Research Article

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Curcumin-removed turmeric oleoresin nano-emulsion as a novel botanical fungicide to control anthracnose (*Colletotrichum gloeosporioides*) in litchi

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Abstract: During curcumin production in Vietnam, curcumin-removed turmeric oleoresin (CRTO) has been considered as a by-product. It costs to treat the by-product to prevent environmental pollution. In this study, the by-product was utilized as an active ingredient for preparing a botanical fungicide-based nano-emulsion and evaluated for its *in vitro* and *in vivo* control efficacy against *Colletotrichum gloeosporioides*, a causal agent of anthracnose of litchi, in the laboratory as well as a field trial. The nano-emulsion is colloidally stable and uniform with particle sizes of 95–250 nm. CRTO nano-emulsion significantly affected various *Colletotrichum* species. Notably, this nano-emulsion showed potent inhibition for the mycelial growth of *C. gloeosporioides* and solidly suppressed the development of anthracnose on litchi fruits. In the *in vitro* inhibition test, the equivalent half-maximal inhibitory concentration of CRTO in nano-formulation was 0.11 mg·mL^{-1}, which was 3.0× and 6.1× lower than IC_{50} values of CRTO alone (0.33 mg·mL^{-1}) and a mixture of curcuminoids (0.48 mg·mL^{-1}), respectively. In the field trial, the litchi anthracnose infection was effectively controlled by nano-formulation. These results suggest that CRTO nano-emulsion could be used as an alternative to harmful synthetic fungicides to control anthracnose on litchi fruits.

Keywords: anthracnose, curcumin, litchi, nano-emulsion, turmeric

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1 Introduction

Litchi chinensis (litchi) is a native plant growing in Southeast Asia. The plant fruit provides a high value in food production, especially in the Vietnam fruit exportation system where fruit production has been mainly exported to China. One of the most serious diseases of litchi is anthracnose caused by the fungus *Colletotrichum gloeosporioides* [1–3]. *C. gloeosporioides* belongs to the class of *Sordariomycetes*, which mostly attaches the leaves, flowers, and fruits of litchi in the period of flowering until the fruit getting half-grown

state and causes anthracnose fruit rot and leaf necrosis [4]. In the past, the anthracnose of litchi was often treated with toxic synthetic chemicals such as carbendazim, mancozeb, and copper fungicides [2,5]. Currently, to meet the criteria in the sustainable management of organic agriculture, the farmers in litchi plantations of Vietnam have tried to replace the harmful chemical pesticides with biopesticides. Among research on active ingredients of biopesticides, substances such as essential oils (EOs), phytochemicals, and chitosan affirmed as generally recognized as safe (GRAS) are considered to apply in control of *Colletotrichum* sp. in fruit preservation and crop protection [6]. The control of *C. gloeosporioides* by GRAS compounds and plant extracts often results in morphological damages and electrolyte leakage for fungi, and their antifungal mechanisms were found to be the inhibition of cellulolytic and pectinolytic enzymes and to induce the defense-related enzymes of host plants [7,8]. Even though several biopesticides have been investigated *in vitro* and *in vivo*, not many of them are considered to be good candidates for in-field treatment. Some commercial bio-fungicides such as antagonistic *Bacillus* species, Milsana (a botanical extract of *Reynoutria sachalinensis*), and EO-based fungicides have been used effectively to treat plant diseases for various crops [8,9]. However, there is no report on the effectiveness of those fungicide treatments on controlling the anthracnose in litchi yet.

Curcuma longa (Turmeric) rhizome is found to contain main constituents such as ar-turmerone, α, and ß-turmerone in the EO, curcuminoids (CURs) (a mixture of curcumin, demethoxylcurcumin (DMC), and bisdemethoxylcurcumin (BDMC) in the yellow pigment part, and starch [10–12]. Turmeric oleoresin extracted from *C. longa* rhizome with organic solvents such as acetone, dichloromethane, hexanes, and short-chain alcohols (methanol and ethanol) comprises EO and CURs [10]. After curcumin is crystallized and separated off from the turmeric oleoresin, the mother liquor named "curcumin-removed turmeric oleoresin" (CRTO) was collected as a by-product [11,12]. In general, the EO from turmeric rhizomes and other EOs are defined as GRAS materials by the US Food and Drug Administration and the Environmental Protection Agency [13]. ar-Turmerone from turmeric has been seen to possess antifungal and insecticidal properties and was confirmed as a good active ingredient for controlling the plant diseases caused by *Phytophthora infestans* and *Erysiphe graminis* [14–16]. Turmeric extracts and CURs have excellent *in vitro* and *in vivo* control efficacies against *Rhizoctonia solani*, *P. infestans*, *Puccinia recondia*, and *Colletotrichum* species [16–18]. However, the solubility of the turmeric extracts and their constituents in water is often low or limited due to their chemical composition, characteristics of molecular structure, lipophilic property, and polarity. This leads to poor bioavailability of the materials during their uses as therapeutic agents in animal models or as fungicides in crop plants.

To enhance the solubility of the oil-soluble botanical materials such as extracts and EOs, encapsulation has been preferred to apply and create good solubility, stability and improve biological effectiveness. Among encapsulation techniques, micro-and nano-emulsions are the easiest to formulate and handle with low-cost performance [13]. In this study, we aim to utilize a by-product of turmeric oleoresin, CRTO, by formulating it into an ecofriendly nano-emulsion fungicide. The nano-formulation was characterized by dynamic light scattering (DLS) and transmission electron microscopy, and its control efficacy was evaluated *in vitro* and *in vivo* against litchi anthracnose as well as assessed through a field trial.

2 Materials and methods

2.1 Materials and testing microorganisms

CRTO was obtained as mother liquor after crystallization of CURs from the CUR production in Vietnam Institute of Industrial Chemistry (VIIC). The major constituents of CRTO were ar-turmerone (35.03%), curcumin (16.89%), DMC (15.22%), and BDMC (14.03%). The minor constituents of CRTO were zingiberene, sesquiphellandrene, α-turmerone, ar-curcumene, and curlone. A mixture of purified CURs including curcumin (79.05%), DMC (7.95%), and BDMC (1.35%) was provided by VIIC. Polysorbate 80 (Tween 80®) was supplied by Anhui BBCA Pharmaceutical Co. Ltd. (China). Ethanol 96%, deionized water, propylene glycol (PG, 99.5%, Sigma-Aldrich), and PEG 400 (Kanto, Japan) were used to formulate the CRTO formulation.

