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An inclusion complex of eugenol into β-cyclodextrin: Preparation, and physicochemical and antifungal characterization

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ABSTRACT

The inclusion of eugenol (EG) into β-cyclodextrin (βCD), its structural characterization and antifungal activity, and mode of action for control of *Peronophythora litchii* in postharvest fresh litchi fruits is described. Nuclear magnetic resonance spectra revealed chemical shifts in H-3 and H-5 protons of βCD, indicating EG inclusion into the lipophilic cavity of βCD. *In vitro* assays showed βCD–EG significantly inhibited *P. litchii* colony growth in a concentration- and time-dependent manner (MIC_{100} = 0.2 g). *In vivo* assays showed βCD–EG significantly ($p < 0.05$) reduced the decay index of treated fresh litchi fruits. After exposure to βCD–EG, the surface of *P. litchii* hyphae and/or sporangiophores became wrinkled, with folds and breakage observed by scanning electron microscopy. Damage to hyphal and/or sporangiophore cell walls and membrane structures post-treatment with βCD–EG was confirmed by transmission electron microscopy. Therefore, βCD–EG shows great potential as a controlled-release agent against *P. litchii*.

1. Introduction

Litchi (*Litchi chinensis* Sonn.) as a tropical and subtropical fruit tree is mainly cultured in China, Vietnam, India, and South Africa. Over time, fresh litchi fruit has been accepted by consumers worldwide for its delicious taste and attractive red color (Ghosh, 2001; Li & Jiang, 2007). *Peronophythora litchii* has been established as a major causal pathogen of litchi downy blight, and it occurs particularly during litchi fruit ripening and postharvest storage (Kao & Leu, 1980). In the provinces of Guangdong, Fujian, and Guangxi in south China, litchi fruit decay is caused largely by the litchi downy blight, leading to significant postharvest loss (Jiang et al., 2006). As a consequence, much research has focused on the control of *P. litchii* (Cao, Wei, Xu, & Tang, 2010; Tang et al., 2011; Xu et al., 2013). Carboxylic acid amide (CAA) fungicides, such as dimethomorph and flumorph, have been used commercially to control the litchi downy blight for more than 10 years in China. However, *P. litchii* has remained capable of developing resistance to CAA under laboratory conditions (Wang, Sun, Stammler, Ma, & Zhou, 2009, 2010). Therefore, for effective and long-term management of *P. litchii*, there is an urgent need to develop new antifungal chemicals to protect against the loss of harvested litchi fruits during their postharvest storage and transportation.

Eugenol (EG) is an essential oil mainly extracted from the dried flower buds of clove, cinnamon and serves as a fungicide with broad-spectrum antifungal activity (Carrasco et al., 2012; Mazzarrino et al., 2015). It was reported that the minimum inhibitory concentrations (MICs) of EG against the growth of *Cladosporium* species and *Trichophyton rubrum* were 350 and 256 μg/mL, respectively (Abbaszadeh, Sharifzadeh, Shokri, Khosravi, & Abbaszadeh, 2014; De Oliveira Pereira, Mendes, & de Oliveira Lima, 2013). Furthermore, EG is an approved food additive by the U.S. Food and Drug Administration, with an LD_{50} of 3000 mg/kg oral mouse (EAFUS, 2013; Jenner, Hagan, Taylor, Cook, & Fitzhugh, 1964). However, under ambient temperature conditions, EG is highly volatile, which renders it impractical and difficult to apply as a postharvest fungicide. The microcapsule is a relatively novel formulation of pesticide that can increase markedly the stability, and extend the life, of actively volatile agents, facilitate their manipulation, and control their application both temporally and spatially (Hammad, Nigam, Tamboli, & Sundara Moorthi Nainar, 2011). Therefore, it can be anticipated that the development of a microcapsule with EG as core agent would be an attractive alternative to organic fungicides treatment and help as an effective control of postharvest disease of litchi fruits caused by *P. litchii*.

β-Cyclodextrin (βCD), which is used as coating material, is a family member of the cyclic oligosaccharides, composed of seven

