contact Angle (CA) and Zeta potential of CZ and CZ@BOS was characterized by using contact angle measuring instrument (JC2000C2, Krus, Germany). Simulaneous thermal analyzer was used to measure thermal stability (TGA, SDT650, Waters, America) of CZ and CZ@BOS. High-speed automated porosity analyzer (NOVA2200e, Quantatech, Hong Kong) was used to measure BET performance.

2.5. Performance measurements

2.5.1. Loading rate

Weigh CZ@BOS and disperse completely in methanol to prepare a concentration of 1000 mg/L. Perform liquid chromatography analysis and calculate the loading rate. The loading rate calculation formula is:

Loading rate (the amount of Boscalid loaded in CZ@BOS)/(the total amount of CZ@BOS) ×100%

2.5.2. Controlled Release Experiments

The release of BOS from CZ@BOS were investigated under different pH (5, 7, 9) and temperature(25℃, 35℃, 45℃). Disperse CZ and CZ@BOS separately in 30 mL of distilled water at room temperature. At each specified time interval, take 0.1 mL of the supernatant from each

sample, filter it through a 0.22 μm membrane filter, and measure the peak area using HPLC. Then, the cumulative release percentage (CRP,%) was calculated using the follow formula(1):

$$CRP_{\%} = \frac{V\sum_{i=1}^{n-1} C_i + V_0 C_n}{m_0} \times 100\% \quad (1)$$

wherein: V (0.1 mL) is the volume of solution taken out; V_0 is the volume of the solution absorbed (30 mL); C_i is Boscalid's mass concentration at the moment before C_n, mg/mL; m_0 is Boscalid's mass adsorbed in CZ@BOS, mg. Release kinetics study was simultaneously conducted, primarily using four models(first-order, Higuchi, zero-order, and Ritger-Peppas) to analyze the release mechanism of Boscalid from CZ@BOS.

2.6. Antifungal properties

Antifungal activity was evaluated using growth rate method. PDA medium containing CZ@BOS preparations at concentrations ranging from 75 mg/L to 175 mg/L was prepared. Aqual volume of sterile water was used as a blank control, and equal concentration of Boscalid was used as an experimental control in PDA medium. The fungal colony diameter was measured by cross method, and the antifungal rate was calculated. All treatments were replicated three times. The antifungal rate was calculated using the corresponding formula (2).

$$\text{Antifungal rate\%} = (D_{Treatment} - D_{CK})/D_{CK} \times 100\%. \quad (2)$$

Herein: $D_{Treatment}$ is diameter of treatment, D_{CK} is diameter of control.

Inoculation experiment of isolated fruits was determined by the spore germination inhibition method. The spore suspension of *Botrytis cinerea* was prepared following the method described in reference [26]. "Jufeng", a grape variety from Qingdao with uniform size and no damage, disinfected with NaClO, rinsed with sterile water, and naturally dried for later use. Make a surface wound (2mm deep×2 mm wide) in the middle of grape fruit with sterile needle, and inject 5 μL spore suspension into the wound (adjust the concentration to 10^5 ea/mL with blood cell counting plate). CZ@BOS was injected into diseased grapes simultaneously with different concentrations of 200 mg/L, 400 mg/L and 600 mg/L. The control groups were injected into 5 μL sterile water. Repeat 3 times for each concentration, with 12 grapes in each repeat. Seal the fruit with plastic wrap, place it into artificial climate box (25 ℃, 80% humidity) for culture, and observe the occurrence of grape fruit diseases every 12 hours. When the diameter of the control lesion is close to that of the grape, count the size of the lesion and measure the disease index using the corresponding formula (3).

$$\text{Disease index\%} = (D/D_{CK}) \times 100\%. \quad (3)$$

Herein: D is disease spot diameter of diseased grape; D_{CK} is control grape disease spot diameter.

2.7. Statistical analysis

The results were analyzed using IBM SPSS Statistics 26.0, and data were reported as the mean of at least three replicates. Analysis of variance ANOVA test was conducted to investigate significant differences which are represented by lowercase letters a, b, c, d ($p<0.05$).