Colletotrichum species such as *C. acutatum* (Ca), *C. gloeosporioides* (Cg), and *C. orbiculare* (Co) were used for *in vitro* and *in vivo* tests. The phytopathogenic strain Cg was isolated from the infected leaves, fruits, and twigs of litchis cultivated in Tan Yen, Bac Giang, Vietnam. In brief, the anthracnose lesions were disinfected with ethanol 70% for 1–2 min, rinsed with distilled water, dried by sterile napkins, and sliced into small sections (×2 mm). The sections were placed on a half-strength potato dextrose agar (PDA) and incubated at 25°C for generating conidia and hyphae. Pure cultures of the Cg isolate were obtained by the

hyphal tip and single germinated conidia isolation. Similarly, the strains of Ca and Co were isolated from the infected tissues of Vietnamese *Panax ginseng* and cucumber, respectively.

2.2 Preparation of CRTO nano-emulsion

The preparation of CRTO nano-emulsion was performed through three steps as described in Figure 1a.

Step 1: The dispersing phase (I) was prepared by mixing CRTO and ethanol at the ratio of 98/60 (w/v) under stirring with a magnetic stirrer at 150 rpm for 3 h to form a homogeneous phase.

Step 2: Phase (II) was made by mixing deionized water with surfactants to get a mixture of PEG400/PG/Tween 80/water ratio of 2.5/20/20.5/40 (w/w/v/v/v) under stirring at 450 rpm for 1 h.

Step 3: The phase (I) was slowly added drop by drop into the aqueous phase (II) containing PEG400/EG/Tween 80/water under stirring at 450 rpm and sonicated during stirring by an ultrasonic bath at 700 W (Elma S120H, Germany) to create a nano-formulation with the ratio of CRTO/ethanol/PEG400/PG/Tween 80/water at 98/60/2.5/20/20.5/40 (w/v/w/w/v/v/v). After completely adding the phase (I), the stirring and sonication remained for 2 h at room temperature and the nano-emulsion was left overnight and tested for morphology characteristic and particle size, and dispensability index measurement [15,16].

2.3 Characterization of CRTO nano-emulsion

The size, zeta potential, and stability of the nanodispersion system in an aqueous solution were investigated by a DLS analysis system (DLS, Zetasizer Ver. 6.20, Malvern Instruments, UK). Before measurement, a portion of CRTO nano-emulsion diluted in water at a concentration of 0.2 mg·mL^{-1} was used for character analysis. The particle's actual size and shape were determined using a transmission electron microscope (high-resolution transmission electron microscopy [HRTEM], JEOL JEM-2100, Japan).

2.4 *In vitro* bioassay for evaluation of antifungal activity against *Colletotrichum* species

The three filamentous fungi *C. acutatum* (Ca), *C. gloeosporioides* (Cg), and *C. orbiculare* (Co), which cause

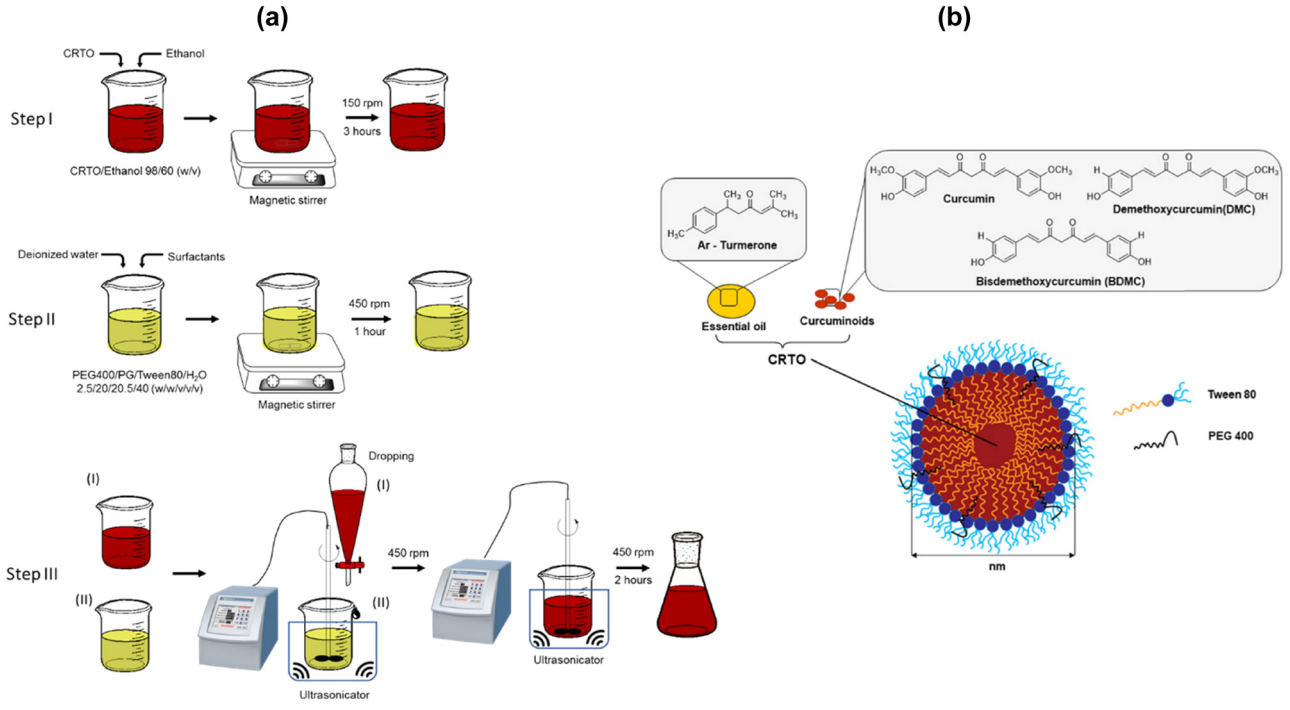

Figure 1: Preparation procedure of botanical nano-emulsion composed of CRTO (a). Schematic of CRTO nano-emulsion droplets and main constituents (b).

