Broad-Spectrum Antimicrobial Activity in vitro of the Synthetic Peptide D4E1

Kanniah Rajasekaran,* Kurt D. Stromberg, Jeffrey W. Cary, and Thomas E. Cleveland

Southern Regional Research Center, Agricultural Research Service, U.S. Department of Agriculture, 1100 Robert E. Lee Boulevard, New Orleans, Louisiana 70124

Broad-spectrum antimicrobial activity of a synthetic peptide, D4E1, is documented in this paper. D4E1 inhibited the growth of several fungal phytopathogens belonging to four classes—Ascomycetes, Basidiomycetes, Deuteromycetes, and Oomycetes, and two bacterial pathogens, *Pseudomonas syringae* pv. *tabaci* and *Xanthomonas campestris* pv. *malvacearum* race 18. The minimum inhibitory concentration (MIC) of D4E1 required to completely inhibit the growth of all fungi studied ranged from 4.67 to 25 μM. Fungal pathogens highly sensitive to D4E1 include *Thielaviopsis basicola*, *Verticillium dahliae*, *Fusarium moniliforme*, *Phytophthora cinnamomi*, and *Phytophthora parasitica*. Comparatively, the least sensitive fungal pathogens were *Alternaria alternata*, *Colletotrichum destructivum*, and *Rhizoctonia solani*. The two bacterial pathogens, *P. syringae* pv. *tabaci* and *X. campestris* pv. *malvacearum* race 18, were most sensitive to D4E1 with MIC values of 2.25 and 1.25 μM, respectively. Microscopic analysis of D4E1 effects on fungal morphology of *Aspergillus flavus* and *R. solani* revealed abnormal hyphal growth and discontinuous cytoplasm. After 8 h of exposure to 25 μM D4E1, *A. flavus* spore germination was reduced by 75%. The suitability of peptide D4E1 to enhance disease resistance in transgenic crop plants is discussed.

Keywords: *Antifungal; antimicrobial; D4E1; disease resistance; phytopathogens; synthetic peptides*

INTRODUCTION

Antimicrobial peptides appear to be ubiquitous in nature, being found in many organisms from bacteria to humans (*1*, *2*). Various plants produce, either a priori or in response to microbial invasion, cysteine-rich antimicrobial peptides such as thionins, defensins, lipid transfer proteins, and hevein- and knottin-type peptides (*3*, *4*). However, these natural defense mechanisms are not sufficient to ward off microbial invasion and, therefore, several laboratories are interested in boosting crop defense mechanisms by overexpression of inducible traits or transgenic introduction of novel antimicrobial peptides from diverse sources including insects (e.g., cecropins and melittins) and amphibians (e.g., magainins). For example, reduced disease severity in transgenic plants expressing cecropin analogues upon infection with bacterial or fungal pathogens has been demonstrated by several laboratories (*5*−*8*). However, tobacco plants expressing a native cecropin did not confer resistance to *Pseudomonas syringae* pv. *tabaci* or *Pseudomonas solanacearum*, presumably due to degradation of the peptide by host proteases (*9*−*11*). Huang et al. (*6*) increased the stability of cecropin B significantly in transgenic tobacco by using a mutant form carrying a single amino acid change. Recently, four synthetic cationic peptides, pep6, pep7, pep11, and pep20, were shown to be effective, both in vitro and in vivo, against potato pathogens *Phytophthora infestans* and *Alternaria solani* (*12*). Osusky et al. (*13*) introduced a synthetic gene encoding an N terminus-modified, cecropin−melittin cationic peptide chimera (MsrA1) into two potato cultivars, and the transgenic plants showed increased resistance to bacterial (*Erwinia carotovora*) as well as fungal (*Fusarium solani* and *Phytophthora cactorum*) pathogens. Transgenic potato plants expressing an alfalfa defensin gene, *alfAFP*, showed increased field resistance to *Verticillium dahliae* (*14*). Arce et al. (*7*) and Reynoird et al. (*15*) also demonstrated improved bacterial resistance in transgenic potato and pear, respectively, through expression of the *attacin* gene from the giant silk moth, *Hyalophora cecropia*.