3. Results and discussion

3.1. SEM, TEM, FTIR, XRD and Particle size

The SEM and TEM images of CZ shows well-defined microspheres (Figure 2A-1 and Figure 2B-1).

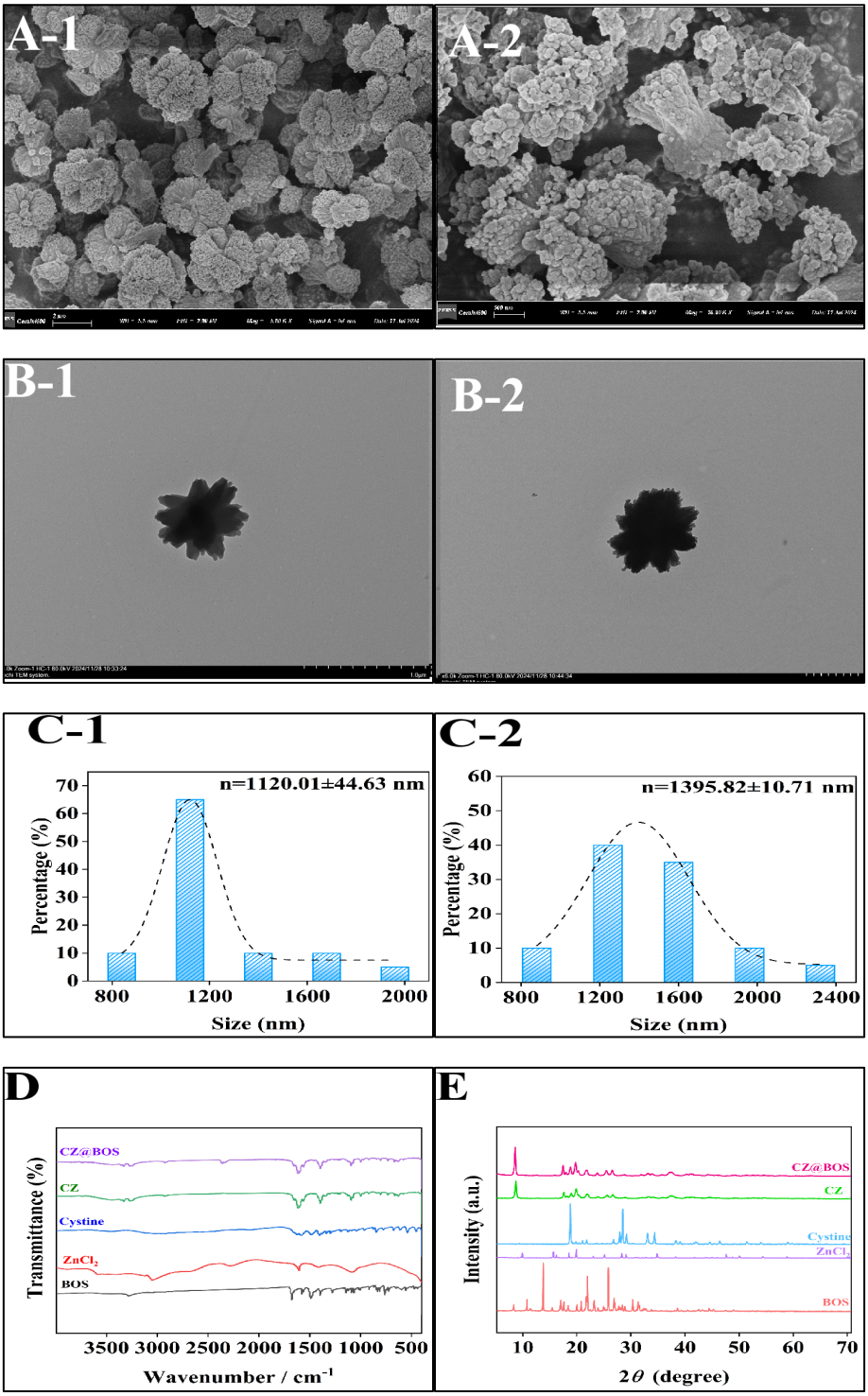

Fig. 2. SEM, TEM, FTIR, XRD and Particle size of samples (A-1、A-2: SEM image of CZ carrier and CZ@BOS；B-1、B-2:TEM of them；C-1、C-2:particle size distribution of them；D:FTIR spectra；E:XRD analysis).