anthracnoses on crop plants, were used in the inhibitory bioassay against the mycelial growth. CRTO and CRTO nano-emulsion were dissolved in DMSO and incorporated with molten PDA medium (at 50–60°C) to reach the working concentrations ranging from 0.15 to 5 mg·mL^{-1}. The antifungal activity of a mixture of CURs composed of curcumin (79.05%), DMC (7.95%), and BDMC (1.35%) was examined at concentrations from 0.125 to 1 mg·mL^{-1}. To inoculate the fungi, 4 mm plugs from the edge of an actively growing mycelium of *Colletotrichum* species were centrally placed in the solidified PDA Petri dishes. The inoculated Petri dishes were incubated at 25°C in the dark for 4 days to reach the edge of the dish. PDA containing 2% DMSO was served as the negative control. A commercial fungicide (Daconil 500 SC containing 500 g·L^{-1} of chlorothalonil) was used as a positive control at the dilution rate of 1,000 fold based on the manufacturer's guide. All treatments were conducted four replicated, and the experiments were repeated twice. Inhibitory efficacy (%) of test materials was expressed as a percentage of inhibition [19].

2.5 *In vivo* antifungal efficacy evaluation on litchi fruits

The fresh litchi fruits with uniform size, shape, and without any signs of damage were harvested and selected to use in the *in vivo* bioassay. *C. gloeosporioides* spore solution was prepared in sterilized water containing 0.2% Tween 80 and filtered through two layers of chest cloth. CRTO nano-emulsion was diluted with sterilized water to get concentrations of 1, 2, 4, and 8 mg·mL^{-1}. Before the test, litchi fruits were washed with sodium hypochlorite (1%) for 3 min, rinsed with deionized water, and allowed to dry at room temperature. A wound (diameter of 4 mm) on each litchi fruit was made using a sterilized needle. *C. gloeosporioides* spores were applied on the wound sites by cotton swabs to form anthracnose infection. The infected fruits were left at room temperature for 3 days until the fungal mycelium appeared. The CRTO nano-emulsion was then sprayed onto the infected fruits and dried at room temperature. The 10 infected fruits in each treatment were placed in sealed and moisture boxes and kept in an incubator at 24–25°C, and it lasted for 7 days. The experiments were repeated twice. The negative control was treated with deionized water alone, and the positive control was treated with Score 250 EC containing 250 g/L of difenoconazole (Syngenta Vietnam Co. Ltd.). The diameter of the infection lesion covered by fungal mycelia was measured at 7 days after being sprayed with CRTO nano-emulsion. The inhibitory efficacy of CRTO nano-emulsion was expressed as the percentage of the fungal infection lesion decreased on the litchi fruit pericarp of treated fruits as compared with those of the negative controls.

2.6 Field trial of CRTO nano-emulsion on litchi plants

The field experiments for evaluating the potential of CRTO nano-emulsion against litchi anthracnose were conducted based on the guide of Vietnam National technical regulation [20]. We selected 130 litchi plants aged from 9 to 16-year-old to evaluate the disease control efficacy of the nano-formulation in the field trial in Tan Yen (65 plants; cultivar: U Hong, 13–16-year-old; from April 8 to May 28, 2020) and Luc Ngan (65 plants; cultivar: Vai Thieu; 9–13-year-old; from April 29 to June 16, 2020) in 2020 in Bac Giang. In each district, all treatments were arranged in a completely randomized design and replicated four times in four plots and each plot has five litchi plants. The nano-emulsion was diluted with water to reach the doses of 5, 7.5, and 10 mg·mL^{-1} and sprayed at a volume of 800 L·ha^{-1} to the infected plants at the interval of 14 days. The last spray was conducted 15 days before harvesting the litchi fruits. The negative control (1 plot) was conducted with water alone. Disease incidence was determined as a percentage of the infected plants, of which leaves and fruits showed at least one lesion, compared with the total number of investigated plants. The disease assessment in the litchi plants was also investigated based on the direct estimation of disease severity on the infected fruits. The disease severity index of anthracnose (%) in litchi plants was measured as $[(n1 \times 1 + n2 \times 2 + n3 \times 3 + n4 \times 4 + n5 \times 5) \times 100]/(5 \times \text{total number of the observed fruits})$, where $n1$–$n5$ are the numbers of observed litchi fruits relative to the disease indices

Table 1: Scores of severity levels for assessment of anthracnose caused by *C. gloeosporioides* in litchi

Score	Damage of fungal infection on litchi fruits
1	Below 5% infection lesion on the observed fruits
2	5–10% of infection lesions on the observed fruits
3	10–20% of infection lesions on the observed fruits
4	20–30% of infection lesions on the observed fruits
5	Higher than 30% of infection lesions on the observed fruits

1–5, respectively (Table 1). The disease control efficacy was expressed as the percentage, according to Henderson–Tilton formula calculation as follows:

$$\text{Control value (\%)} = 1 - \left(\frac{T_a \times C_b}{C_a \times T_b}\right), \quad (1)$$

where T_a is disease severity of the plants in the treatment plots after spraying with CRTO nano-emulsion; T_b is disease severity of the plants in the treatment plots before spraying with CRTO nano-emulsion; C_a is disease severity of the plants in the negative control plots after spraying with water; C_b is disease severity of the plants in the negative control plots before spraying with water [20,21].

2.7 Statistical analysis

All bioassay experiments were performed at least in triplicate. The data were subjected to analysis of variance (ANOVA) by using IRRISTAT 5.0 and WINPEPI software version 11.63. The data were presented as means ± standard deviation (SD) and Duncan's multiple range test ($P \leq 0.05$) were carried out for all experiments. IC_{50} values were calculated based on the Probit analysis by WINPEPI software [19].

