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ORIGINAL ARTICLE

Improvement of antifungal activity of carboxin by inclusion complexation with cucurbit[8]uril

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Abstract Interaction between cucurbit[8]uril (Q[8]) with a fungicide, carboxin in aqueous solution, was investigated by ^{1}H-NMR, electronic absorption spectroscopy, and fluorescence spectroscopy. Spectroscopy analysis established a basic interaction model which formed an inclusion complex with a host:guest ratio of 1:1. ^{1}H-NMR showed that Q[8] encapsulated the phenyl ring into its cavity and the rest of the guest molecule stayed outside the host. Comparative in vitro evaluations of the growth inhibitory effects of the inclusion complex solution toward *Rhizoctonia solani* demonstrated appreciable improvements in the antifungal activity of carboxin through the addition of Q[8]. In comparison with the positive control, improvement was evaluated in terms of area covered by the mycelia of *R. solani* and their growth inhibition rate. Inclusion complexation of carboxin with Q[8] suggests a potential means for production of an environmentally friendly carboxin-based fungicide to counteract *R. solani*.

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Introduction

Carboxin is a systemic anilide fungicide. It is used as a seed treatment for control of smut, rot, and blight on barley, oats, rice, cotton, vegetables, corn and wheat. It is also used to control fairy rings on turf grass. The product may be used to prevent the formation of these diseases or may be used to cure existing plant diseases [1].

Sheath blight symptom and leaf rot caused by the fungus *Rhizoctonia solani* is destructive to many fruit and trees species including cucumber, barley, oats, apple tree and mulberry tree. The disease is spread worldwide, including Europe, Russia, the United States and Canada. This fungal disease, which spreads rapidly and is very difficult to prevent, has done great damage to commercially grown grapevines, fruits and trees, and other crops. In China, it spreads all over the nursery and orchard, and 1–2 L m^{-2} of fungicide (active ingredient: carboxin, concentration: 75%, dosage form: wettable powder) is sprayed on the soil to control the disease [2]. However, this level of fungicide use has raised concerns about soil pollution.

The cucurbit[*n*]uril (Q[*n*]) family of macrocycles comprise *n* glycoluril units, and have the ability to form inclusion complexes with various molecules [3, 4]. They have great potential in contemporary science, and could be used in such areas as molecular machines, chemical sensors, catalysts, and drug delivery vehicles [5–8]. Q[*n*] can entrap industrial pollutants, such as acetonitrile and dioxane [9, 10], and remove heavy metals, aromatic substances and dyes [5]. Studies on effect of Q[*n*] on pesticides have been few in number. This motivated us to investigate the

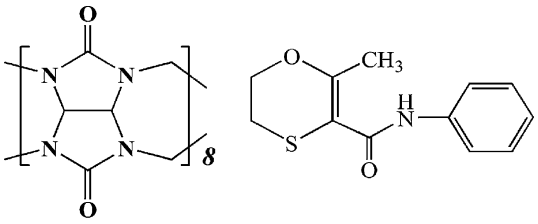

Fig. 1 Structures of the host Q[8] and carboxin

complexing properties of Q[*n*] as hosts for pesticides (carboxin was used as a guest model), which could add a new carrier to improve the activity of pesticides.In this study, the potential of Q[8] as a delivery system for carboxin (Fig. 1) was investigated. Moreover, the effect of host–guest complexation on the growth of *Rhizoctonia solani* was also investigated to demonstrate the improved antifungal activity by Q[8]. These studies were intended to provide fundamental information for production of an environmentally friendly yet effective carboxin-based fungicide against *R. solani*.

Experimental

Materials

Q[8] was prepared and purified according to the literature method [11]. Carboxin was obtained from Jiangsu Yongcheng Chemical Co., Ltd., at analytic grade, and was used without further purification. *R. solani* in hot pepper from Guizhou Key Laboratory of Agricultural Bioengineering was maintained on potato dextrose agar (PDA) (200 g potato infusion, 20 g glucose, and 20 g agar in 1 L water) slants at 25 °C, and each PDA plate that measured 9 cm in diameter contained approximately 20 mL PDA medium. Sterile water was used throughout the study. Unless otherwise stated, all of the chemicals were of analytical reagent grade and were used as received.