The magnified SEM images showed that CZ@BOS were constructed radially, and there were some cauliflower-like structures, which also confirmed that CZ@BOS were composed of nanorods(μm), indicating that the CZ@BOS were very dense (Figure 2A-2 and 2B-2). Figure 2C-1 and 2C-2 displayed the uniform particle size of samples and showed that the particle sizes of CZ and CZ@BOS are 1120.01 nm and 1395.82 nm, respectively, which indicate that the particle sizes of CZ were increased after loading with Boscalid. The CZ sample was characterized by FTIR in the wave number range of 400 cm^{-1}-4000 cm^{-1}. The FTIR spectra of Boscalid samples in Figure 2D showed the stretching vibration absorption peak of the N-H bond in the methylene group was observed at 3282 cm^{-1}, while the C-H bond stretching vibration appeared at 1494 cm^{-1}, the absorption peak in CZ is mainly attributed to the vibration of Cysteine. The absorption peak near 500-600 cm^{-1} is attributed to the S-S stretching vibration from Cysteine, while the peak in the range of 1723 cm^{-1} is assigned to the C=O stretching vibration of the carboxyl group. These absorption peaks can be found in CZ. The above FTIR spectral data show that the CZ carrier was successfully synthesized. XRD shows strong and narrow peaks (Figure 2E). Cystine samples have obvious absorption peaks at 19.8° and 29.1°, and Boscalid samples have narrow peaks at 14.3°, 22.1° and 27.4°, indicating that it has good crystallinity. CZ and CZ@BOS have broad peaks at 21.4° and 22.0°, respectively, which are different from cystine crystals, probably because the packing arrangement of cystine molecules is modulated by the zinc ions. All the above results indicate that Boscalid and CZ are successfully combined.

3.2. TGA, Zeta, Contact Angle (CA)and BET

Figure 3A shows the TGA curves of different samples. For CZ, the decomposition begins at 240 ℃, because the high temperature destroys the link between Cysteine and Zinc, resulting in weight loss.

For CZ@BOS, the weight began to decrease at 250 ℃ because of the decomposition of Boscalid and the destruction of CZ carrier structure. At 700 ℃, the weight losses between 100 ℃ and 700 ℃ were 71.45%, 78.95%, and 62.30% for CZ, Boscalid, and CZ@BOS, it is proved that CZ and Boscalid are successfully combined, respectively. It was also observed that CZ exhibited a higher thermal decomposition temperature than Cysteine. This phenomenon is primarily attributed to enhanced thermal stability induced by chemical grafting. Due to the successful loading of Boscalid, the absolute value of the Zeta potential(ζ) of CZ@BOS is greater than that of CZ(Figure 3B), and the absolute Zeta potential(ζ) of CZ@BOS is greater than -15mV, indicating that it can be well dispersed to achieve better wetting and drug efficacy. The contact angle can also directly reflect the wettability of the sample. Therefore, the wettability of the pesticide was characterized by measuring the static contact angles of different sample solutions on cabbage leaves. Figure 3C and Figure 3E shows the contact angles of deionized water, Boscalid, CZ, and CZ@BOS droplets on the surface of cabbage leaves. Compared with deionized water, the contact angle of Boscalid water dispersion is significantly reduced, mainly because the addition of water for dispersion and the additives in water-dispersible granules reduces the surface tension of the system. Compared with CZ, the contact angle of CZ@BOS is reduced by 12.8%, mainly because there is electrostatic attraction between Cysteine and cabbage leaves, resulting in increased wettability. Meanwhile, as shown in Figure 3D, the pore sizes of CZ@BOS is significantly smaller than that of CZ, indicating successful adsorption of BOS on the CZ carrier.