3 Results

3.1 Characterization of CRTO nano-emulsion

The formulation of CRTO using the encapsulation technique resulted in a milky and dark nano-emulsion. The characterization of CRTO nano-emulsion was determined by DLS and HRTEM methods. The means of droplet size (Z-Ave), zeta potential, and polydispersity index (PDI) of the CRTO nano-emulsion were determined by using a particle size analyzer. The Z-Ave of the CRTO nano-emulsion was 225 nm (Figure 2a). As for the stability of nano-emulsion, the zeta potential of the CRTO nano-emulsion diluted at a concentration of 0.2 mg·mL^{-1} archived a high value (–32.7 mV), which confirmed the good stability of the nanoformulation (Figure 2b). Marzuki et al. [22] mentioned that the higher zeta potential values lead to a more stable emulsion than the lower values. The small value of PDI (0.206) of CRTO nano-emulsion indicated that the nano-emulsion is more physically stable and uniform (Table 2). The morphology of the CRTO nano-emulsion was studied by HRTEM, at which the characterization of CRTO nano-emulsion that included the actual size and shape of nanoparticles was observed (Figures 1b and 3). The CRTO droplets in the nano-emulsion were found to appear as dark droplets with particle sizes in the range of 95–250 nm under HRTEM observations (Figure 3).

3.2 *In vitro* inhibitory activity of CRTO nano-emulsion against the mycelial growth of *Colletotrichum* species

The *in vitro* antifungal activity of CRTO nano-emulsion was evaluated against the mycelial growth of *Colletotrichum* species. Both CRTO and CRTO nano-emulsion showed significant inhibitions for the mycelial growth of the fungi tested (Table 3 and Figure 4). The positive control with CUR showed antifungal activities against Ca (IC_{50} = 0.13 mg·mL^{-1}), Cg (IC_{50} = 0.48 mg·mL^{-1}), and Co (IC_{50} = 0.51 mg·mL^{-1}). The strain Cg was less sensitive compared with the other fungi tested; it was suppressed by 50% growth when exposed to CRTO

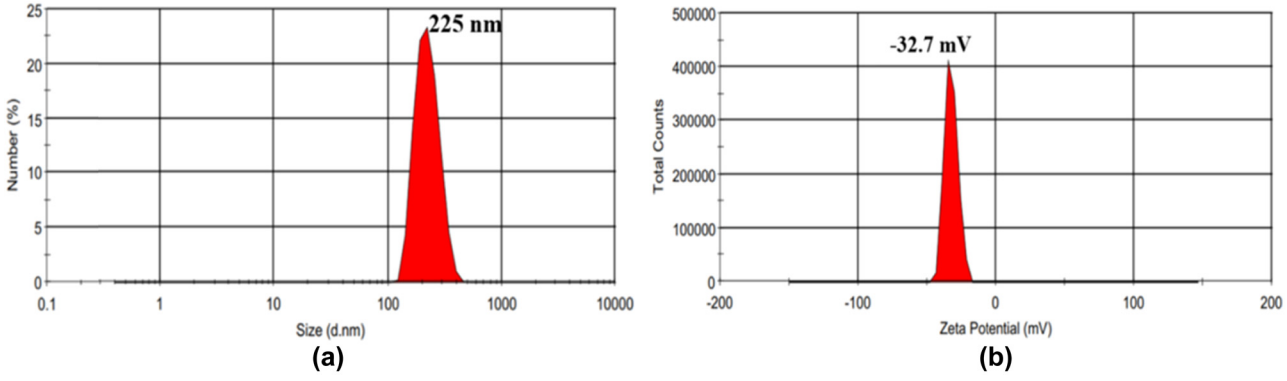

Figure 2: Droplet size distribution (a) and zeta potential (b) of CRTO nano-emulsion.

Table 2: Other characterized parameters of CRTO nano-emulsion

PDI	Zeta potential (mV)	Dispersant refractive index	Viscosity (centipoise)	Dispersant dielectric constant
0.206	−32.7	1.33	0.8872	78.5

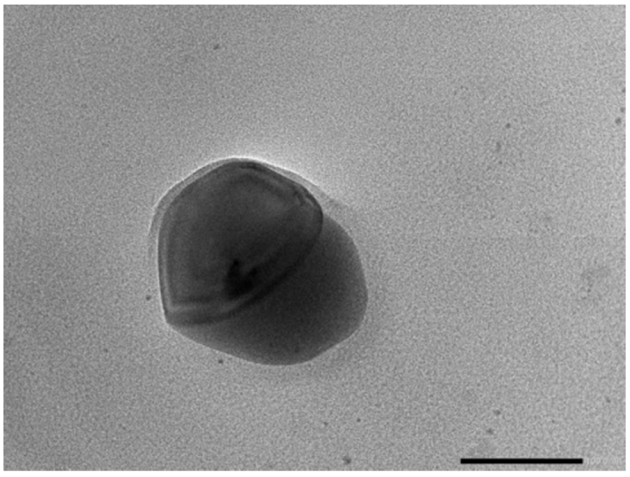

Figure 3: HRTEM image of CRTO nano-emulsion. The scale bar is at 100 nm.

Table 3: The half-maximal inhibitory concentration (IC_{50}, mg·mL^{-1}) of test samples against the mycelial growth of *Colletotrichum* species *in vitro*

	The half maximal inhibitory concentration (IC_{50}) (mg·mL^{-1})		
Fungi	CRTO nano-emulsion[a]	CRTO	CUR
Ca[b]	1.15 ± 0.08a[c]	0.64 ± 0.05a	0.13 ± 0.09b
Cg	0.28 ± 0.03b	0.33 ± 0.06b	0.48 ± 0.14a
Co	0.19 ± 0.03b	0.46 ± 0.18ab	0.51 ± 0.09a

[a] CRTO: curcumin removed turmeric oleoresin; CRTO nano-emulsion contains 40.1% of CRTO as an active ingredient. CUR: a mixture of curcuminoids composed of curcumin (79.05%), DMC (7.95%), and BDMC (1.35%) was used as a positive control.
[b] Ca: *C. acutatum*; Cg: *C. gloeosporioides*; and Co: *C. orbiculare*. The fungi were cultured in Petri dishes and incubated at 25°C for 4 days in the dark.
[c] Means in the same row followed by the same lowercase letter are not significantly different according to Duncan's multiple range test ($P \leq 0.05$).