Recent advances in automated peptide synthesis and computer-assisted combinatorial peptide chemistry have made it possible to rapidly formulate, synthesize, and screen large numbers of peptides for their ability to inhibit the growth of target microbial pathogens (*16*−*18*). Plants do not produce linear amphipathic antimicrobial peptides (*3*, *19*), and the availability of synthetic peptides provides a safe and effective complement to natural antimicrobial proteins and peptides for genetic engineering of crop plants for combating phytopathogens. These linear, synthetic peptides often can be less than half the size (10−20 amino acids) of their native counterparts and many times more stable and potent without the concomitant toxicity to host tissues. One such synthetic peptide, D4E1, has been shown to inhibit the growth of mycotoxin-producing *Aspergillus* and *Fusarium* fungi (*20*), and transgenic tobacco plants expressing D4E1 demonstrated increased resistance to *Aspergillus flavus*, *Verticillium dahliae*, *Colletotrichum destructivum* (*21*), and other fungal pathogens (K. Rajasekaran, unpublished results). In this paper, we report the broad-spectrum effects in vitro of this synthetic peptide on several fungal and bacterial phytopathogens, which are among the most devastating pathogens of cultivated crops.

* Author to whom correspondence should be addressed [e-mail krajah@srrc.ars.usda.gov; fax (504) 286-4217; telephone (504) 286-4482].

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Table 1. Inhibitory Concentrations of Antimicrobial Peptide D4E1 on the Growth of Several Phytopathogens

phytopathogen	source	IC_{50}[a] (μM)	IC_{50} 95% confidence interval	MIC[b] (μM)
Alternaria alternata	SRRC 1107[c]	12.39	(11.71, 13.09)	25.0
Aspergillus flavus 70-W	A. DeLucca, SRRC	7.75	(7.23, 8.30)	25.0
Aspergillus flavus 70-GFP	J. Cary, SRRC	11.01	(9.94, 12.18)	25.0
Cercospora kikuchii	SRRC 1078	8.67	(6.59, 11.34)	25.0
Colletotrichum destructivum	ATCC 42492	13.02	(9.94, 15.95)	25.0
Claviceps purpurea	SRRC 118	1.60	(1.24, 2.01)	20.0
Fusarium graminearum	SRRC 1042	2.10	(1.92, 2.29)	25.0
Fusarium moniliforme	SRRC 1082	0.88	(0.81, 0.95)	12.5
Fusarium oxysporum	SRRC 187	2.05	(1.74, 2.38)	12.5
Penicillium italicum	SRRC 2352	5.92	(5.28, 6.23)	25.0
Phytophthora cinnamomi	E. L. Stromberg, Virginia Tech	ND[d]		4.67
Phytophthora parasitica	E. L. Stromberg, Virginia Tech	ND		4.67
Pseudomonas syringae pv. *tabaci*	K. Damann, Louisiana State University	0.52	(0.47, 0.57)	2.25
Pythium ultimum var. *sporangiforium*	E. L. Stromberg, Virginia Tech	ND		13.33
Rhizoctonia solani	SRRC 1443	ND		26.7
Thielaviopsis basicola	SRRC 1277	0.52	(0.47, 0.57)	6.0
Verticillium dahliae	SRRC 1030	0.60	(0.54, 0.67)	5.25
Xanthomonas campestris pv. *malvacearum* race 18	K. El-Zik, Texas A&M	0.19	(0.10, 0.29)	1.25

[a] IC_{50} defined as the concentration of D4E1 (μM) that inhibits the growth of 50% of the spores or bacterial cells relative to a control treatment (0 μM D4E1). The IC_{50} value was estimated using the dose−response package in GraphPad Prism software. Two separate experiments were used to generate dose−response curves and estimate the IC_{50}. [b] The MIC is defined as the minimum concentration of D4E1 required to completely inhibit the growth of fungal or bacterial cells. [c] Numbered SRRC cultures were obtained from the repository maintained by M. Klich. [d] Not determined.