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α-1,4-linked glucopyranose subunits (Bender & Komiyama, 1978). βCD exhibits a hydrophobic cavity and hydrophilic external surfaces, suitable for the integration of guest molecules into its unique structure. Thus, βCD can be used as material for the generation of microcapsules. It is generally accepted that the guest molecule is encapsulated into the cavity of βCD (the host) without altering the chemical structure of the host. Interestingly, it has been found that a number of chemical and physical properties of the guest molecule are optimized during the complex formation, such as an increase in its water solubility, greater molecular stability in varying light, heat, and oxidation conditions, the masking of certain undesirable physiological effects, such as odors, and a reduction in volatility (Farcas, Jarroux, Farcas, Harabagiu, & Guegan, 2006). As reported in the published literature, the inclusion of essential oils into βCD dramatically increases the solubility, stability, and meanwhile increases the antimicrobial and antioxidant activities of these compounds (Arana-Sánchez et al., 2010; Hill, Gomes, & Taylor, 2013; Kfoury, Auezova, Greige-Gerges, Ruellan, & Fourmentin, 2014; Weisheimer, Miron, Silva, Guterres, & Schapoval, 2010). Thus, it can postulated that βCD would be suitable as coating material in the development of controlled-release microcapsule formulations aimed at combating postharvest disease of litchi fruits.

The aims of our study were to prepare an inclusion complex by incorporating EG into βCD, to determine the structural and morphological characteristics of the βCD–EG inclusion complex, to assess the antifungal activity of the complex through inhibition of mycelia growth of *P. litchii* both *in vitro* and *in vivo*, and to elucidate the antifungal characteristics based on the microstructure of the complex. The findings from this study would help in the development of a new handling agent aimed at reducing postharvest loss of fresh litchi fruits.

2. Materials and methods

2.1. Materials

βCD was purchased from Mengzhou Huaxing Biochemistry Co., Ltd. (Guangzhou, China), and 1,3,4-EG (98% purity) was obtained from Yuanye Biotechnology Co., Ltd. (Shanghai, China). Of note, 1,3,4-EG was used without further purification. All other reagents were from Guangzhou Chemical Reagents Co. (Guangzhou, China) and were of analytical grade. Distilled water was obtained from a Milli-Q UV system (Millipore, Darmstadt, Germany) and sterilized prior to use.

2.2. Preparation of the βCD–EG inclusion complex

The βCD–EG inclusion complex was prepared by the method of saturated aqueous solution, as described by Balmas, Delogu, Sposito, Rau, and Migheli (2006), with minor modification. Two equivalents (6.50 mmol) of βCD were dissolved in 160 mL of distilled water at 70 °C, with continuous stirring for 1 h. The EG solution, containing one equivalent (3.25 mmol) in a ratio of ethanol: EG = 1:1, was added and mixed by stirring at 60 °C for 12 h. The water was evaporated by vacuum, and the inclusion complex was dried at 60 °C for 24 h. The βCD–EG inclusion complex was recovered as a pale yellow solid, with a yield of >90%.

2.3. NMR measurements

^{1}H NMR and ^{13}C NMR spectra of the βCD–EG inclusion complex and βCD, as well as ^{1}H NMR spectra of EG, were recorded in D_2O solution, using a Bruker Avance III 500 Hz spectrometer (Bruker, Switzerland). ^{1}H and ^{13}C chemical shifts, shown as ppm (δ), were given by comparing the peak relative to D_2O (4.70 ppm) and acetone (30.89 ppm), respectively. Multiplicities were shown by means of d (doublet), t (triplet), m (multiplet), or dd (double of doublets). ^{1}H NMR and ^{13}C NMR spectra were recorded using a 5-mm tube in D_2O without degassing. 2D NMR spectra of the βCD–EG inclusion complex were analyzed in D_2O by nuclear Overhauser enhancement spectroscopy (NOESY), using the Bruker Avance III 500 Hz spectrometer. Mixing and delay times of the NOESY test were 1.00 and 1.7 s, respectively.

2.4. Bioassays

2.4.1. In vitro assay

A monosporic isolate of *P. litchii* (kindly provided by Dr. Rong Zhang, College of Horticulture, South China Agricultural University, Guangzhou, China) was cultured on potato dextrose agar (PDA) at 25 °C in a dark room. Growth inhibitory effect of the βCD–EG inclusion complex on *P. litchii* was performed using 9 cm culture dishes, each with one cross-wall to form two cabinets. One cabinet was inoculated with an assay disc (0.4 cm in diameter) cut from the periphery of the 7-day-old culture of *P. litchii*, and the other cabinet was treated with the βCD–EG inclusion complex at different concentrations of 1, 0.8, 0.4, 0.2, 0.1 and 0.05 g and for different storage periods of 1, 30, and 60 days. βCD only was used as the negative control. The prepared culture dishes were left lidless and kept aseptically in a 5 L jar sealed with multilayer films. In a separate and parallel bioassay, culture dishes were stored aseptically with unsealed lids on. All incubations were carried out in a dark room at 25 °C for 5 days. The percent inhibition rate of radial growth of *P. litchii* was calculated by the following formula: percent inhibition rate = $(CK - T)/CK \times 100$, where CK is the colony diameter (cm) of the control samples, and T is the colony diameter (cm) of the samples post-treatment with the βCD–EG inclusion complex. The MIC was measured through no visible growth of *P. litchii* under treatment conditions, compared to the control set. All experiments were carried out in triplicate.

