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Evaluation and mechanism of antifungal effects of carbon nanomaterials in controlling plant fungal pathogen

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ABSTRACT

The antifungal activity of six carbon nanomaterials (CNMs, single-walled carbon nanotubes (SWCNTs), multi-walled carbon nanotubes (MWCNTs), graphene oxide (GO), reduced graphene oxide (rGO), fullerene (C_{60}) and activated carbon (AC)) against two important plant pathogenic fungi (*Fusarium graminearum* (*F. graminearum*) and *Fusarium poae* (*F. poae*)) was evaluated. SWCNTs were found to show the strongest antifungal activity, followed by MWCNTs, GO, and rGO, while C_{60} and AC showed no significant antifungal activity. The antifungal mechanism of CNMs was deduced to target the spores in three steps: (i) depositing on the surface of the spores, (ii) inhibiting water uptake and (iii) inducing plasmolysis.

1. Introduction

The plant fungal pathogens *Fusarium graminearum* (*F. graminearum*) and *Fusarium poae* (*F. poae*) are the causative agents of Fusarium head blight (FHB), a global serious plant disease affecting wheat and other cereal (e.g. maize) productions [1,2]. Between 1998 and 2000, direct and secondary economic losses due to FHB for all crops in the Northern Great Plains and Central United States were estimated to be about 3 billion U.S. dollars [3,4]. Furthermore, the *F. graminearum* and *F. poae* not only cause significant losses in crop yield and quality, but also produce mycotoxins that ruin almost all cereals produced in the infected fields [2,5]. Currently, plant cultivars highly resistant to FHB or tolerant of the mycotoxins are not available and the breeding processes are tedious and laborious [6]. Therefore, chemical treatment to these fungi is the main method for plant protection. However, synthetic fungicides are known to be associated with great concerns about risk of fungi resistance, instability, uncontrolled release of anti-infective agent, toxicity to human cells, and eventual depletion of the anti-infective agent [7,8]. In view of limitations in current control measures and the severe impact of FHB on important economical crops, developing alternative agents for effective control of these diseases has become urgent.

Carbon nanomaterials (CNMs) such as graphene oxide (GO) and carbon nanotubes (CNTs) are of particular interest to researchers due to their amazing thermal, mechanical, and electrical properties as well as a wide range of technological applications [9,10]. Recently, the antibacterial activity of CNMs has attracted great attention in the research field [11,12]. Single-walled carbon nanotubes (SWCNTs) and multi-walled carbon nanotubes (MWCNTs) were found to inhibit the growth of gram-negative bacteria and gram-positive bacteria [8], with a minimal cytotoxicity to mammalian cells [12]. One recent study indicated that GO and reduced graphene oxide (rGO) have presented a noticeable cytotoxicity to bacteria but a minimal cytotoxicity to A549 cells [11]. Moreover, we have demonstrated that CNMs such as SWCNTs, MWCNTs, GO and rGO can significantly inhibit the growth of copper-

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resistant *Ralstonia solanacearum* [13]. These findings implied that CNMs with superior inhibition ability to bacteria growth can be considered as potential antibacterial agents.

The antifungal effect of CNMs has received only a marginal attention from researchers and few studies have been published in this field [14]. Only one report was published in 2012 about the rGO activity against three fungi, namely *Aspergillus niger* (*A. niger*), *Aspergillus oryzae* (*A. oryzae*) and *Fusarium oxysporum* (*F. oxysporum*) [14]. Moreover, to date, several explorations have been conducted regarding the positive effects of CNMs on host plants [15,16]. Both SWCNTs and MWCNTs are reported to be able to significantly stimulate cell growth and enhance seed germination and plant growth [16,17]. Our previous study also demonstrated positive effects of MWCNTs and GO on the germination and growth of wheat plants [18]. For these reasons, we have been motivated to explore the antifungal properties of CNMs against various pathogenic fungal diseases.

The aim of the current study was to evaluate the antifungal activity of CNMs against two fungi — *F. graminearum* and *F. poae*. First, a comprehensive study was conducted on the antifungal effects of CNMs (SWCNTs, MWCNTs, GO, rGO, C_{60} and AC) on the mycelial growth and spore germination of *F. graminearum* and *F. poae*. Subsequently, *F. graminearum* was used to explore the key factors that may influence the antifungal activity of CNMs. Finally, the direct contact interaction mechanism between CNMs and spores of *F. graminearum*, and ultra-structural changes in the morphology of spores were examined. To the best of our knowledge, this is the first attempt of its kind to address this issue. The experimental results will form the basis of further research about the growth inhibition mechanisms of fungi by CNMs.