MATERIALS AND METHODS

Fungal and Bacterial Cultures. Economically important plant pathogenic fungi and bacteria were obtained from several sources (Table 1). Fungal isolates were stored at 4 °C on potato dextrose agar (PDA; Difco, Detroit, MI) slants except for *Phytophthora* and *Pythium* species, which were stored on colonized hemp seeds in sterile water, and *Rhizoctonia solani*, which was kept on PDA plates at room temperature. Bacterial cultures of *Pseudomonas syringae* pv. *tabaci* and *Xanthomonas campestris* pv. *malvacearum* (race 18) were stored at −80 °C as suspensions in a 20% (v/v) glycerol solution. To visualize the anti-fungal effect of D4E1 on spores, we used an *A. flavus* strain expressing the green fluorescent protein (GFP) from the jellyfish, *Aequorea victoria* (*22*). This strain was constructed by placing the EGFP gene (ClonTech, Palo Alto, CA) under the control of the constitutively expressed *Aspergillus nidulans* glyceraldehyde phosphate dehydrogenase (*gpd*) gene promoter and the *Aspergillus parasiticus nmt-1* gene transcriptional terminator. All of these elements were subcloned into the plasmid vector pBCKS (Stratagene, La Jolla, CA) to produce the vector gpd-EFGP. Plasmid gpd-EGFP was cotransformed with the vector pSL82 harboring the *A. parasiticus nia*D gene into the *nia*D mutant of *A. flavus* 70. An isolate stably expressing high levels of GFP, designated *A. flavus* 70-GFP, was used in all experiments.

Germinated Spore Antifungal Bioassays. *A. flavus* isolates, *Cercospora kikuchii*, *Claviceps purpurea*, *Fusarium graminearum*, *Fusarium moniliforme*, *Fusarium oxysporum*, *Penicillium italicum*, *Thielaviopsis basicola*, and *V. dahliae* were grown on PDA slants for 7 days at 30 °C. Similarly, cultures of *Cercospora kikuchii*, *Claviceps purpurea*, *T. basicola*, and *V. dahliae* were grown at 22 °C. *C. destructivum* was grown on Czapek yeast autolysate agar (CYA), and *A. alternata* was grown on V-8 juice agar for 7 days at 22 °C to induce conidiation. Conidia were harvested by the addition of 1% (w/v) potato dextrose broth (PDB; pH 6.0) to slants or plates, and conidia were removed by scraping the mycelial surface gently with a sterile pipet. Conidial suspensions were diluted with 1% PDB to 10^4 conidia/mL (*Fusarium* spp.) or 10^5 conidia/mL with the aid of a hemocytometer. Conidial suspensions were incubated either for 8 h at 30 °C (*A. flavus*, *Fusarium* spp., and *Penicillium italicum*) or overnight at 22 °C prior to assay to obtain germinated conidia.

D4E1 solutions were prepared in 1% PDB. Control samples consisted of 270 μL of 1% PDB and 30 μL of germinated conidial suspension. Test samples consisted of 30 μL of germinated conidial suspension, an appropriate volume of D4E1 stock solution to yield the desired final D4E1 test concentration ranging from 0.25 to 25.0 μM, and enough 1% PDB to yield a final volume of 300 μL. All samples were then incubated for 60 min at either 30 °C (*A. flavus*, *Fusarium* spp., *Penicillium italicum*) or 22 °C. Four 50 μL aliquots of each sample were spread on PDA plates (9 cm diameter). Fungal colonies were enumerated following incubation at either 30 or 22 °C for 24−48 h. Each fungus was tested at least twice.

Microplate Antifungal Bioassay. The efficacy of D4E1 to inhibit the growth of *Phytophthora cinamomi*, *Phytophthora parasitica*, *Pythium ultimum* var. *sporangioforum*, and *R. solani* was assessed using a microplate assay. Fungi were grown in 50 mL of 1% PDB at 28 °C with shaking at 90 rpm for 48 h prior to assay. Mycelia were then harvested by straining the culture through sterile cheesecloth, placed in a Waring blender with 25 mL of 1% PDB, and blended on high speed for 30 s. The concentration of mycelial fragments was adjusted to ~10^5 fragments/mL with 1% PDB and the aid of a hemocytometer. All experiments were conducted in 24 well microplates. Control samples consisted of 100 μL of mycelial fragments and 1900 μL of 1% PDB. Test samples consisted of 100 μL of mycelial fragments, an appropriate volume of D4E1 stock solution to yield the desired final D4E1 test concentration ranging from 2 to 40 μM, and enough 1% PDB to yield a final volume of 2 mL. Microplates were incubated for 48 h and at 90 rpm prior to recording of the minimum D4E1 concentration that completely inhibited fungal growth (MIC). Each experiment was replicated three times.