2.4.2. In vivo assay

The *in vivo* assay of the βCD–EG inclusion complex against *P. litchii* was performed, as previously described (Xu et al., 2013). In brief, a 5 mm wide and 2 mm deep cross-like wound was made on the base of the litchi pericarp, followed by inoculation with 10 μL of spore suspension (1×10^6 spores/mL) of *P. litchii*. The βCD–EG inclusion complex at 1, 0.5, 0.25 or 0.125 g and was packaged with gauze and then used for incubation with 30 litchi fruits, each fruit in duplicate. Each inclusion complex concentration was used in triplicates. 1 g βCD was used as negative control-treatment. Litchi fruits incubated with the βCD–EG inclusion complex were placed in 0.02-mm polyethylene plastic bags and then stored at 28 °C for 5 days. The extent of decay of litchi fruits was monitored according to an organoleptic test, as follows: 1 = no decay, 2 = slight decay, 3 = 25–50% decay, and 4 ⩾ 50% decay. The decay index was further calculated by the formula: Σ (decay scale × proportion of fruits corresponding to each scale)/$5n \times 100\%$ (where n = the number of fruits investigated).

2.5. Electron microscopy observation

SEM and transmission electron microscopy (TEM) were used to assess the morphological structures of *P. litchii* after treatment with the βCD–EG inclusion complex. The mycelium, which was cut from the *P. litchii* colony pretreated with 0.2 g of 30-day βCD–EG inclusion complex and incubated for 5 days, as described in Section 2.4.1, was infiltrated with 2.5% glutaraldehyde (prepared in 0.1 M phosphate buffer, pH 7.2) and kept at 4 °C overnight. The samples were rinsed with 0.1 M phosphate buffer (pH 7.2) three

times (10 min each time) and then postfixed in 1% OsO_4 (Osmium tetroxide) solution for 2 h at 4 °C. The fixed materials were dehydrated through a graded ethanol series from 10% to 90% in 10% incremental steps, followed by 100% ethanol with three changes and 10 min per change. For SEM, the samples were freeze-dried using tertiary butyl alcohol and then examined using a JSM-6360LV scanning electron microscope (NEC, Tokyo, Japan) operated at 25 kV. In the case of TEM, the samples were successively treated with epoxypropane, epoxypropane:resin (3:1), epoxypropane:resin (1:1), and epoxypropane:resin (1:3) for 1 h, then soaked in 100% resin overnight, and finally embedded into resin at 60 °C for 36 h. Ultrathin sections were made using Leica UC6 ultramicrotome (Leica Microsystems, Wetzlar, country) and mounted on copper grids. The sections were stained with 2% uranyl acetate and lead citrate for 30 min and then examined using a JEM-1010 transmission electron microscope (JEOL, Tokyo, Japan) at an accelerating voltage of 100 kV.

In addition, the morphological structures and sizes of the βCD–EG inclusion complex and free βCD were also examined using the JSM-6360LV scanning electron microscope. The samples were evenly distributed on a brass stub using double-sided aluminum tape. To improve conductivity, the samples were gold-coated by a JFC-1600 fine coater (JEOL, Tokyo, Japan). Micrographs were obtained at an accelerating potential of 15 kV under low vacuum, and the particle sizes were measured using Smile View image processing software (NEC, Tokyo, Japan).

2.6. Statistical analyses

All experimental design was fully randomized, and data presented as the means ± standard errors of three replicate experiments. Sigma Plot 12.5 software (Systat Software Inc, CA, USA) was used to perform the analysis of variance. *T*-tests were used, with $p < 0.05$ indicating statistical significance.