2. Experimental

2.1. Carbon nanomaterials

C_{60} (purity: >99.9%) and AC (black powder, purity: >99.5%, moisture content: ⩽10%, suitable for plant cell culture) products were purchased from Sigma–Aldrich. SWCNTs (purity: >99%, OD × length 1–2 nm × 30 μm, –COOH content: 2.83 wt.%), and MWCNTs (purity: >99%, OD × length 8 nm × 30 μm, –COOH content: 3.86 wt.%) were purchased from Shenzhen Nanotech Port Co., Ltd. (China). GO and rGO were prepared as described in the Supporting Information. All suspensions of CNMs were obtained by 30 min sonication using a sonicator bath (Elamsonic, S60H) at 37 kHz less than 550 W without adding any dispersant. The pH values for SWCNTs, MWCNTs, GO, rGO, C_{60} and AC were 6.24, 6.39, 6.46, 7.13, 6.6, and 6.34, respectively. Particle size distributions of CNMs were measured using a Zetasizer Nano ZS90 dynamic light scattering (DLS) system (Malvern, England). Micrographs of CNMs were taken with a digital camera connected to a Leica microscope (Leica, Germany).

2.2. Fungal strains

F. graminearum and *F. poae* were obtained from the State Key Laboratory of Agricultural Microbiology of Huazhong Agricultural University. The fungal cultures were maintained on a potato dextrose agar (PDA) slant at 4 °C. The old cultures were transferred to a fresh slant every 2 months to avoid a decline in strain viability [2].

2.3. Effect of CNMs on mycelial growth of *F. graminearum* and *F. poae*

The antifungal activity on mycelial growth of *F. graminearum* and *F. poae* was tested by following standard procedures as previously reported [19]. Briefly, *F. graminearum* and *F. poae* were inoculated onto solid PDA containing 62.5–500 μg mL^{-1} CNMs or left untreated. After an incubation of 72 h (for *F. graminearum*) and 120 h (for *F. poae*) at 24 ± 2 °C, the mycelial growth and mycelial biomass of pathogenic fungi were observed in each Petri dish. The inhibition percentage of growth (*I*, %) was calculated as follows:

$$I = (1 - dt/dc) \times 100\% \quad (1)$$

where dc is the fungal colony diameter measured in control sets, and dt is the fungal colony diameter measured in treatment sets after 72 h and 120 h of incubation. The antifungal effect was measured under a totally random design with four replicates. The dry weight of mycelia was measured after the mycelial pellets were washed repeatedly with distilled water and dried overnight at 70 °C to a constant weight.

2.4. Spore germination and CNM treatment

For spore germination studies, *F. graminearum* spores were prepared as described previously [20]. Spores incubated in 3% green bean soup liquid medium for 5 days were harvested by centrifugation at 3500 rpm for 5 min and washed twice with sterile distilled water. The spores of *F. poae* fungi were obtained by washing their mycelium with DI water and filtering the resulting suspension through gauze. Both spore suspensions were first adjusted to a concentration of 5×10^5 spores mL^{-1}, and then 80 μL suspensions of spores were mixed with 80 μL of CNMs in the tubes to obtain CNMs at a final concentration of 62.5, 125, 250 and 500 μg mL^{-1}. Control samples containing 80 μL suspensions of spores were mixed with 80 μL DI water. 30 μL mixture with a different concentration of CNMs was transferred onto a concave slide for further incubation at 28 °C for 5 h (for *F. graminearum*) and 12 h (for *F. poae*) in complete darkness. Five concave slides were prepared for each treatment and the mean values were compared. Micrographs were taken with a digital camera connected to a Leica microscope. Spore germination rate (%) was calculated as follows: (the number of germinated spores)/(total number of spores).

2.5. Fluorescence imaging and analysis

The fresh spores were treated with CNMs for 3 h and stained with 4′-6-diamidino-2-phenylindole (DAPI, 3.0 μg mL^{-1}) for 10 min in the dark. Fluorescence images were taken on an Olympus BX40 fluorescence microscope [21].

2.6. Structural and morphological characterization

The spores treated with CNMs for 3 h were fixed with 2.5% glutaraldehyde with a vacuum pump in an ice bath for 30 min, followed by 4 h incubation at 4 °C and three times of