Spore Germination Assays. The effect of D4E1 on the ability of fungal spores to germinate was determined by exposing freshly prepared suspensions of nongerminated conidia (10^6 conidia/mL) of *A. flavus* 70-GFP to concentrations ranging from 0 to 25 μM of D4E1 prepared in 1% PDB. Conidial suspensions were then incubated at 30 °C for 8 h prior to the determination of the proportion of germinating conidia for each concentration of D4E1 studied. Germ tube morphology was also examined microscopically using an Olympus microscope equipped with a 200 W high-pressure lamp providing light for GFP fluorescence (excitation at 480 nm and emission at 530 nm). The experiment was repeated twice.

Antibacterial Bioassays. Suspensions of *P. syringae* pv. *tabaci* and *X. campestris* pv. *malvacearum* were grown in 50 mL of nutrient broth (NB; Difco) overnight at 28 °C and 225 rpm. Bacterial suspensions were then diluted to 10^4 colony-forming units/mL with sterile NB with the aid of a spectro-

photometer. D4E1 stock suspensions were prepared as above except D4E1 was dissolved in NB. Control samples included 450 μL of NB and 50 μL of bacterial suspension. Test samples consisted of 50 μL of bacterial suspension, an appropriate volume of D4E1 stock solution to yield final D4E1 test concentrations from 0.25 to 4.5 μM, and NB to bring the final volume to 500 μL. All samples were then mixed and incubated for 60 min at 28 °C. Four 100 μL aliquots from each D4E1 test concentration were then spread onto NB plates. NB plates were incubated for 24−48 h at 28 °C and bacterial colonies enumerated. Both bacterial species were tested twice.

Statistical Analyses. Fungal and bacterial colony data were expressed as percent mortality compared to the control treatment in each experiment. The dose−response curve-fitting package in a commercial statistics software (GraphPad Prism, San Diego, CA) was used to generate the best fitting dose−response curve (*23*) and estimate the IC_{50} of D4E1 for each fungus or bacterium tested. IC_{50} values indicate the concentration of D4E1 required to inhibit the growth of 50% of germinating fungal spores or bacterial cells.

The effect of D4E1 on the germination of *A. flavus* 70-GFP spores was determined using the one-way ANOVA, and mean separation was performed using the method of Tukey (*24*).

RESULTS

Antifungal Bioassays. D4E1 inhibited the growth of all fungal phytopathogens studied. The fungal phytopathogens belonged to four classes of fungi including Ascomycetes, Basidiomycetes, Deuteromycetes, and Oomycetes. IC_{50} values of D4E1 were in the low micromolar range (0.52−13.02 μM) for all fungal phytopathogens (Table 1). MIC values of D4E1 ranged from 4.67 to 26.7 μM. *T. basicola*, *V. dahliae*, *F. moniliforme*, *P. cinnamomi*, and *P. parasitica* were highly sensitive to the antimicrobial activity of D4E1 as indicated by low MIC values (6.0, 5.25, 12.5, 4.67, and 4.67 μM, respectively),and low IC_{50} values of 0.52, 0.60, and 0.88 μM for the first three species, respectively (Table 1). The least sensitive fungal phytopathogens to the antimicrobial effects of D4E1 were *A. alternata*, *C. destructivum*, and *R. solani* with relatively high MIC values of 25.0, 25.0, and 26.7 μM, respectively, and IC_{50} values of 12.39 and 13.02 μM for the first two species, respectively (Table 1).

Hyphal growth characteristics of *R. solani* in the presence of a sublethal concentration (20 μM) of D4E1 were analyzed microscopically (Figure 1). *R. solani* hyphae growing in the presence of 20 μM D4E1 appeared to be more jagged, rougher, and thinner (Figure 1B) than hyphae not exposed to D4E1 (Figure 1A). Extracellular material was also present surrounding some *R. solani* hyphae grown in the presence of 20 μM D4E1 (Figure 1B). This extracellular material was not observed surrounding hyphae not exposed to D4E1 (Figure 1A).