3. Results and discussion

3.1. Structural characterizations of the βCD–EG inclusion complex

Due to the presence of the aromatic ring, the inclusion of aromatic compounds typically has a pronounced effect on the chemical shifts of protons in the βCD cavity (Schneider, Hacket, Rüdiger, & Ikeda, 1998). In the present study, the formation of the βCD–EG inclusion complex by insertion of the aromatic ring of EG into the βCD lipophilic cavity was clearly demonstrated by the chemical shifts of H-3 and H-5 resonances of the βCD. As shown in Fig. 1, significantly high field shifts ($\Delta\delta$) of H-3 and H-5 of βCD proton resonance in the βCD–EG inclusion complex were indicated by −0.081 and −0.132, respectively. Consistent with our results, studies on the inclusion complex of βCD with rutin or doxepin also found that the H-3 and H-5 resonances of βCD caused significant chemical shifts (Cruz, Becker, Morris, & Larive, 2008; Ding, Chao, Zhang, Shuang, & Pan, 2003). The chemical shifts of βCD carbon atoms due to the interaction with EG were altered slightly, although significant downfield changes were observed for C1, C3 and C5 (Table 1). 2D NOESY NMR is a powerful technique to study the internal interactions of inclusion complexes, clearly distinguishing between inclusion complexes and other possible interactions (Jahed, Zarrabi, Bordbar, & Hafezi, 2014). In our study, the 2D NMR spectra of the βCD–EG inclusion complex showed that cross-peaks obtained between H-2 and H-5 of EG and H-5 and H-3 of βCD, respectively (Fig. 2A), were in accordance with the ^{1}H NMR spectra. Thus, it can be deduced that the formation of the βCD–EG inclusion complex involved interaction of the hydrophobic aromatic ring side of EG with the lipophilic cavity of βCD, as shown in Fig. 2B. In agreement with our results, Jahed et al. (2014) reported that an inclusion complex of βCD and curcumin showed a cross-peak between H-3 proton of βCD and the aromatic ring group of curcumin, thus indicating that the hydrophobic interactions occurred between the aromatic rings of curcumin and the cavity of βCD.

SEM was used to study the microscopic structure both of βCD and the βCD–EG inclusion complex. Although this method is not a direct analytical technique to understand the interaction forces in the formation of the inclusion complex, nevertheless, it helps to reveal the presence of individual components in a preparation product (Mura, 2015). At 300-fold magnification, βCD was shown as irregular quadrilateral or spherical particles with a mean size of 20.7 μm, whereas the βCD–EG inclusion complex was present as smaller-sized particles (4.2 μm) with a tendency to form aggregates. However, at 2000-fold magnification, we observed a compact crystalline structure of the βCD–EG inclusion complex, with drastic change in its morphology; in contrast, βCD was present as an amorphous shape. These changes in the particle shape, size, and structure provided evidence for a maximum or complete complex formation.

3.2. Antifungal activity of the βCD–EG inclusion complex against P. litchii in vitro and in vivo

It is well known that essential oils exhibit excellent antibacterial and antifungal activities (Azarakhsh, Osman, Ghazali, Tan, & Adzahan, 2014; Prakash, Kedia, Mishra, & Dubey, 2015). However, their application as food preservative is challenged by their high volatility that leads to their quick release from the application point. In the present study, we described the methodology for the inclusion of EG into βCD, together with the mycelia growth inhibition activity of the inclusion complex against *P. litchii*. As shown in Fig. 3, mycelia growth of *P. litchii* was strongly inhibited by the βCD–EG inclusion complex. At a microdosage of 0.05 g, >50% growth inhibition was obtained, compared to the control. This result also showed that the MIC of freshly prepared βCD–EG inclusion complex against *P. litchii* was 0.2 g. After 30 days of storage, the MIC of the inclusion complex decreased to 0.4 g. However, a strong growth inhibition was observed against *P. litchii* at a concentration of 0.2 g. For example, 0.2 g of the βCD–EG inclusion complex after storage for 30 and 60 days showed 92.6% and 87.4% growth inhibition against *P. litchii* in culture dishes with unsealed lids, respectively. Shah, Davidson, and Zhong (2013) reported that EG solubility and antimicrobial activity against *Escherichia coli* O157:H7 and *Listeria monocytogenes* can be improved markedly in bovine milk, by encapsulating EG in nanocapsules prepared with conjugates of whey protein isolate and maltodextrin. Another study was performed by incorporating EG into polyhydroxybutyrate films that synergized the antimicrobial activity of EG against food spoilage microorganisms in conjunction with pediocin (Narayanan, Neera, & Ramana, 2013). Our study demonstrated, for the first time, that the βCD–EG inclusion complex significantly inhibited colony growth of *P. litchii* in a concentration- and time-dependent manner (Fig. 3). However, under our experimental conditions, the volume of outside space may be little effect on antimicrobial activity of the βCD–EG inclusion complex against *P. litchii*, as there was no significant difference in the growth inhibition rate between samples kept in the 5-L sealed jar or culture dishes with unsealed lids (Fig. 3).

