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Anti-*Rhizoctonia solani* activity by polymeric quaternary ammonium salt and its mechanism of action

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ABSTRACT

Polymeric quaternary ammonium salts (PQASs) exhibit antibacterial action and are less toxic, less stimulatory to the human body and have easier-to-modify functionalities than small molecular antibacterial agents. However, few studies on the structure-activity relationship and toxicity mechanism of PQASs against fungi have been reported. We previously described the synthesis of a novel PQAS, namely, a homopolymer of (2-methacrylamido)propyltetrabenzyldimethylammonium chloride (PQD-BC), and discovered that the polymer exhibits antifungal activities not only against *Fusarium oxysporum* f. sp. *Cubense* tropical race 4 (Foc 4), the pathogen of banana wilt, but also against *Rhizoctonia solani* (*R. solani*), the pathogen of rice sheath blight (RShB). Furthermore, we studied the mechanism of action of PQD-BC against Foc 4, which is markedly different from *R. solani* in morphology and life cycle. Therefore, the structure-antifungal activity relationship and toxicity mechanism of PQD-BC against *R. solani* were extensively studied in this work and compared with those of the low-molecular-weight quaternary ammonium salt benzyldimethyldodecylammonium chloride (BC), and the results play an important role in identifying long-term and low-toxicity fungicides that can suppress the sclerotia of *R. solani*. The results showed that PQD-BC and BC can destroy the structural integrity and morphology of a cell, such as by loss of the cell wall and plasma membrane integrity, leading to the release of intracellular contents and can induce mitochondrial dysfunction and interference with genomic DNA and inhibit the formation of sclerotia. However, PQD-BC showed a special mechanism for causing the lipid peroxidation of the cell membrane; this mechanism was not observed with BC. The newly elucidated mechanism accounts for differences between polymers and small-molecule compounds and provides a theoretical basis for further application of PQAS against fungi and sclerotia.

1. Introduction

Rice, one of the primary staple food on earth, is subject to many diseases that often place major biological constraints on production. Rice sheath blight (RShB), a pervasive rice disease occurring throughout temperate and tropical production systems, is caused by *Rhizoctonia solani* (*R. solani*) Kühn, among which AG-1(IA) is the strongest and most harmful strain. Although the pathogen is soil borne, RShB develops into a major production limitation in an alarmingly brief timespan [1]. It has been reported to reduce yield by 15% - 44% in Texas, USA [2]. The most important factor for RShB epidemic is the formation of sclerotia. As the dormant structures of fungi, sclerotia are hard, asexual and resistant to unfavorable conditions as well as to chemical and biological degradation [3–6]. The high capability of sclerotial survival may be related to its special double layer [7] and secreted melanin [8]. The mycelia of *R. solani* forms sclerotia to survive in soil when host plants are removed and the sclerotia germinate and infect new plants in the next growing season [3–6]. This cycle can follow on all the while. Without breaking the cycle, RShB is difficult to eradicate.

To date, the methods of controlling RShB mainly involve agricultural, biological and chemical managements. Agricultural managements which control RShB directly, such as salvage sclerotia [9], crop rotation [10] and duck-rice system [11,12], require a lot of time and manpower. Biological managements exploit microbes, such as *Trichoderma* [13], *Bacillus* [14] and *Pseudomonas* [15], to inhibit mycelial growth of *R.solani*. Their inhibiting effects are reported as evident and friendly to environment. However, the field application of microbes tends to be susceptible to other factors, and hard to achieve the laboratory effect. Chemical antimicrobial agents are the most adopted. Management of RShB using chemical agents has focused on killing hypha and preventing the formation of sclerotia, such as New-Ag-

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antibiotic 702 [16] and validamycin [17]. Nevertheless, no methods have been reported to completely inhibit the growth of mycelia and the germination of *R. solani* sclerotia, especially from the aspect of taking advantage of functional polymers.

Quaternary ammonium salts (QASs) have been widely used as small molecular biocides with several advantages over other antibacterial agents, including excellent cell membrane penetration properties, lower toxicity, less skin irritation, better environmental stability, extended residence time and enhanced biological activity. Polymeric quaternary ammonium salts (PQASs) exhibit greater antimicrobial activities than the corresponding small molecular QASs. The higher activity of polymeric PQASs has been interpreted as follow: the net positive charge of PQASs and the net negative charge of bacterial cell membranes provide a stronger driving force for the initial attraction of the PQASs to the cell surface. After the PQASs bind to the negatively charged phospholipid, their hydrophobic moieties interact with the inner hydrophobic core of the bacterial membrane, leading to a disruption of the cytoplasmic membrane and a release of potassium and other constituents, which eventually causes cell death. Therefore, PQASs can be firmly adsorbed onto the surface of negatively charged surfaces, effectively restraining bacteria [18–20].

In addition, compared with small molecular compounds, functional polymers' properties can be manipulated through changes in their structure. PQASs can then be grafted from a hydrophilic group or hydrophobic groups and endowed with amphiphilicity. These materials not only deliver steric stabilization in solid dispersions and generate controlled surface structures upon arrangement of materials but also modify interfacial properties such as wetting and lubrication [21,22]. These properties might offer a path for inhibiting the germination of sclerotia, thereby controlling RShB epidemics.