(0.33 mg·mL^{-1}), CRTO nano-emulsion (0.28 mg·mL^{-1}), and CUR (0.48 mg·mL^{-1}). The nano-formulation displayed the lowest IC_{50} value of 0.28 mg·mL^{-1} for Cg, equivalent to 0.11 mg·mL^{-1} of CRTO in the nanoformulation. The equivalent concentration of CRTO (0.11 mg·mL^{-1}) was 3.0× and 6.1× lower than IC_{50} values of CRTO (0.33 mg·mL^{-1}) and CUR (0.48 mg·mL^{-1}), respectively (Table 3). Similarly, in the bioassay against Co strain, the CRTO nano-emulsion with IC_{50} of CRTO equivalent to 0.08 mg·mL^{-1} also exhibited the best effectiveness; it causes a comparable inhibition to the uses of CRTO and CUR at the 5.7× and 6.3× higher IC_{50} values (Table 3).

3.3 *In vivo* antifungal efficacy evaluation on litchi fruits

The previous results of *in vitro* test proved that CRTO nano-emulsion affected mycelial growth on PDA medium. To rigorously evaluate the antifungal efficiency *in vivo*, we conducted the tests of CRTO nano-emulsion on litchi fruits that were artificially infected with the fungus. Results demonstrated that fungal mycelium decreased gradually with spraying at the highest concentration (8 mg·mL^{-1}). After 7 days of treatment at 8 mg·mL^{-1}, the fungal mycelium was significantly inhibited at the infection sites (73.4% inhibition, Figure 5). In parallel, in the control samples that were sprayed with sterile water instead, the mycelium showed no sign of decreasing the infection lesion (Figure 5a). However, the remarkable results were observed only at the concentrations 2, 4, and 8 mg·mL^{-1} of CRTO nano-emulsion (equivalent to 0.26, 1.30, and 3.26 mg·mL^{-1} of CRTO). From the *in vitro* results, it was found that at the lower concentrations than 1 mg·mL^{-1}, the antifungal effect observed was not clear or not significant in the control of the anthracnose on litchi fruits.

3.4 Field trial of CRTO nano-emulsion on litchi plants

The assessments of disease incidence and disease severity and disease control efficacy of CRTO nano-emulsion against litchi anthracnose in field trial were based on the guide of Vietnam National technical regulation [20]. Disease incidence values of the test plants were from 0.9 to 1.02% before treatment. All plants treated with CRTO nano-emulsion showed no phytotoxicity symptom (data not shown).

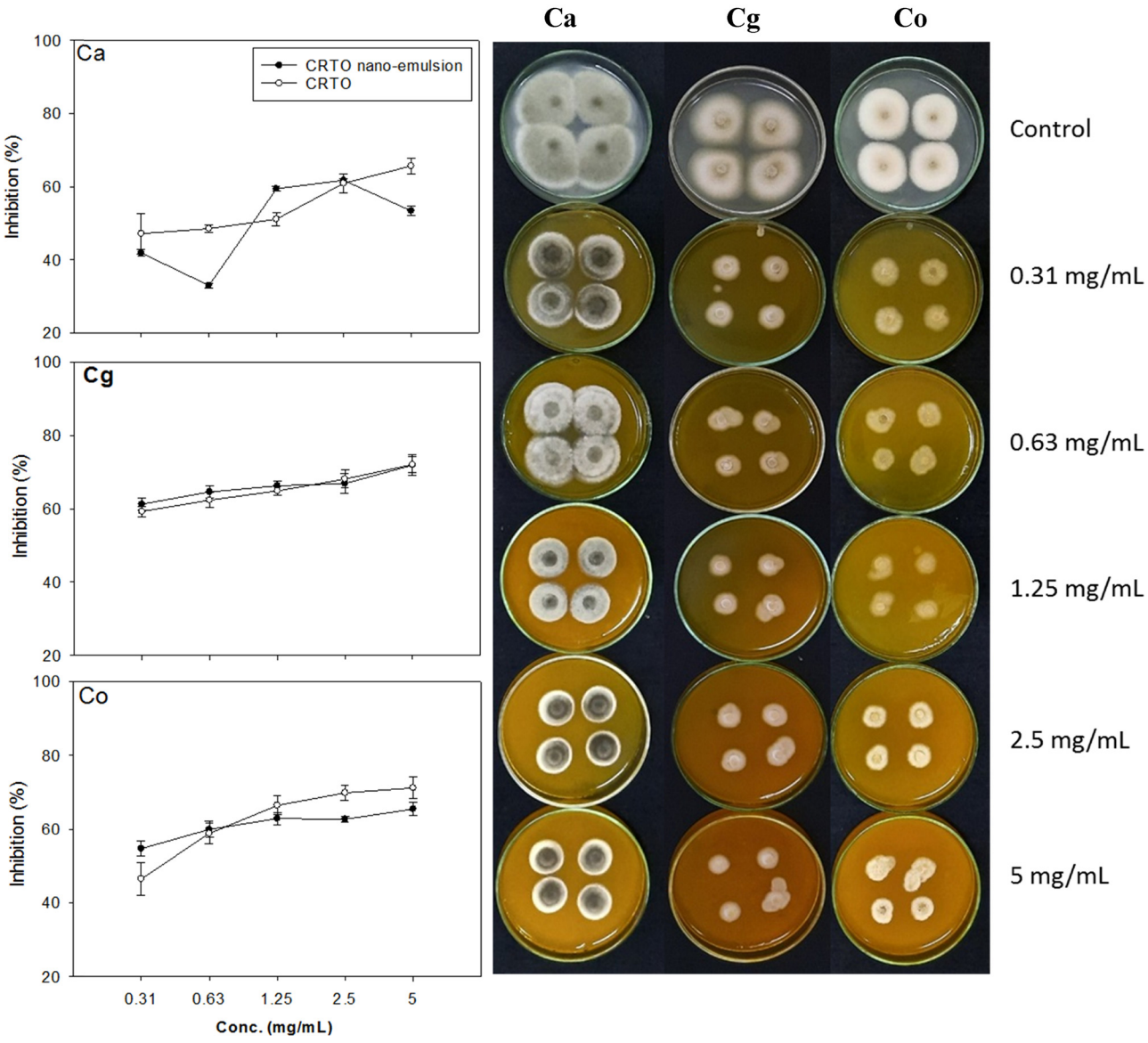

Figure 4: The *in vitro* effects of different concentrations of CRTO nano-emulsion and CRTO on the mycelial growth of *Colletotrichum* species. CRTO: curcumin removed turmeric oleoresin; CRTO nano-emulsion contains 40.1% of CRTO as an active ingredient; Ca: *C. acutatum*; Cg: *C. gloeosporioides*; and Co: *C. orbiculare*. The fungi were cultured in Petri dishes and incubated at 25°C for 4 days in the dark.