Furthermore, litchi fruits rapidly decayed when inoculated with the *P. litchii* spores. Application of the βCD–EG inclusion complex significantly reduced the decay index of litchi fruit during storage. The decay index was 53.2% in control fruits by the end of the storage period. Following βCD–EG inclusion complex treatment at 0.5 and 1 g, the decay indices decreased to 28.5% and 17.4%,

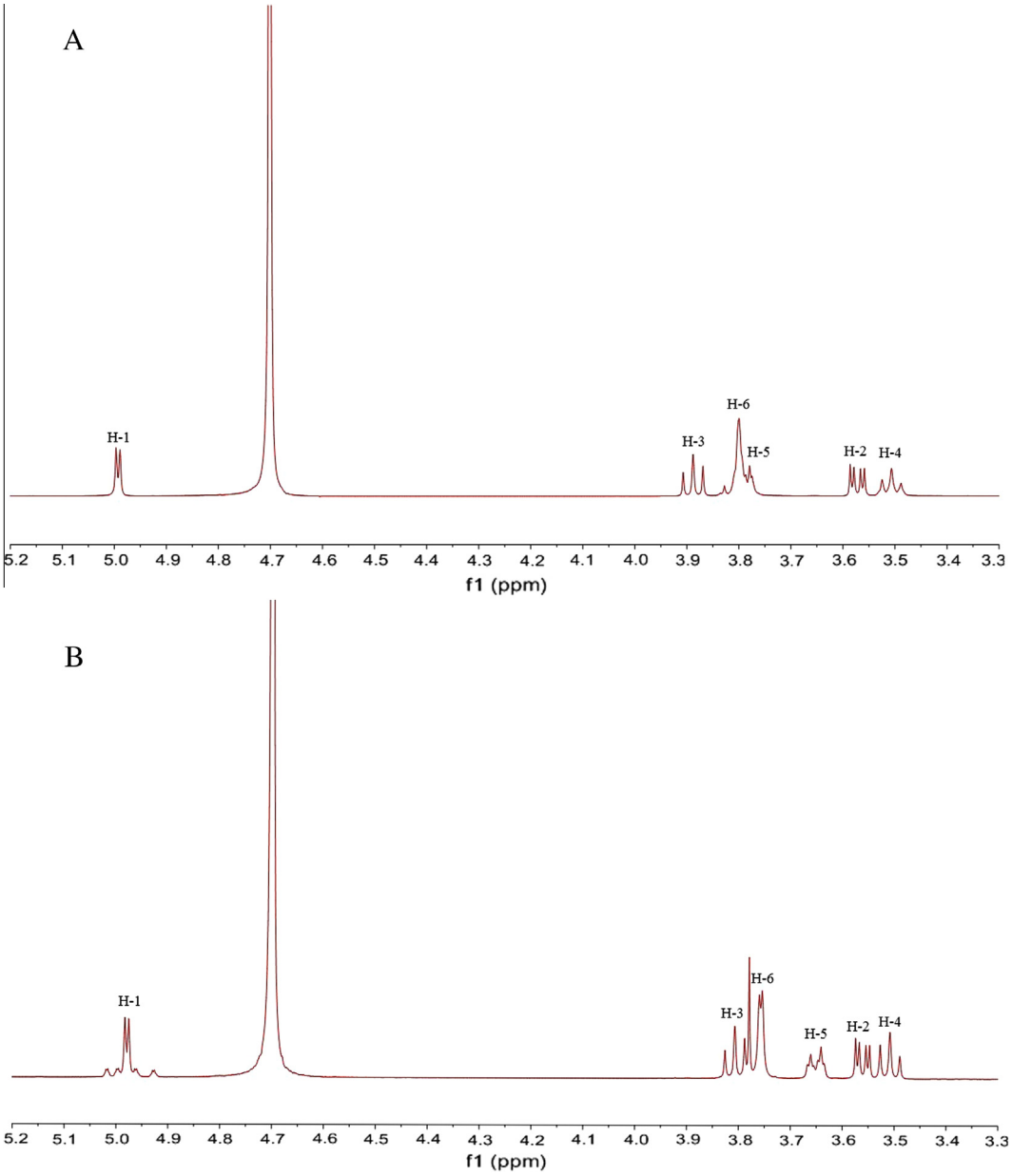

Fig. 1. ^{1}H nuclear magnetic resonance spectra of βCD (A) and βCD–EG inclusion complex (B).

Table 1
^{1}H and ^{13}C nuclear magnetic resonance shifts of the βCD–EG inclusion complex and βCD free in D_2O. The corresponding shifts ($\Delta\delta$) represent the chemical shift differences (ppm) between the two states. Negative values indicate shift to high field.

