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Fungicidal activities of camptothecin semisynthetic derivatives against *Colletotrichum gloeosporioides in vitro* and in mango fruit

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ABSTRACT

Increasing attention to the resistance of plant pathogenic fungi to fungicides and their residues impels the development of more efficient fungicides with novel mechanisms of action. Camptothecin (CPT-1) is a naturally occurring quinoline alkaloid with significant antineoplastic and pesticidal activities. To evaluate the anti-fungicidal activities of CPT-1 and its derivatives against postharvest mango anthracnose disease and their potential as a lead compounds for fungicide development, CPT-1 and its semisynthetic derivatives (CPT-2–15) *in vitro* and *in vivo* against *Colletotrichum gloeosporioides* were tested. Five of the agents, CPT-1, 16a-thiocamptothecin (CPT-2), 7-ethyl-camptothecin (CPT-6), 9-methoxycamptothecin (CPT-11) and 7-benzyl-chloro-camptothecin (CPT-15) at doses of 20 mg L^{-1} produced the effective mycelial growth inhibition of *C. gloeosporioides*. Among these, CPT-11 exhibited the strongest inhibition, with EC_{50} and EC_{90} values of 1.79 and 7.37 mg L^{-1}, respectively. At a dose of 100 mg L^{-1}, 10 of the tested derivatives inhibited the germination of *C. gloeosporioides* spores. In addition, CPT-1, −2, −6, −11 and −15 showed different abilities to inhibit appressorium formation. Dipping treatment with CPT-11 at 500 mg L^{-1} exhibited an equivalent efficiency in suppressing postharvest anthracnose in three different cultivated varieties of mango fruit when compared with the commercial fungicide carbendazim at the same concentration, but it was less effective than prochloraz. Scanning and transmission electron microscopy observations revealed that CPT-11 caused alterations in the hyphal morphology and ultrastructures of *C. gloeosporioides*, including swelling, abnormal branching, and the rupturing and thickening of cell walls. These findings indicated that CPT-11 could be a potential antifungal lead compound for controlling postharvest mango anthracnose disease through a different mode of action than camptothecin.

1. Introduction

Colletotrichum gloeosporioides is an important pathogen worldwide that infects more than 1000 plant species (Phoulivong et al., 2010). Varieties of important tropical and subtropical crops, such as mango (Zhang et al., 2013), citrus (Boonruang et al., 2017), papaya (Ong and Ali, 2015), cashew (Uaciquete et al., 2013), avocado (Malick et al., 2016) and cacao (Falcäo et al., 2014), are susceptible to anthracnose caused by *C. gloeosporioides*, which has led to reduced yields and enormous economic losses. The pathogen *C. gloeosporioides* can either infect crops during growth and development or attack them during the after-harvest storage period (Yong et al., 2013). Control of anthracnose disease is usually achieved by applications of fungicides, such as benomyl, carbendazim, mancozeb and prochloraz (Zhou et al., 2016). A mixed and rotational use of fungicides with different mechanisms of action has been employed to delay the development of resistance and to acquire a desired control efficiency against plant anthracnose diseases (Xu et al., 2014). However, recent studies have shown that *C. gloeosporioides* has developed middle to high levels of resistance against many different fungicidal active ingredients, including benzinidazoles (Chung

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et al., 2010), dithiocarbamate and azoles (Xu et al., 2014; Osorio et al., 2014). Thus, the identification and exploitation of new and safe products as an alternative strategy to traditional fungicides has been proposed.

Various plant extracts, such as essential oils and natural compounds, have been isolated from medicinal plants, herbs and antagonistic microorganisms and have antifungal impacts against *C. gloeosporioides in vitro* and *in vivo* (Zhang et al., 2014; Deressa et al., 2015; Hong et al., 2015; Chávez-Magdaleno et al., 2018). Camptothecin (CPT-1) is a naturally occurring quinoline alkaloid in the wood and bark of the tree *Camptotheca acuminata* (Wall et al., 1966). CPT-1 exhibits strong anticancer, antivirus, insecticidal and antimicrobial activities (Oberlies and Kroll, 2004; Li et al., 2012; Yang et al., 2014; Dong et al., 2016). CPT-1 has prominent inhibitory activities against a broad spectrum of phytopathogen fungi, such as *Botrytis cinerea*, *Rhizoctonia solani*, *Alternaria alternata*, *Epicoccum nigrum*, *Pestalotia guepinii* and *Fusarium avenaceum* (Kulkarni et al., 2015; Mao et al., 2015; Dai et al., 2017). CPT-1 causes alterations in the mycelial morphology and ultrastructures of *B. cinerea*, and enhanced the electrolyte leakage, peroxidase activity and the expression of the *cytb* gene, which encodes an 11-subunit mitochondrial respiratory enzyme. The antifungal effects of CPT-1 and its derivatives on *C. gloeosporioides* isolated from harvested fruit have not been documented, and the fungicidal mechanisms of CPTs against plant pathogenic fungi are still unknown.

In the present study, we conducted a fungicidal bioassay study of CPT-1 and its 14 semisynthetic derivatives against *C. gloeosporioides* to investigate its fungicidal performance *in vitro*, assessed their *in vivo* antifungal activities and observed their hyphal morphology and ultrastructures using scanning electron microscopy and transmission electron microscope, respectively. This study also helps understand the structure activity relationship and antifungal mechanism of CPT derivatives against *C. gloeosporioides*.