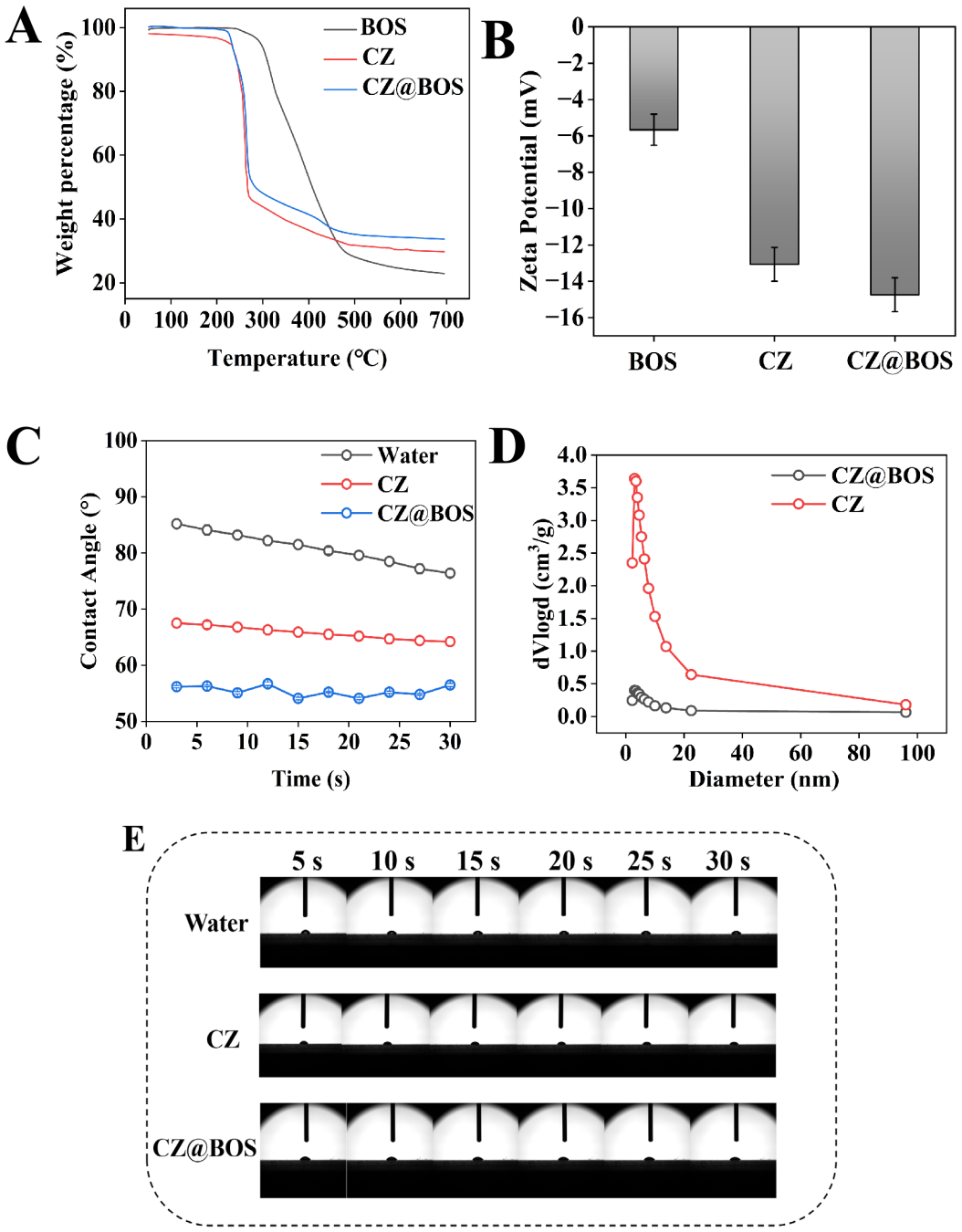

Fig. 3. TGA, Zeta potential , Contact Angle (CA)and BET of samples (A:TGA curve; B:Zeta potentials； C、E:Contact angle change within 30 s； D:BET pore size distribution).