In the field trial from April to May 2020 in Tan Yen district, the development of infection on the control plants was observed with an initial disease incidence of 0.96% at the first stage and developed the infection to the incidence values of 7.16% at the third assessment. At all of the three assessments, the litchi plants treated with CRTO nano-emulsion at 5, 7.5, and 10 mg·mL^{-1} (equivalent to 2.03, 3.05, and 4.07 mg of CRTO/mL) showed disease incidences lower than that of the negative control plants. The treatment of CRTO nano-emulsion with a dose of 10 mg·mL^{-1} caused the best suppression for litchi anthracnose, at which the disease incidence archived 1.61% (Table 4). The disease severity indices of the treated plant and negative control experiment were presented in Table 4. At the third assessment, the disease severity index of the treated plants at 10 mg·mL^{-1} was 0.34%, while the negative control group showed the highest value of 2.05% (Table 5).

In the Luc Ngan field trial, CRTO nano-emulsion was sprayed on 65 litchi plants belonging to the cultivar of Vai Thieu. After the first application, the disease incidence of anthracnose in the untreated plants (negative control) was 2.52%; whereas, CRTO nano-emulsion reduced the disease incidences by 1.37–1.95%. The

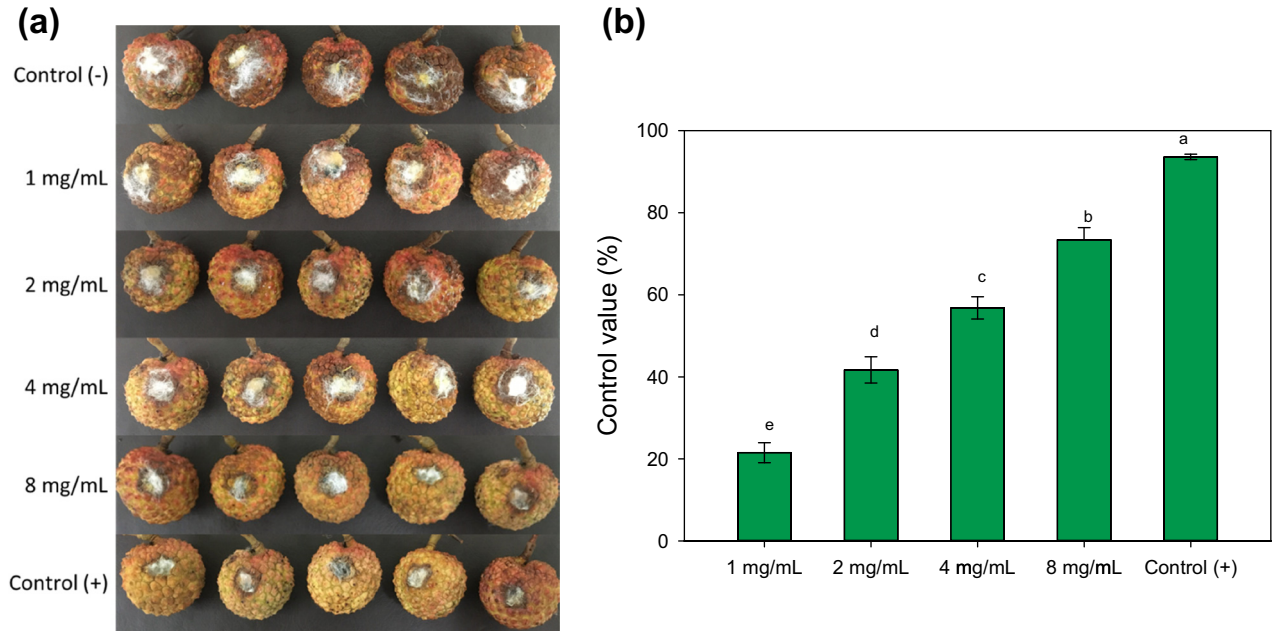

Figure 5: Lesion of anthracnose on litchi fruits treated with CRTO nano-emulsion (a) and percentage of inhibition of anthracnose on litchi fruits *in vivo* (b). The CRTO nano-emulsion was sprayed onto the infected fruits, and the treated litchi fruits were kept in an incubator at 24–25°C for 7 days. The measurements of the fungal infection lesion were recorded at 7 days after treatment.

Table 4: Disease incidence (%) of anthracnose on litchi plants 14 days after applications in field trials from April to June 2020

Conc. ($mg \cdot mL^{-1}$)	Conc. of AI ($mg \cdot mL^{-1}$)	Disease incidence (%)							
		Litchi plants in Tan Yen district				Litchi plants in Luc Ngan district			
		Before treatment	14 DAT after 1st application	14 DAT after 2nd application	14 DAT after 3rd application	Before treatment	14 DAT after 1st application	14 DAT after 2nd application	14 DAT after 3rd application
5	2.03	0.9a	1.95ab	2.67b	3.19b	0.76a	1.95ab	2.51b	2.89b
7.5	3.05	1.02a	1.88ab	2.43b	2.77b	0.87a	1.88ab	2.29b	2.50b
10	4.07	0.91a	1.37b	1.99b	1.61c	0.86a	1.37b	1.76b	1.58b
Control		0.96a	2.52a	5.12a	7.16a	0.75a	2.52a	4.85a	6.54a
$LSD_{5\%}$		0.32	0.68	0.84	1.36	0.14	0.68	0.89	1.73
CV%		17.9	18.9	14.6	19.7	12.5	18.9	16.5	27.2

AI: active ingredient of CRTO of nano-emulsion. DAT: days after treatment. Means in the same column followed by the same letter are not significantly different (assuming significance at $P \leq 0.05$). The field trials in Tan Yen and Luc Ngan were performed from April to May 2020 and April to June 2020, respectively.

disease incidence from the negative controls increased by 4.85% and 6.54% at 14 days after the second and third applications, respectively. All treatments with CRTO nano-emulsion resulted in a significant reduction against anthracnose in disease incidences from 1.76% to 2.51% in the second assessment and from 1.58% to 2.89% in the third assessment (Table 4). The disease severity indices and disease control efficacies of each treatment with CRTO nano-emulsion at doses of 5, 7.5, and 10 $mg \cdot mL^{-1}$ are presented in Tables 5 and 6.