Absorption and fluorescence studies

UV–visible (UV–vis) absorption spectra of the host–guest complexes were recorded on an Agilent 8453 spectrophotometer at room temperature. Fluorescence spectra of the host–guest complexes were recorded on a Varian RF-540 fluorescence spectrophotometer. Aqueous solutions of carboxin were prepared with a concentration of 5×10^{-4} mol L^{-1}. Aqueous solutions of the host were prepared with a concentration of 1×10^{-4} mol L^{-1}. These stock solutions were combined to give solutions with a guest:Q[8] ratio of 0, 0.2:1, 0.4:1, 1:1, 1.5:1, 2:1 for absorption and fluorescence spectra determination.

^{1}H-NMR measurements

To study the host–guest complexation of Q[8] and the guest, 2.0×10^{-3} to 2.5×10^{-3} mmol Q[8] in 0.5–0.7 mL D_2O with a Q[8]:guest ratio of 0:1 and 0.6:1 was prepared, and the corresponding ^{1}H-NMR spectra were recorded at 20 °C on a VARIAN INOVA-400 spectrometer.

Antifungal activity determination

The in vitro fungicidal activities against *R. solani* were tested according to the reported method [12], with some modifications. An aqueous solution of carboxin was prepared with a concentration of 1.0×10^{-4} and 2.0×10^{-4} mol L^{-1}. The aqueous solution of Q[8] was prepared with a concentration of 1.0×10^{-4} mol L^{-1}. Inclusion complex solutions were combined to give solutions with a guest:Q[8] ratio of 1:1 and 2:1. The inocula, 8 mm in diameter, were removed from the margins of actively growing colonies of mycelium, and placed on the right side of a Petri dish. Filter papers (6 mm in diameter) were placed on the left side of the dish, and 15 μL Q[8], carboxin and the previously prepared inclusion complex solutions was soaked onto the filter papers after autoclaving, when the agar had cooled to 45–50 °C. The sterilized water was used as a negative control. After that, the plates were covered, sealed with parafilm, and incubated for 48 h in a 25 °C incubator. Each treatment was prepared in triplicate. The diameter of the mycelium was measured at 48 h. The growth inhibition rate was calculated according to the formula (1).

$$Y = (H1 - H2) / H3 \times 100\% \quad (1)$$

where *Y*(%) is the growth inhibition, H1(cm) the distance from the center of inoculum to the roots of the mycelium. H2 (cm) the radius of the inoculum, and H3 (cm) the distance from the center of the filter paper to the center of the inoculum, excluding the radius of the filter paper and bacterial cake.

Results and discussion

Spectrophotometric analysis of the interaction between Q[8] and carboxin

To quantify the interaction between Q[8] and carboxin in solution, a ratio-dependent study was pursued by monitoring electronic absorption and fluorescence spectra at pH 5.8. Usually, the host Q[8] shows no absorbance at $\lambda > 210$ nm, and the free carboxin shows maximum absorption at λ_{max} 249 nm. Fig. 2a shows the variation in the UV spectra obtained with aqueous solutions that