3.3. Release performance

The loading capacity of Boscalid in CZ@BOS was determined to be 20.5% as described in Section 2.5.1 and Figure 4 investigates the release behavior of Boscalid in CZ@BOS. As shown in Figure 4A, release behavior of Boscalid in CZ@BOS under different pH conditions was studied at 25 ℃. In the initial release period (the first 200 min), the CZ@BOS system showed rapid release, which was due to the release of unencapsulated Boscalid. After 400 min of continuous

release, the release of Boscalid was stable. At this time, the cumulative release percentage of CZ@BOS reached 100%. The reason for this may be that the carrier is more hydrophilic, has better swelling in aqueous solution, and is easier to release Boscalid; it may also be that the amino group has a strong electrostatic adsorption effect on Boscalid, and Boscalid adsorbed on the surface of the carrier is easier to release into the external environment. The experimental data also reveal that the CZ@BOS system exhibits excellent pH-responsive drug release properties. In CZ@BOS system, Zn^{2+} forms coordination bonds with cysteine via carboxyl ($-COO^-$) or amino ($-NH_2$) groups. Under acidic conditions, H^+ protonates the carboxyl group ($-COO^- \rightarrow -COOH$), leading to the cleavage of the coordination bonds. The dissociation of the coordination structure generates soluble Zn^{2+} and free amino acids, facilitating the diffusion and release of the Boscalid. Additionally, the acidic secretions from *Botrytis cinerea* [27] may directly trigger the breakdown of Zn^{2+} coordination bonds, resulting in BOS release. The alkaline environment (pH 8-10) in the midgut of lepidopteran pests [28] can also activate the carrier response. Under alkaline conditions, Zn^{2+} may form $Zn(OH)_2$ precipitates or hydroxyl complexes, indirectly promoting Boscalid release.