Proton	βCD	βCD–EG	$\Delta\delta$	Proton	βCD	βCD–EG	$\Delta\delta$
H1	4.993	4.979	−0.014	C1	102.50	102.59	0.09
H2	3.572	3.560	−0.012	C2	72.69	72.70	0.01
H3	3.888	3.807	−0.081	C3	73.73	73.87	0.14
H4	3.507	3.508	0.001	C4	81.74	81.73	−0.01
H5	3.783	3.651	−0.132	C5	72.47	72.58	0.11
H6	3.800	3.772	−0.028	C6	60.87	60.76	−0.11

respectively (Fig. 4). Plant essential oils, such as EG, have been shown to have high antioxidant effectiveness by reducing the production of reactive oxygen species (Horvathova et al., 2014). Rapid postharvest pericarp browning is one of the most important limitations in the litchi industry that lowers the commodity value of litchi fruits (Jiang, Duan, Joyce, Zhang, & Li, 2004). Our study, however, showed the effect of EG on pericarp browning of the litchi fruits was not significant (data not shown), possibly due to a lack of inhibition of polyphenol oxidase activity which was reported to be involved in pericarp browning of litchi fruits (Wang, Liu, Xiao, Li, & Sun, 2014).

3.3. Action of the βCD–EG inclusion complex in the control of *P. litchii*

Recently, there has been increasing interest in the use of plant-derived antimicrobial compounds as natural preservatives, but little is known about their mode of action (Calo, Crandall, O'Bryan, & Ricke, 2015). Bhatia et al. (2012) reported the fungicidal activity of two essential oil components against *Candida* species through H^+/ATPase-mediated inhibition. In addition, the tolerance of yeast to EG was evaluated by studying the calcium ion influx via a plasma membrane calcium channel (Roberts, McAinsh, Cantopher, & Sandison, 2014). In the present study, the effects of the βCD–EG inclusion complex on the hyphal morphology of *P. litchii* were

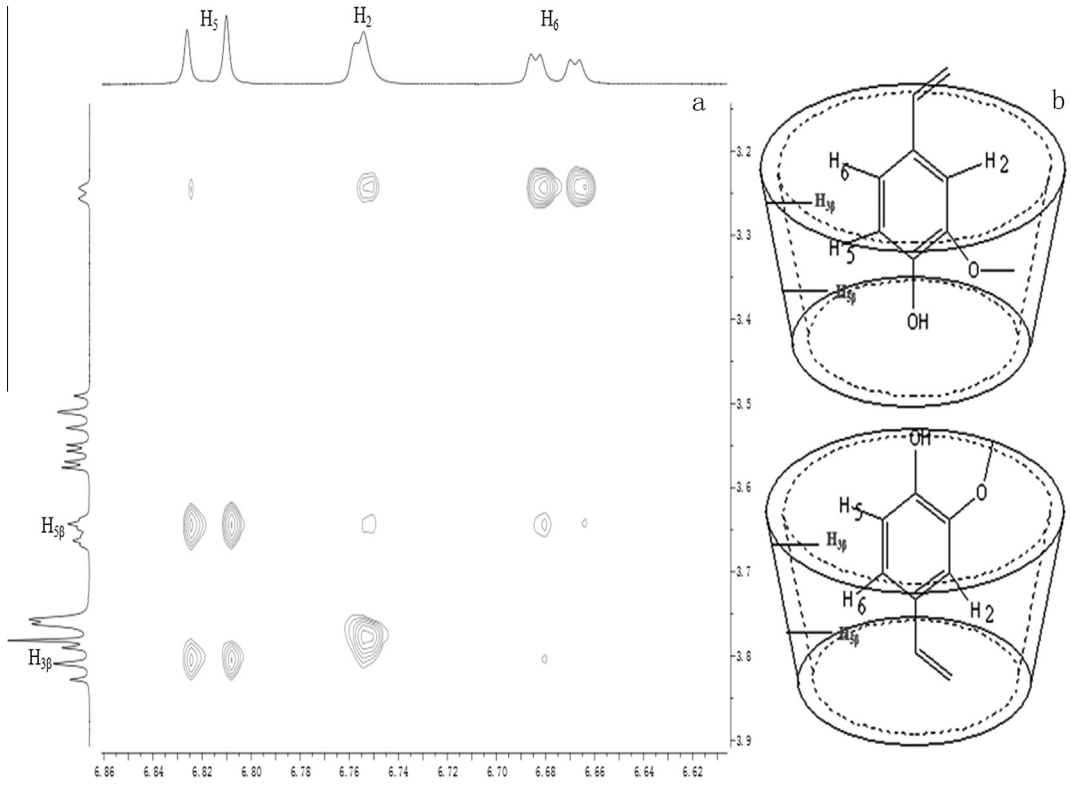

Fig. 2. NOESY spectrum of the βCD–EG inclusion complex (A) and the possible inclusion mode (B).