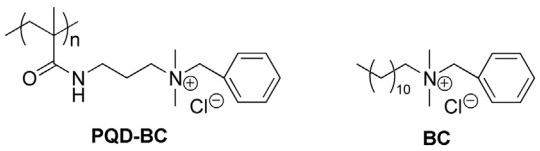
Previously, we have revealed the antifungal activity of polymeric QAS against phytopathogenic fungi, including *Fusarium oxysporum* f. sp. *Cubense* tropical race 4 (Foc 4) and *R. solani* [19,20,23]. Foc 4 is the pathogen of fusarium wilt of banana, which can produce spores by asexual reproduction [23,24]. However, *R. solani* and Foc 4 belong to different classes and have different morphologies and life cycles. *R. solani* strains are reported to be tiny, multinucleated, seldom producing spores and infecting host plants by mycelia and sclerotia [1]. Therefore, the antifungal mechanism of the functional polymer against *R. solani* remains undiscovered, as well as the difference in the antifungal mechanism between PQASs and small molecular counterparts against filamentous fungi.

In this paper, poly((2-methacrylamido)propyltetrabenzyldimethylammonium chloride (PQD-BC) is synthesized (Scheme 1) [19], and its activities and mechanisms against the mycelial growth and sclerotial formation of *R. solani* AG-1(IA) are investigated and compared with those of a commercially available small-molecular QAS, benzalkonium chloride (BC), to provide a clearer direction for the application of polymeric antifungal agents and new choices for controlling RShB.

2. Experimental

2.1. Materials

Bovine serum albumin (BSA) was provided by Hangzhou Sijiqing Biological Engineering Materials Co. Ltd. (Hangzhou, China). 2,3,5-Triphenyltetrazolium chloride (TTC) and an Ezup Column Fungi Genomic DNA Purification Kit were purchased from Sangon Biotech (Shanghai) Co., Ltd. (Shanghai, China). Propidium iodide (PI) was supplied by Aladdin Reagent Co. Ltd. (Shanghai, China). The fungal strain used in this study is *Rhizoctonia solani* Kühn AG-1(IA), which was donated by the Fungi Laboratory at the South China Agriculture University and was maintained on potato dextrose agar (PDA). *R. solani* mycelial suspensions were obtained from the surface of the agar after culturing for 2 days at 28 °C. The concentration of the mycelial suspensions was determined using a hemocytometer. Benzyldimethyldodecylammonium chloride (BC) with a purity > 99% was purchased from Shanghai Aladdin Bio-Chem Technology Co., Ltd. (Shanghai, China). Alkaline phosphatase (ALP), malondialdehybe (MDA) and succinatedehydrogenase (SDH) kits were purchased from the Nanjing Jiancheng Institute of Bioengineering (Nanjing, Jiangsu, China). A mitochondrial isolation kit and GoldView™ were purchased from the Beijing Solarbio Science & Technology Co. Ltd., Beijing, China. PQD-BC was synthesized using radical polymerization according to the method described in our previous study [19], the structure of PQD-BC and BC were shown in Scheme 1.



Scheme 1. Chemical structures of PQD-BC and BC.

2.2. Antifungal bioassay

2.2.1. Mycelial growth inhibition

The antifungal efficiency of PQD-BC and BC on the mycelial growth of *R. solani* was measured using an *in vitro* mycelial growth inhibition assay [19]. Briefly, the compounds (PQD-BC and BC) were sterilized by passage through a 0.45 μm Millipore filter, diluted in different concentrations (PQD-BC: 0.25, 0.50, 0.75, 1.00, 1.25 and 1.5 mg/mL; BC: 0.015, 0.03, 0.045, 0.06, 0.075 and 0.2 mg/mL) and then mixed with molten agar (at a temperature > 60 °C); agar was diluted with distilled water for the control. Mycelial pieces (6 mm diameter) obtained from the periphery of 2-day-old cultures of *R. solani* were inverted on the center of each plate. Treated and untreated plates were incubated at 28 °C in triplicate. The efficacy of the treatment was determined by computing the average of two perpendicular diameters of each colony. The percent mycelial inhibition of the radial growth of the fungi by the compounds compared with the effect of the control was calculated at day 2 using the following formula:

$$\text{Percentage mycelial inhibition} = (D_c - D_t)/D_t \times 100\% \quad (1)$$

where D_c is the mean colony diameter of the control group and D_t is the mean colony diameter of the treatment group. Each measurement consisted of at least three replications.

2.2.2. Minimum inhibitory concentration (MIC)

The antimicrobial properties of the synthesized compounds were studied by the conventional procedure of broth microdilution with TTC. The TTC reagent is colorless; however, it gives off a bright-red color when reduced, indicating the presence of live fungi [25,26]. The fungistatic activity was characterized by the minimum inhibitory concentration (MIC) corresponding to the lowest serial dilution that resulted in the lack of visible microorganism growth. The synthetic compounds against *R. solani* were determined using *R. solani* cultured in sterile potato dextrose broth (PDB) medium for 3 days. Afterwards, the mycelia were suspended using a homogenizer. The sample solution (100 μL), in concentrations ranging from 5×10^{-3} to 0.3 mg/mL, was added to 96-well plates. The same volume of mycelial suspension containing approximately 10^5–10^6 CFU/mL was incubated at 28 °C for 2 days using a hemocytometer. Two control tests containing PDB medium supplemented with a tested strain and an equal volume of sterile PDB medium (negative control) were also performed. Then, 50 μL (5.0 mg/mL) of TTC solution (in PDB) was added to every well and the mycelia were cultured in the dark at 28 °C for another 2 h. The visual color changes were recorded before and after incubation to determine the MIC (mg/mL, present in the well). The color changes present in the well matched that in the blank well that was taken as the MIC for each fungus.