4 Discussion

The treatment of anthracnose with synthetic fungicides led to toxic residues in litchi fruits and serious harm to humans and the environment. To alternate the toxic fungicides, the research of ecofriendly fungicides from natural sources has been considered. The antifungal activity and spore germination of the individual CUR against Ca and Co were described by Cho et al. [23]. In our study, CRTO and CRTO nano-emulsion were active against

Table 5: Disease severity index (%) of anthracnose on litchi plants 14 days after applications in field trials from April to June 2020

Conc. ($mg·mL^{-1}$)	Conc. of AI ($mg·mL^{-1}$)	Disease severity index (%)							
		Litchi plants in Tan Yen district				Litchi plants in Luc Ngan district			
		Before treatment	14 DAT after first application	14 DAT after second application	14 DAT after third application	Before treatment	14 DAT after first application	14 DAT after second application	14 DAT after third application
5	2.03	0.18a	0.40b	0.64b	0.70b	0.15a	0.32b	0.58b	0.64b
7.5	3.05	0.20a	0.39b	0.60b	0.62b	0.17a	0.29b	0.52b	0.57b
10	4.07	0.18a	0.27b	0.43b	0.34b	0.18a	0.27b	0.35b	0.35b
Control		0.19a	0.55a	1.64a	2.05a	0.15a	0.44a	1.51a	1.87a
$LSD_{5\%}$		0.06	0.14	0.27	0.44	0.33	0.11	0.29	0.58
CV%		17.5	19.1	17.6	25.3	14.7	19.3	21.2	30.7

AI: active ingredient of CRTO of nano-emulsion. DAT: days after treatment. Means in the same column followed by the same letter are not significantly different (assuming significance at $P \leq 0.05$). The field trials in Tan Yen and Luc Ngan were performed from April to May 2020 and April to June 2020, respectively.

Table 6: Disease control efficacy of CRTO nano-emulsion against anthracnose on litchi plants 14 days after applications in field trials from April to June 2020

Conc. ($mg·mL^{-1}$)	Conc. of AI ($mg·mL^{-1}$)	Disease control efficacy (%)					
		Litchi plants in Tan Yen district			Litchi plants in Luc Ngan district		
		14 DAT after 1st application	14 DAT after 2nd application	14 DAT after 3rd application	14 DAT after 1st application	14 DAT after 2nd application	14 DAT after 3rd application
5	2.03	22.9a	58.4b	63.3b	29.6a	62.7c	66.5b
7.5	3.05	34.2a	65.9ab	71.4ab	44.1a	70.5b	74.0b
10	4.07	47.2a	72.3a	82.4a	49.7a	80.8a	84.6a

AI: active ingredient of CRTO of nano-emulsion. DAT: days after treatment. Means in the same column followed by the same letter are not significantly different (assuming significance at $P \leq 0.05$). The field trials in Tan Yen and Luc Ngan were performed from April to May 2020 and April to June 2020, respectively.

Colletotrichum species *in vitro*; this is due to the occurrence of turmeric oil and CUR in these materials. In a previous study, the turmeric oil yielded from CRTO by *n*-hexane partition was reported to contain major constituents ar-turmerone (21.4%), α-zingiberene (15%), and aromatic curcumene (10.3%) and to exhibit *Fusarium moniliforme*, *Aspergillus flavus*, *A. parasiticus*, and *Penicillium digitatum* at 3–6 $mg·mL^{-1}$ [11]. In comparison with the previous results, the antifungal effects of the turmeric oil were less than that of CRTO nano-emulsion.

Nano-emulsion of EOs was described to enhance insecticidal activity due to the reduction of droplet size. The nano-emulsions of eucalyptus oil (droplet sizes 4.04 and 2.27 nm) killed the grain store insects *Sitophilus oryzae* and *Tribolium castaneum*. In comparison with the lone EO, the nano-emulsion of eucalyptus oil has stronger insecticidal activity and was 1.4–1.6× effective against *S. oryzae* [24]. Citral, *Syzgium aromaticum*, and *Scaligera tripartite* oils showed an inhibition for *C. gloeosporioides* with MIC 75, 50, and 20 $μg·mL^{-1}$, respectively. However, compared to their nano-emulsion, the EO has weaker effects [25,26]. A nano-emulsion of eugenol (sizes 50–120 nm) also abated the size and number of conidiospores and reduced the pigmentation in hyphae of *Fusarium oxysporum* [27]. The enhancement of the antimicrobial activity of some EO nano-emulsions compared to its raw active ingredients was also observed for cinnamon oil, tea tree oil, manuka oil, thyme oil, and citral [15,27]. The nano-emulsions of thyme oil and manuka oil with droplet sizes ranging from 95 to 98 nm showed 100% inhibition for *Scopulariopsis brevicauslis*, *F. culmorum*, *Phytophthora cactorum*, *Trichophyton mentagrophytes*, and *Microsporum gypseum*. The manuka oil nano-emulsion at 0.5% exhibited similar effectiveness in manuka oil caused at 5× higher concentration [15]. The nano-emulsion of crude neem oil (droplet size of 17.8 nm) and the nano-emulsion of citronella

oil (droplet size of 8.7 nm) displayed EC_{50} values of 13.67 and 25.64 μg·mL^{-1} for *R. solani* and 14.71 and 20.88 μg·mL^{-1} for *S. rolfsii*, respectively [16].