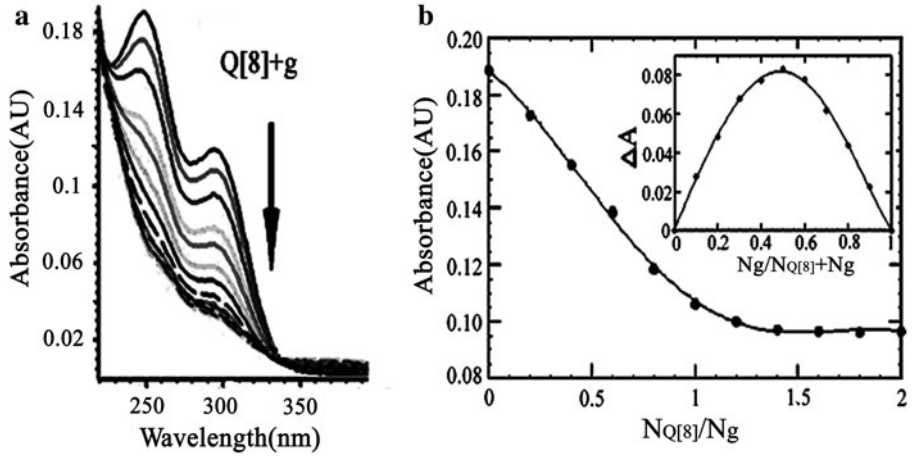

Fig. 2 **a** UV absorption spectra of carboxin in the presence of increasing concentrations of Q[8]; **b** corresponding A versus $N_{Q[8]}/N_g$ curve and the *inset* ΔA versus $[N_{Q[8]}/(N_{Q[8]} + N_g)]$ curve at $\lambda_{max} = 249$ nm

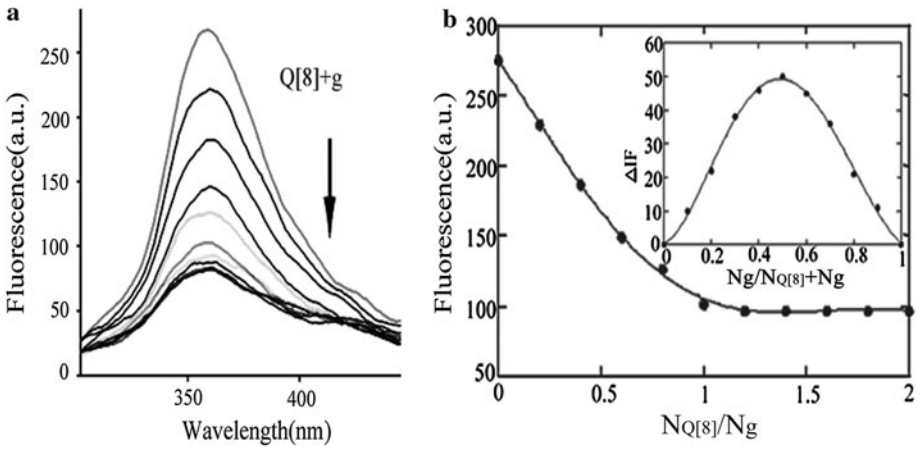

Fig. 3 Fluorescence emission spectra of carboxin in the presence of increasing concentrations of Q[8] (**a**) and corresponding I_f versus $N_{Q[8]}/N_g$ curve and ΔI_f versus $[N_{Q[8]}/(N_{Q[8]} + N_g)]$ (*inset*) at $\lambda_{max} = 360$ nm (**b**)

contained a fixed concentration of carboxin (32 mM) and variable concentrations of Q[8]. The absorption band of the guest carboxin exhibited a progressively lower absorbance with a 4-nm red shift as the ratio of $N_{Q[8]}/N_g$ increased. The absorbance (A) versus ratio of moles of the host Q[8] and guest carboxin data can be fitted to a 1:1 binding model for the Q[8]–carboxin system at λ_{max} 249 nm (Fig. 2b). The insert shows the absorbance change (ΔA) versus ratio of $[N_{Q[8]}/(N_{Q[8]} + N_g)]$ data, which can also be fitted to a 1:1 binding model. The corresponding formation constants (K) are 2.78×10^5 and 5.65×10^5 L mol^{-1} respectively.