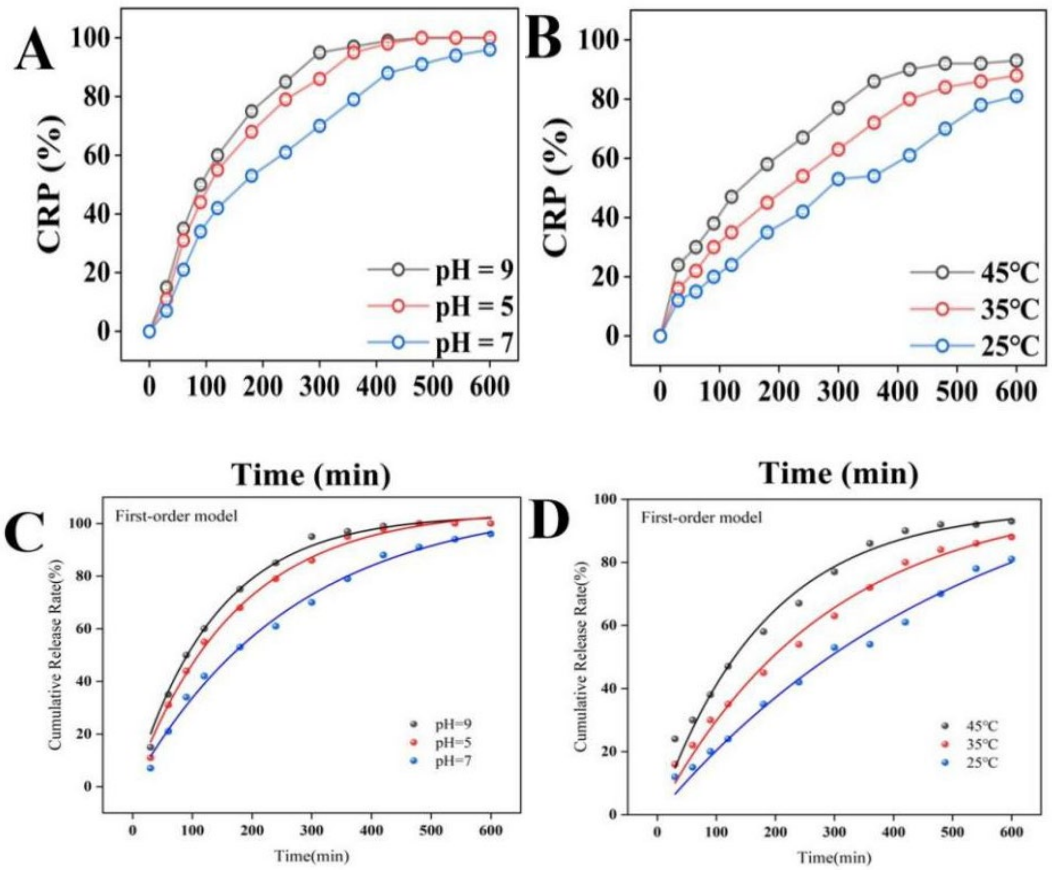

Fig. 4. Release performance of CZ@BOS (A:pH; B:Temperature; C:pH release fitting model; D:Temperature release fitting model).

Furthermore, the released Zn^{2+} itself exhibits antifungal activity by disrupting the cell membranes of pathogens, thereby synergistically enhancing the antifungal efficacy of Boscalid.

Thus, the CZ@BOS controlled-release system developed in this study shows potential as a carrier for insecticides, and further research will be conducted in this regard. As shown in Figure 4B, at different temperatures (25 °C, 35 °C and 45 °C), the cumulative release percentage of CZ@BOS reaches equilibrium after 300 h, and the cumulative release percentage of CZ@BOS can reach 98.2%. Compared with different temperatures, there is not much difference in the cumulative release rate, which can indicate that the CZ carrier is less affected by temperature in application.

As shown in Table 1, the criterion for judgment is the fitting coefficient($R^2 > 0.9$) to evaluate the degree of conformity of the Boscalid release curve with the fitting model. Obviously, the sustained release behavior of Boscalid from CZ@BOS is highly consistent with the Frist-order model (Figure 4C and 4D). These results show that the release curve of the system conforms to the Frist-order model, which means that the release rate of Boscalid is linearly related to the concentration of Boscalid, and the higher the concentration of Boscalid, the faster the release rate.

Table 1. Fitting results of CZ@BOS release kinetics model.

Release model	pH	K_1	K_2	R^2
First-order	5	105.541	0.006	0.994
	7	108.221	0.004	0.993
$y=K_1*[1-\exp(-K_2t)]$	9	103.434	0.007	0.995
First-order	45℃	97.275	0.006	0.979
	35℃	101.079	0.003	0.989
$y=K_1*[1-\exp(-K_2t)]$	25℃	117.876	0.002	0.988

3.4. Antifungal properties

The antifungal results are shown in Figure 5A-5B, it can be seen that with increasing concentration of CZ@BOS, the antifungal property tends to increase, which is consistent with the research results of Kumar. Kumar[29] proposed that the concentration is particularly important for the stability of particles. Low concentration will make the particles unstable, while high concentration will cause the particles to aggregate, thus the antifungal property is better. At the same concentration, CZ@BOS has a stronger antifungal effect than Boscalid and BOS-WG. At a concentration of 175 mg/L, the inhibition rate of CZ@BOS against *Botrytis cinerea* can reach 86%, while the inhibition rate of BOS-WG is only 60%. It can be concluded from Table 2 that the EC50 of CZ@BOS is 96.35 mg/L, which is 30.9% higher than that of Boscalid and 18.7% higher than that of BOS-WG. This may be due to the negative charge of CZ, which can significantly reduce the electrostatic repulsion of fungi, forming smaller particles that are easier to penetrate, this result is consistent with previous literature report[30] that the amino acid Schiff base as a drug carrier enhances the lipid water partition coefficient of benzyltin, making it easier to penetrate the biofilm and reach the target site, thereby increasing drug efficacy. And from the inoculation experiment of “Jufeng” grapes, the disease index of CZ@BOS is significantly lower than that of BOS and BOS-WG are shown in Figure 5C-5D, indicating that CZ@BOS has significant control effect on *Botrytis cinerea*.