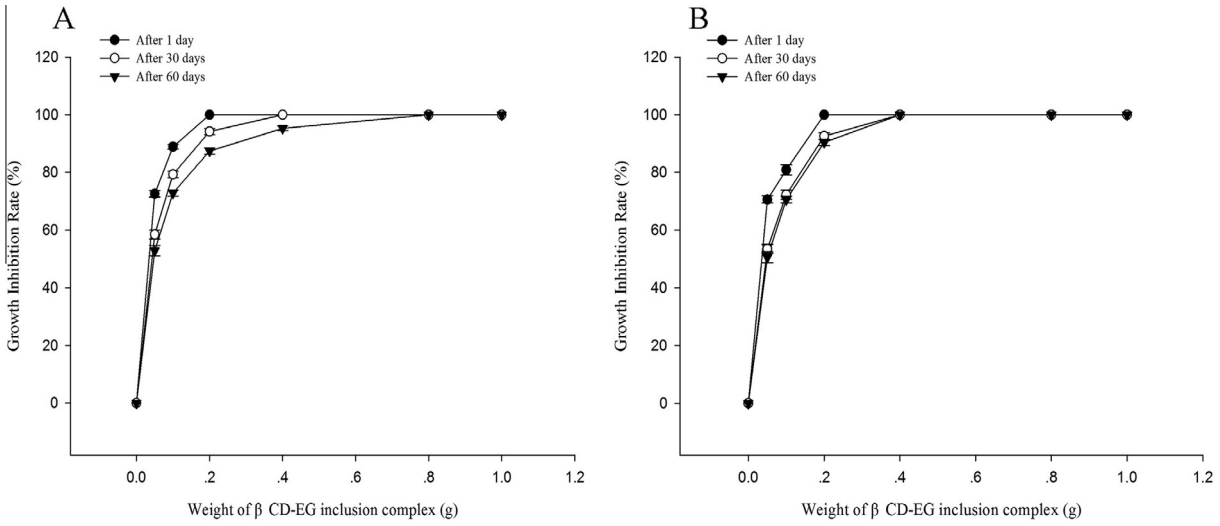

Fig. 3. Growth inhibition rate of the βCD–EG inclusion complex against *P. litchii* measured from samples kept in a 5-L sealed jar (A) or culture dishes with unsealed lids (B). The storage periods of the βCD–EG inclusion complex were 1, 30, and 60 days.

investigated by SEM. As shown in Fig. 5A, mycelia grown on PDA containing only βCD had a linear and completely tubular shape, and sporangia and sporangiophores were normal with a smooth surface. Following treatment of *P. litchii* hyphae, with the βCD–EG inclusion complex, sporangia and sporangiophores displayed aberrant morphologies, including surface shrinkage, partial distortion, and wrinkling (Fig. 5B). Furthermore, treatment with the βCD–EG inclusion complex resulted in ultrastructural changes of *P. litchii*, including disruption of the cellular walls and endomembrane system, especially the plasma membrane, mitochondria, and vacuoles, leading to cellular leakage and loss of cellular integrity (Fig. 5E and F). Similar results were found by others. Zore, Thakre, Jadhav, and Karuppayil (2011) reported that terpenoid inhibited the growth of *Candida albicans* by altering the membrane integrity of the fungus, which resulted in leakage of cellular materials. Hypothemycin caused hyphae deformation of *P. litchii* and the formation of evident craters on the surface of the fungus (Xu et al., 2013). Shao et al. (2015) reported that the antifungal activities of chitosan, combined with clove oil, against citrus green mold could be attributed to the disruption of the cellular membrane. Our study is the first to explore the antifungal mechanism of EG against *P. litchii* in relation to changes in the fungal microstructures.

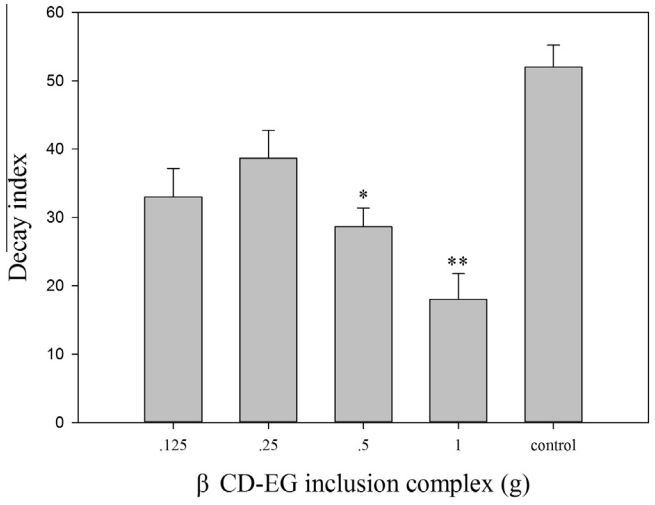

Fig. 4. Effects of the βCD–EG inclusion complex at different concentrations on decay index of litchi fruits. Statistical analysis was performed using Sigma Plot 12.5 (mean ± standard error, with $^{*}p < 0.05$ and $^{**}p < 0.001$).