Using fluorescence spectroscopy, similar experiments were performed. Fig. 3a shows emission spectra of the carboxin obtained with aqueous solutions that contained a fixed concentration of carboxin (32 μM) and variable concentrations of Q[8] at $\lambda_{Ex}/\lambda_{Em} = 317/360$ nm. The emission spectra of the carboxin exhibited a progressive change in fluorescence intensity with a violet shift as the ratio of $N_{Q[8]}/N_g$ increased. Both the curves of fluorescence intensity (If) versus $N_{Q[8]}/N_g$ and ΔI_f versus $[N_{Q[8]}/(N_{Q[8]} + N_g)]$ could also be fitted to a 1:1 binding model for the host–guest system (Fig. 3b), which was consistent with those from the absorption spectrophotometric analysis. The corresponding formation constants (K) were: 3.31×10^5 and 4.97×10^5 L mol^{-1}, respectively. They were close to those obtained by absorption spectrophotometric analysis.

^{1}H-NMR study of the inclusion complexes

Figure 4 shows the ^{1}H-NMR spectra of free carboxin (a) and carboxin with Q[8] in D_2O. Generally, Q[8] is insoluble in neutral aqueous solution, no any proton peaks can not be observed in ^{1}H NMR spectra. However, when it interacts with guest, in particular, the amines, Q[8] become soluble in aqueous solution and the corresponding proton resonances [δ, 4.0(d), 5.3(s) and 5.6(d)] can be observed. Compared to the size of the guest, the cavity of Q[8] is too large to hold the guest tightly, a fast exchange process on the NMR time scale lead to the broadness of the guest resonances, so that the proton resonances of the included guest can not be observed clearly, except the proton resonances of the free guest. The molar ratio of the dissolved Q[8] to carboxin is only 0.12. The small peaks at $\delta \sim 6.6$

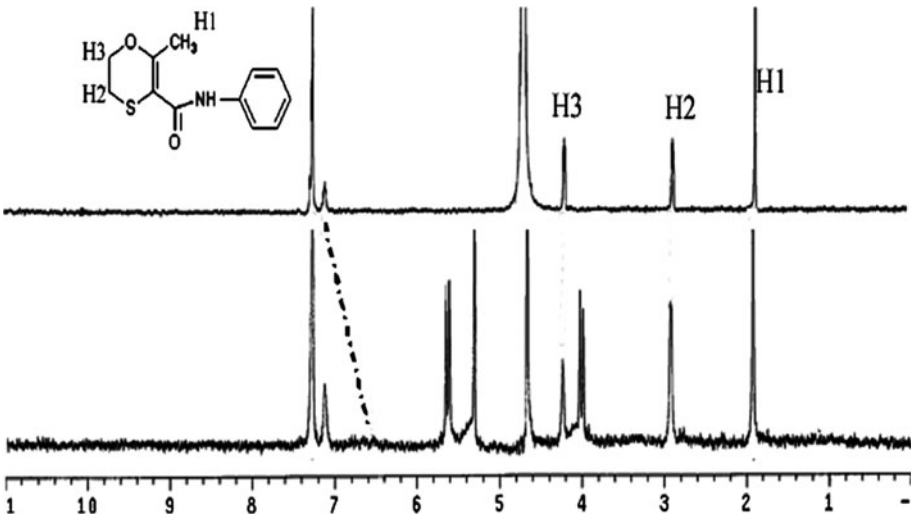

Fig. 4 ^{1}H-NMR spectra of the Q[8]–guest system in the absence (**a**) and in the presence (**b**) 0.12 equiv. of Q[8]