Microscopic examination of spore germination of *A. flavus* 70-GFP in 25.0 μM D4E1 indicated that spore germination was reduced by >75% (Figure 2). Spore germination was not significantly reduced ($P > 0.05$) by exposing *A. flavus* 70-GFP spores to 12.5 μM of D4E1 compared to the control, although this concentration of D4E1 was greater than the IC_{50} of D4E1 (Figure 2B and Table 1) used to test pregerminated spores. GFP microscopy indicated that many of the germ tubes examined from *A. flavus* 70-GFP spores germinating in 12.5 μM of D4E1 exhibited breakage or a high degree of vacuolization as seen by the nonfluorescent regions in the germ tubes or spore (Figure 2B). These nonfluorescent regions were not seen in germ tubes of spores germinated in 0 μM (Figure 2A) or 6.0 μM D4E1 (data not shown). *A. flavus* 70-GFP spores exposed to 25.0 μM D4E1 either did not germinate or possessed short and thickened germ tubes (Figure 2C). These data and observations suggest D4E1 does not significantly reduce spore germination at lower concentrations (~IC_{50}) but inhibits fungal growth by interacting with fungal germ tubes. However, high concentrations of D4E1 inhibit fungal growth by retarding spore germination.

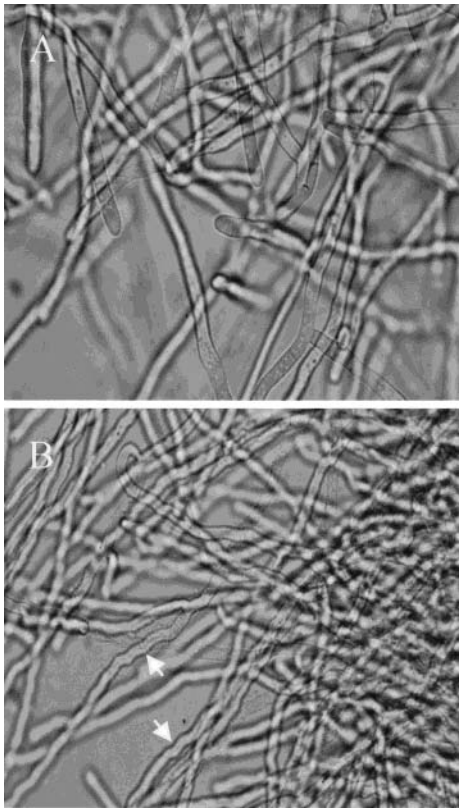

Figure 1. Effect of antimicrobial peptide D4E1 on the growth characteristics of *R. solani* hyphae after 48 h in the presence of 0 μM (A) and 20 μM (B) D4E1. Arrows indicate morphological changes in hyphal growth habit in the presence of 20 μM D4E1. Magnification 300×.

Antibacterial Bioassays. The two Gram-negative bacteria studied were the most sensitive phytopathogens to D4E1. IC_{50} values for D4E1 for *P. syringae* pv. *tabaci* and *X. campestris* pv. *malvacearum* were 0.52 and 0.19 μM, respectively, whereas the MIC values were 2.25 and 1.25 μM, respectively.

DISCUSSION

We have demonstrated the broad-spectrum activity of the synthetic peptide D4E1 against several phytopathogens (Table 1). Transgenic plants expressing D4E1 demonstrated significant antifungal activity in vitro and in planta (*21*). In our laboratory, we are interested in producing disease-resistant cotton and corn primarily to control or eliminate preharvest aflatoxin contamination caused by the fungus *A. flavus*. Aflatoxin contamination significantly reduces the value of grain as an animal feed and as an export commodity and, more importantly, poses food and feed safety-related threats to humans and farm animals.

The precise mode of action of D4E1 in transgenic plants is yet to be elucidated. Suggested modes of action of antifungal peptides include lysing fungal cells (*25*,