Table 2. EC50 of BOS、BOS-WG、CZ@BOS

	EC_{50}(mg/L)
BOS	139.44(134.22-144.66)
BOS-WG	118.56(113.81-123.31)
CZ@BOS	96.35(92.86-99.84)

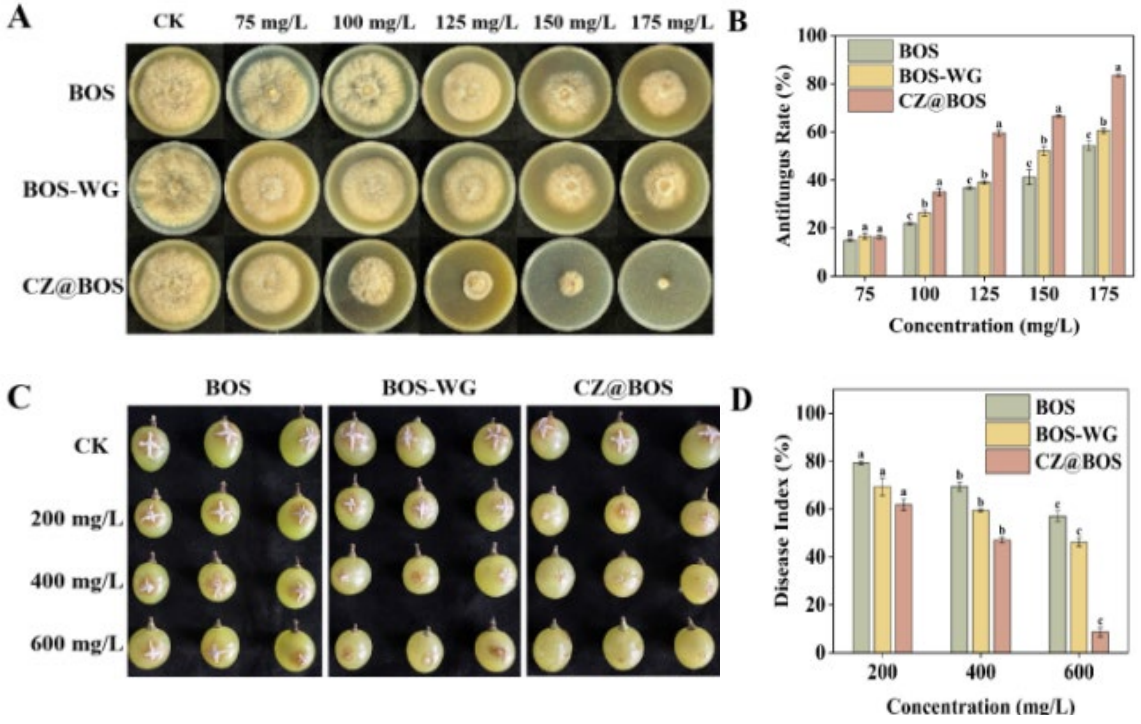

Fig. 5. Antifungal activity (A, B:Plate antibacterial of CZ@BOS, Boscalid, and BOS-WG, C, D:Inoculation experiment of grape).

4. Conclusions

In conclusion, this study successfully prepared a pH-responsive controlled-release fungicide, CZ@BOS, via self-assembly technology, with the aim of more effectively controlling *Botrytis cinerea* in agriculture. Specifically, Cysteine was first grafted to zinc to synthesize the CZ carrier, and then Boscalid was loaded to obtain the CZ@BOS pesticides. Characterization results indicated that the particle size of CZ@BOS was approximately 1395.82 nm. Simultaneously, the release profile of CZ@BOS was found to follow the first-order kinetic model and possessed pH-responsive characteristics. Most notably, the antifungal efficacy of CZ@BOS was significantly

superior to that of commercial Boscalid water-dispersible granules (BOS-WG). Therefore, this work is the first to utilize the combination of Cysteine and zinc to develop an efficient Cysteine-functionalized delivery system with pH-sensitive controlled-release fungicide delivery property, and its outstanding efficacy against *Botrytis cinerea* highlights its considerable potential for mitigating the challenges caused by this disease.

Acknowledgements

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