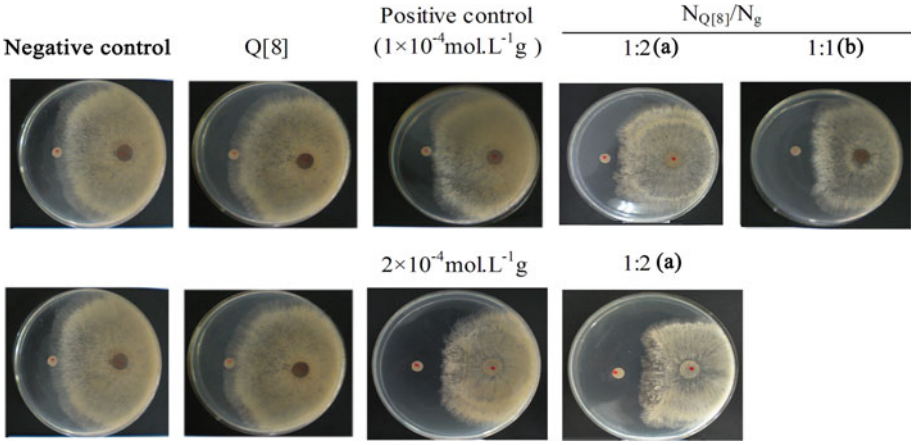

Fig. 5 Improvement of growth inhibitory effect of carboxin on *R. solani* with increased Q[8] concentration in inclusion complex solution. **a** 0.5 molar ratio; **b** 1 molar ratio

seem to show that the protons on the phenyl ring moiety of carboxin are shifted upfield by ~0.6 ppm, which suggests that Q[8] encapsulates the phenyl ring into its cavity and the rest of the guest molecule stay at the portal of Q[8].

Effect of carboxin on growth of *R. solani*

Figure 5 illustrates the inhibitory effect of carboxin and its inclusion complex solutions with Q[8] on the mycelial growth of *R. solani*. The leftmost column was the negative control treatment, which contained no carboxin, the second column from the left was the Q[8] control, and the third column from the left was the positive control treated with 1×10^{-4} moL L^{-1} carboxin concentrate. The rest were those treated with Q[8]–guest inclusion complex solutions with molar ratios of Q[8] to carboxin of 0.5 and 1 arranged in ascending order from the left. Except for the negative control, all of the treatments involved addition of an identical amount of 1×10^{-4} moL L^{-1} carboxin.

In the negative control and 1×10^{-4} mol L^{-1} Q[8] control, the fungus was visible to the naked eye, and covered the majority of the surface of PDA medium after 48 h incubation. Although fungal growth could be observed after that, changes were minimal. In the positive control with 1×10^{-4} moL L^{-1} carboxin concentrate, a clear fungal circle was observed on the right of the plates, and the inhibition rate was 9.09%. Treatment with aqueous mixtures of Q[8] and carboxin at molar ratios of 0.5 induced growth retardation; the fungus was observed to reduce in quantity and the inhibition rate was 15.15%. As the molar ratios increased above 1, the inhibition rate increased to 21.21% and the mycelial growth of *R. solani* was further retarded. The increase in the amount of Q[8] improved the inhibitory effect of carboxin on mycelial growth of *R. solani*.

After treatment with 2×10^{-4} mol L^{-1} carboxin, the relative mycelium-covered area was reduced by half compared to that with 1×10^{-4} mol L^{-1} carboxin. The inhibition rate for each treatment is summarized in Table 1. Addition of Q[8] reduced the mycelium-covered area to the greatest extent compared with the positive control samples. The growth inhibitory effect of carboxin increased in a

Table 1 Growth inhibition rate (Y) of carboxin and inclusion complex solutions

Solutions	Inhibition rate (%)
1×10^{-4} mol L^{-1} g	9.09
0.5×10^{-4} mol L^{-1} Q[8] + 1×10^{-4} mol L^{-1} g	15.15
1×10^{-4} mol L^{-1} Q[8] + 1×10^{-4} mol L^{-1} g	27.27
2×10^{-4} mol L^{-1} g	21.21
1×10^{-4} mol L^{-1} Q[8] + 2×10^{-4} mol L^{-1} g	51.52
0.5×10^{-4} mol L^{-1} Q[8]	0
1×10^{-4} mol L^{-1} Q[8]	0
Negative control	0

dose-dependent manner in the positive treatment and inclusion complex solutions.

Conclusions

Complexation of carboxin with Q[8] promoted the inhibitory effect of carboxin on *R. solani*. Nevertheless, the mechanisms of action are still unclear, and a detailed study is necessary to elucidate the entire process. Inclusion complexation of carboxin with Q[8] provides a potential means through which the fungicidal action of carboxin could be improved, along with alleviation of the environmental burdens of synthetic agrochemicals.

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