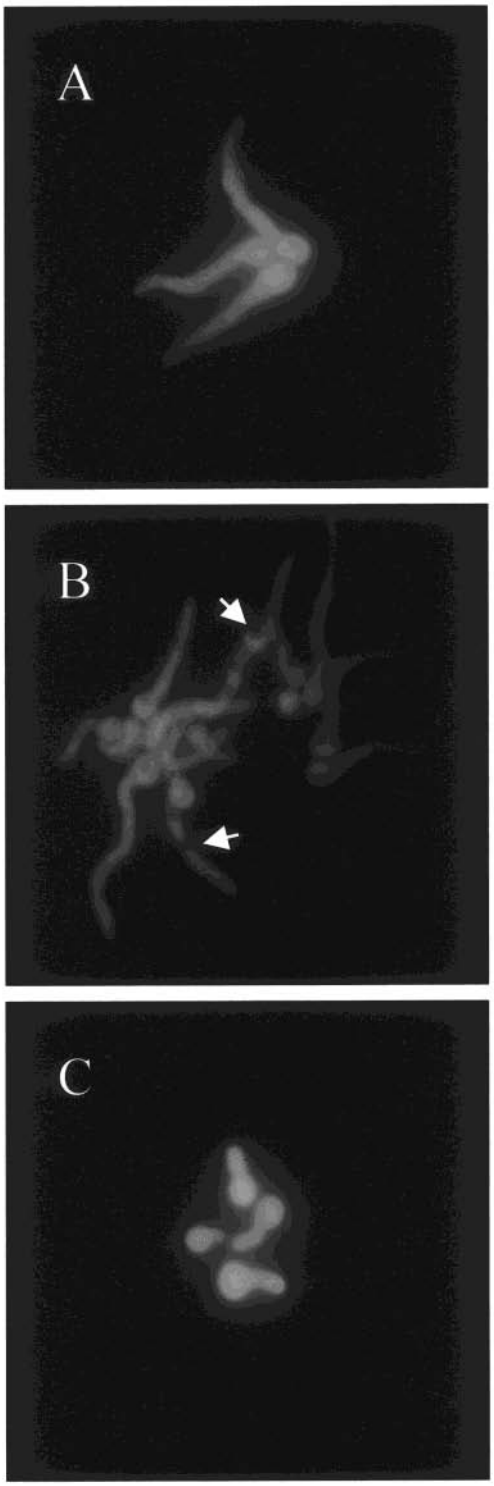

Figure 2. Effect of antimicrobial peptide D4E1 on germinating conidia of *A. flavus* 70-GFP. Conidia were germinated for 8 h in 1% (w/v) PDB containing 0 μM (A), 12.5 μM (B), and 25 μM D4E1 (C). Note the high occurrence of vacuoles and discontinuous cytoplasm (arrows) in germ tubes from conidia germinated in the presence of 12.5 μM D4E1 (B) and the short thickened germ tubes from conidia germinated in the presence of 25 μM D4E1 (C). Magnification 600×.

26), interfering with cell wall synthesis (*27*), inhibiting mitochondrial energy production, releasing cytochrome *c* resulting in apoptosis, or inhibiting DNA/RNA synthesis (*28*). According to DeLucca et al. (*20*) the lethality of D4E1 appears to be due to its binding to the sterols present in the fungal conidial surface. Our light microscopic studies have indicated abnormal hyphal growth (Figure 1B), discontinuous cytoplasm in germ tubes (Figure 2B), or reduction in spore germination (Figure 2C) in D4E1-treated *R. solani* and *A. flavus* spores, respectively, compared to controls (Figures 1A and 2A). Similar observations were made with cultures of *V. dahliae* and *Thielaviopsis basicola* (data not shown), two of the most important cotton pathogens. Antimicrobial peptides are known to induce abnormal morphological changes in the hyphae of test fungi (*12*, *29*, *30*). The broad-spectrum antimicrobial activity of D4E1 (*20*, *21*, this paper) along with its low toxicity to plants and animals (J. Jaynes, personal communication) makes it an ideal candidate for microbial control. However, the high cost of producing large quantities of synthetic antimicrobial peptides prohibits their direct application for the purpose of pathogen control. Instead, transgenic approaches are being undertaken to provide the amount of antimicrobial peptides necessary to impart improved resistance in planta to microbial pathogens. We reported previously that successful transgenic expression of D4E1 resulted in the inhibition of fungal growth in vitro and in planta against mycotoxigenic *A. flavus* and other fungal pathogens (*21*). Decreased disease incidence in transgenic potatoes expressing antifungal peptides has also been reported recently from two separate laboratories (*13*, *14*).

SAFETY

Standard safety procedures for the handling of fungal or bacterial cultures and aseptic culture media in a biosafety cabinet are noted; otherwise, no special precautionary measures are required for the materials and laboratory methods presented here.

ABBREVIATIONS USED

CYA, Czapek yeast autolysate agar; GFP, green fluorescent protein from jellyfish; IC_{50}, concentration at which 50% inhibition of the growth of spores or bacterial cells relative to a control occurs; MIC, minimum inhibitory concentration required to completely inhibit the growth of fungal or bacterial cells; NB, nutrient broth; PDA, potato dextrose agar; PDB, potato dextrose broth.

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