2. Materials and methods

2.1. Chemicals and stock solutions

The structures of CPT-1 and its derivatives tested in this study are shown in Fig. S1. Camptothecin (CPT-1), 10-hydroxycamptothecin (CPT-4) and 9-methoxycamptothecin (CPT-11) were isolated from the Chinese medicinal plant *C. acuminate*, and CPT-1 served as the starting material for the preparation of the derivatives. Its derivatives, 16a-thiocamptothecin (CPT-2), 9-nitrocamptothecin (CPT-3), CPT-5–10, 9-amino-campothecin (CPT-12), and 7-benzyl-chloro-camptothecin (CPT-15), were synthesized using previously published procedures from our laboratory (Liu et al., 2010). Purification was achieved by silica gel column chromatography. The chemical structures of the compounds synthesized in this study were confirmed by direct comparison with an authentic sample and spectral data reported previously (Liu et al., 2010; Zhang et al., 2011; Li et al., 2012).

The commercial fungicide carbendazim (non-formulated analytical grade, 98% purity) (Jiangsu Bailing Agrochemical Co., Ltd., Wuxi, China) and prochloraz (non-formulated analytical grade, 98% purity) (Jiangsu Huifeng Agrochemical Co., Ltd., Yancheng, China) were used as positive controls. GelBond® Film was purchased from Lonza Rochkland (Lonza Rockland, ME, USA). *C. gloeosporioides* was provided by Postharvest Pathology and Preservation Laboratory, Environment and Plant Protection Institute of Chinese Academy of Tropical Agricultural Science. It was isolated from harvested mango fruit in Hainan Province, China that had medium sensitivity to carbendazim.

2.2. In vitro effects of CPT derivatives on the mycelial growth of C. gloeosporioides

The effects of these CPT derivatives were assessed using the Poison Food Technique on solid media (Dhiangra and Sinclair, 1986). The compounds were dissolved in dimethyl sulfoxide (DMSO) and diluted using acetone, followed by a mixture with sterile molten potato dextrose agar (PDA) to obtain final concentrations ranging from 0.625 to 20.0 mg L^{-1}. Prochloraz was chosen as a fungicidal control, and its test concentrations were 0.0125, 0.025, 0.05, 0.10, 0.20 and 0.40 mg L^{-1}. The PDA was poured into 9-cm Petri plates (15 mL) that were then inoculated with 4-mm plugs of *C. gloeosporioides*. Each treatment contained three replicate plates. PDA containing corresponding concentrations of acetone and DMSO in water served as solvent controls, while PDA alone was used as the normal growth control. After the fungal growth in the normal control had completely covered the petri dishes, mycelial growth diameters were measured and inhibition percentages relative to the control with acetone were calculated using the formula of Agarwal et al. (2001) as follows:

$$I\ (\%) = [(C - d) - (T - d)]/(C - d) \times 100, \quad (1)$$

where d represents the diameter of the cut fungus (4 mm), I represents the inhibition (%), and C and T represent the average colony diameters of the mycelia of the control and treatment, respectively.

2.3. Spore germination assay

The inhibitory effects of compounds (CPT-1–15, carbendazim and prochloraz) on the spore germination of *C. gloeosporioides* were assessed by microscopic observation as described by Zhang et al. (2014). Spore suspensions (1×10^5 spores/mL) were prepared by seeding conidia in a 0.05% Tween-80 solution. The acetone solution of the test compounds were diluted with the conidial suspension to obtain final concentrations of 100 mg L^{-1}. Then, 30 μL of these cultures were placed on concave slides and incubated in a humidity chamber at 28 °C with 90% relative humidity. Three replicates were performed, and a conidial suspension containing a corresponding concentration of acetone in water was used as a control. After incubation at 28 °C for 12 h, the number of germinated spores and the lengths of germ tubes were counted and measured, respectively, approximately 100 conidia in each field of three randomly selected fields under a biological microscope with a photographic system at 400× magnification. The experiments were repeated three times.

2.4. Appressorium formation assay

The effects of CPT derivatives on appressorium formation during spore germination of *C. gloeosporioides* were assessed using the methods described in Section 2.3, with a slight modification. In this test, the conidial suspensions were placed on the hydrophobic side of GelBond® Film. The experiments were repeated three times.

2.5. In vivo trials on mango fruit

Three cultivated varieties of mango (*Mangifera indica* L.) fruit at physiological maturity were harvested from the main mango growing areas in China. Among them, cultivar 'Zill' was obtained from the Mango Standard Production Model Base at the Ministry of Agriculture, which is located in Panzhihua City, Sichuan Province, China, on July 25, 2015. The cultivar "Hong-Yu" was gathered from Dunchang City, Hainan Province, China, on July 16, 2018, and cultivar "Gui-Qi" was harvested from Baise City, Guangxi Zhuang Autonomous Region, China, on August 2, 2018. CPT-11, carbendazim and prochloraz were dissolved in 5 mL of 10% DMSO, 0.1 M HCl and acetone, respectively, and then diluted with sterile distilled water to a final concentration of 500 mg L^{-1}. The surfaces of the mango fruit were sterilized by immersion in 70% ethanol for 1 min, and then two wounds with 2–3-mm radii (1-mm deep) were inflicted on the equivalent side of each fruit with a sterile needle. Then, 5 μL of spore suspension (10^4 spores/mL) of *C. gloeosporioides* were inoculated into the wound sites. For the curative