The *in vivo* bioassay of botanical materials against anthracnose caused by *C. gloeosporioides* on litchi fruit was rarely reported in previous publications. Recently, the turmeric rhizome extract was also reported to suppress anthracnose (*C. gloeosporioides*) in dragon fruits in Southeast Asia at 10 mg·mL^{-1} [28]. The strawberry anthracnoses caused by *C. acutatum*, *C. gloeosporioides*, *C. fragariae*, and *C. graminicola* were also treated with *trans*-cinnamic acid, ferulic acid, and *p*-coumaric acid at 5, 10, and 50 nM, respectively [29]. In our study, the treatment of CRTO nano-emulsion on litchi fruits was proved to be effective against anthracnose *in vivo*. Through *in vivo* evaluation, this nanoformulation caused the best suppression against anthracnose on litchi fruits at a dose of 8 mg·mL^{-1}.

Our study is also the first report on the potential of CRTO nano-emulsion against litchi anthracnose in field trials. Anthracnose caused by *C. gloeosporioides* was commonly found to attack the leaves and fruits in the young fruiting stage of litchi plants growing in Tan Yen and Luc Ngan, Bac Giang province in Vietnam. Based on our observation, CRTO nano-emulsion even at the lowest dose of 5 mg·mL^{-1} (equivalent to 2.03 mg·mL^{-1} of CRTO) suppressed the anthracnose in litchi with controls value of 58.4% and 62.7% at 14 days after the second application in Tan Yen and Luc Ngan, respectively. In all of the field trials, the control efficacy of CRTO nano-emulsion was better when increasing the doses from 5 to 10 mg·mL^{-1}. The CRTO nano-emulsion also displayed a dose–response

Figure 6: Litchi plant treated with CRTO nano-emulsion observed at 14 days after third application (a) and negative control (b) observed at 14 days after the third application. The field trials were conducted from April to May in Tan Yen (6.1) and from April to June in Luc Ngan (6.2) districts of Bac Giang province in Vietnam.

disease control efficacy at all of the observations. At the observation of the third application in Tan Yen, the development of anthracnose in the plants treated with CRTO nano-emulsion was controlled by 63.3% at 5 mg·mL^{-1}, by 71.4% at 7.5 mg·mL^{-1}, and by 82.4% at 10 mg·mL^{-1}, respectively (Table 6 and Figure 6a). However, the disease control efficacy at 10 mg·mL^{-1} was not significantly different from those of the group treated at 7.5 mg·mL^{-1} at 14 days after the third application (Table 6). The treatments of CRTO nano-emulsion in Luc Ngan showed better disease control efficacy in the same doses. After the second application in Luc Ngan, all of the means of control efficacy values were separated based on the significant differences at doses of 5 mg·mL^{-1} (62.7%), 7.5 mg·mL^{-1} (70.5%), and 10 mg·mL^{-1} (80.8%). Notably, at 10 mg·mL^{-1}, CRTO nano-emulsion displayed the highest disease control efficacy (84.6%) against litchi anthracnose and no phytotoxicity was observed at 14 days after the third application (Table 6 and Figure 6b).

In conventional litchi disease management, anthracnose is managed by burning affected plant parts and using Bordeaux mixture or Capton (30% wettable powders). In the case of a high severity index in litchi plants, the disease is controlled with copper oxychloride (0.25%), chlorothalonil (0.15%), or carbendazim (0.1%) [2]. However, these fungicides are considered as high toxic pesticides for humans and the environment; for example, carbendazim has been banned in use for fruits and vegetables in many countries including Vietnam [5]. The potential enhancement of litchi fruit quality in food management and preservation requirements of the use of bio-fungicide as botanical materials for controlling litchi anthracnose has been considered recently. The extract of *Areca catechu* pericarp contains fernenol, arundoin, and stigmasterol, and sitosterol has effects on mycelial growth, germ tube elongation, and spore germination of *C. gloeosporioides* isolated from mango fruits [30]. Pseudolaric acids A and B purified from the barks of *Pseudolarix amabilis* inhibited *C. gloeosporioides* with EC_{50} values of 1.62 and 1.07 μg·mL^{-1}, respectively [31]. When treated with pseudolaric acids B, the lesion diameters caused by anthracnose on mango fruits reduced 87.61% in comparison with those in negative controls [31]. The mycelial growth and spore germination of *C. gloeosporioides* were also significantly affected by the leaf extracts of *Andrographis paniculata*, *Azadirachta indica*, *Cymbopogon citratus*, *Ocimum sanctum*, and *Plumbago zeylanica* [9].

However, to the best of our knowledge, no field trials of botanical fungicides on litchi have been reported until now. CRTO is a by-product of curcumin production and contains CUR and EO. In fact, it costs to treat the by-product to prevent environmental pollution. In this study, the by-product was utilized to prepare a botanical nano-fungicide and applied for controlling litchi anthracnose for the first time. The production of CRTO nano-emulsion may generate added values for the manufacturing of turmeric products, and its application may bring cost benefits to the fruit production of litchi. In future studies, the mode of action and antimicrobial effectiveness of CRTO nano-emulsion against other plant pathogenic fungi and bacteria causing litchi diseases should be conducted.

5 Conclusion

CRTO nano-emulsion was prepared as a botanical fungicide for controlling the litchi anthracnose for the first time. The CRTO droplets in the nano-emulsion with particle sizes of 95–250 nm appear as dark droplets, uniform, and colloidally stable. The antifungal activity of CRTO nano-emulsion against *C. gloeosporioides* was better than that of CRTO in *in vitro* bioassay. In the *in vitro* inhibition test against litchi anthracnose pathogen agent *C. gloeosporioides*, the equivalent IC_{50} of CRTO in nano-formulation was 0.11 mg·mL^{-1}, which was 3.0× and 6.1× lower than the concentrations of CRTO (0.33 mg·mL^{-1}) and CUR (0.48 mg·mL^{-1}), respectively. At a dose of 8 mg·mL^{-1}, the best suppression against anthracnose on litchi fruits was observed. In the field trial, the litchi anthracnose was suppressed significantly when sprayed with CRTO nano-emulsion at 5, 7.5, and 10 mg·mL^{-1}. Disease incidences and severity indexes in all of the anthracnose treatments were significantly lower than those in the negative control groups. The promising results of *in vitro* and *in vivo* bioassays and field trial experiments in our study indicated that CRTO nano-emulsion was effective at controlling litchi anthracnose and could be used as an alternative to harmful synthetic fungicides.

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