4. Conclusions

In this study, we produced a βCD–EG inclusion complex, analyzed its structural characteristics, and demonstrated its antifungal mode of action by showing its disruptive effects on the morphology and ultrastructure of *P. litchii* post-treatment. Taken together, our findings indicate that the antifungal activity of the βCD–EG inclusion complex shows great promise for the control of postharvest litchi downy blight. Therefore, we believe that the βCD–EG inclusion complex could be a strong candidate as a safe and highly effective fungicidal microcapsule, which can be used in the control against *P. litchii*. However, further studies of its application on a commercial scale are required.

Acknowledgments

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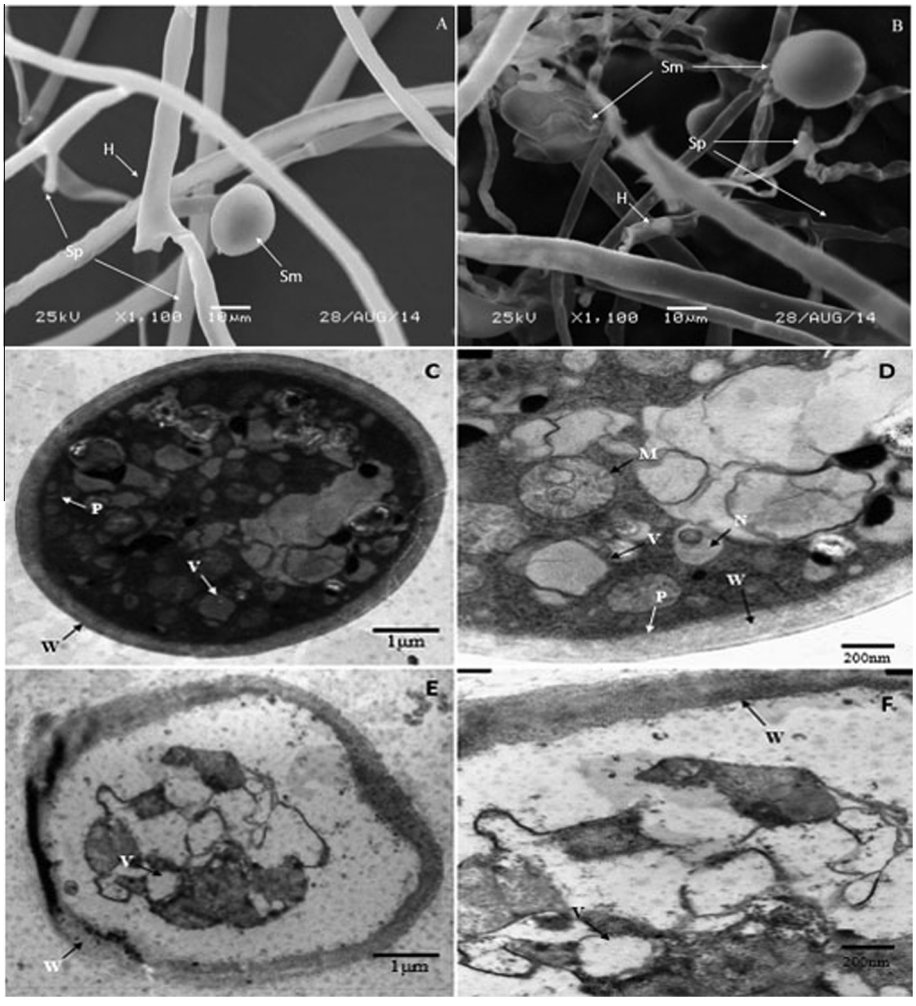

Fig. 5. Scanning electron micrographs of hyphae or sporangia of *P. litchii* grown for 5 days on PDA plates treated without (A) or with (B) the βCD–EG inclusion complex. H, hyphae; Sm, sporangium; and Sp, sporangiophore. Transmission electron micrographs of longitudinal sections through the hyphae of *P. litchii*. (C) and (D) show the control group; (E) and (F) show samples treated with the βCD–EG inclusion complex. M, mitochondria; N, cell nucleus; P, plasma membrane; V, vacuoles; and W, cell wall.

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