RESEARCH ARTICLE

Efficacy of Diphenyleneiodonium Chloride (DPIC) Against Diverse Plant Pathogens

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ABSTRACT

Many of the fungicides and antibiotics currently available against plant pathogens are of limited use due to the emergence of resistant strains. In this study, we examined the effects of diphenyleneiodonium chloride (DPIC), an inhibitor of the superoxide producing enzyme NADPH oxidase, against fungal and bacterial plant pathogens. We found that DPIC inhibits fungal spore germination and bacterial cell proliferation. In addition, we demonstrated the potent antibacterial activity of DPIC using rice heads infected with the bacterial pathogen *Burkholderia glumae* which causes bacterial panicle blight (BPB). We found that treatment with DPIC reduced BPB when applied during the initial flowering stage of the rice heads. These results suggest that DPIC could serve as a new and useful antimicrobial agent in agriculture.

1. Introduction

Plants are continuously exposed and attacked by a variety of pathogenic microorganisms, including bacteria, fungi, and viruses, in their environments, and these infections lead to severe crop losses worldwide [1–3]. The pathogenic fungi that cause the most severe losses are in the genera *Fusarium*, *Botrytis*, *Magnaporthe*, *Colletotrichum*, and *Cylindrocarpon* [4,5]. Bacteria in the genera *Burkholderia*, *Pseudomonas*, and *Ralstonia* are major plant pathogens that exist widely in the environment [6–8]. Recently, it has been shown that *Fusarium graminearum*, which causes Fusarium head blight (FHB) and *Burkholderia glumae*, which causes bacterial panicle blight (BPB), interact cooperatively in their development and dispersal on rice [9]. This finding suggests that comprehensive studies are required to control plant diseases in fields.

Many of the antimicrobial agents currently used in agriculture are highly toxic and non-biodegradable and often cause long-term environmental pollution [10–12]. Moreover, the continuous use of the antimicrobial agents frequently results in the occurrence of antimicrobial-resistant strains [10,13], for example, antimicrobial-resistant fungi have emerged worldwide even though azole-type fungicides have broad spectrum antifungal activity and an improved safety profile [14,15]. Thus, we need to develop new alternative compounds that are not toxic to environment and that minimize the occurrence of resistant strains.

Diphenyleneiodonium chloride (DPIC) not only affects NADPH oxidase but also interferes with other flavoenzymes, including nitric oxide synthase and xanthine oxidase [16–18]. DPIC inhibits superoxide production in intact mitochondria and cell redox metabolism and blocks ion channels nonselectively [19]. DPIC also shows potent and broad spectrum antimicrobial activity against human pathogenic bacteria *in vitro* [20]. In addition, DPIC prevents the proliferation of cancer cells and may be non-toxic to healthy tissues in animals [21]. In plants, DPIC inhibits the accumulation of hydroxycinnamic acid, which is caused by wounds, in carrots [22] and inhibits suberization and lignification in potatoes, thus DPIC can also improve the quality of agricultural products [23,24].

In this study, we found that DPIC has potent antifungal and antibacterial activities against phytopathogenic fungi and bacteria. We tested the efficacy of DPIC against *B. glumae in vitro* and *in vivo*, and we found that rice heads treated with DPIC had significantly reduced disease severity caused by *B. glumae*. DPIC showed an intense dissolution mode and powerful antimicrobial effects suggesting that it is a good candidate for the control of plant pathogens.

2. Materials and methods

2.1. Fungal and bacterial strains

The fungal and bacterial strains used in this study were obtained from the Centre for Fungal Genetic Resources (Seoul National University, Seoul, Korea) and the Korean Agricultural Culture Collection (National Agrobiodiversity Center, Jeonju, Korea). *F. graminearum*, *Fusarium oxysporum*, *Fusarium fujikuroi*, *Fusarium solani*, *Colletotrichum gloeosporioides*, *Cylindrocarpon destructans*, *Botrytis cinerea*, and *Magnaporthe oryzae* were inoculated on potato dextrose agar (PDA) at $25\,°C$. Spore production was induced in carboxymethyl cellulose (CMC) [25] or carrot agar (CA) [26], and spore germination was tested in minimal medium (MM). *B. glumae*, *Bacillus megaterium*, *Escherichia coli*, *Pseudomonas putida*, and *Ralstonia solanacearum* were cultured in lysogeny broth (LB) [27] at $30\,°C$ with shaking at 200 rpm for 24 h. All of the strains were stored in 15% (v/v) glycerol at $-70\,°C$.

2.2. Spore preparation and antifungal activity assay

To induce spore production, strains in the genus *Fusarium* were incubated in 50 ml of CMC medium as previously described [25], *B. cinerea* and *M. oryzae* were incubated in CA for 7 days at $25\,°C$ under a blue light, and *C. destructans* was incubated in 50 ml of PDB + G (potato dextrose broth supplemented with ginseng powder) as previously described [28]. The cultures were filtered with two layers of miracloth (Calbiochem, La Jolla, CA), and the spores were harvested by centrifugation at 13,000 rpm. The harvested spores were washed twice with distilled water and resuspended in 1 ml of MM. To test effects of DPIC (Sigma-Aldrich, St. Louis, MO) on spore germination, spores of each fungal strain were incubated at a final concentration of 10^6 spores/ml in 20 ml of liquid MM containing 0 or 0.1 mM DPIC, and germination was observed at 4, 8, and 12 h. To determine whether the inhibition of germination was temporary, spores were treated with DPIC for 24 h, centrifuged at 13,000 rpm for 5 min, the supernatant was removed, and the pelleted spores were resuspended in distilled water; this procedure was repeated two more times. Then, the pelleted spores were resuspended in fresh liquid MM and the rate of spore germination was determined by counting the number of germinated and total spores every 12 h.

2.3. Antibacterial activity assay

Bacteria were cultured overnight at $30\,°C$ in liquid LB for the preparation of cell suspensions. Each cell suspension was adjusted spectrophotometrically to approximately 10^4 CFU/ml, and 100 μl of each bacterial cell suspension was added to 20 ml of liquid MM containing 0.1 mM DPIC. The cultures were incubated at $30\,°C$ with shaking at 200 rpm, and cell growth (OD_{600}) was measured every 4 h for 20 h.

2.4. Rice seedling growth assay

Rice seeds were soaked in 1% (w/v) sodium hypochlorite for 5 min, rinsed in sterile water for 5 min, and then these sterilized rice seeds were germinated in distilled water at $28\,°C$ for 2 days. The pre-germinated seeds were incubated in 10 ml of distilled water or DPIC solution (0.1 mM) for 1 h in an orbital shaker (200 rpm), were dried on a clean bench for 1 h, and then the seeds were transplanted on a seedbed with filter paper. Growth was determined by measuring shoot and root lengths after incubation at $28\,°C$ with high relative humidity (close to 100%) for 7 days. The experiments were repeated three times with three replicates, and the Tukey test in the R software package version 3.1.2 was performed to evaluate significant differences ($p < 0.05$).

2.5. Efficacy assay of DPIC against FHB and BPB on rice heads

The effects of DPIC treatment on rice heads exposed to *F. graminearum* or *B. glumae* were tested using the "Dongjin" rice cultivar at initial- or mid-anthesis. Rice heads were dipped in 30 ml of distilled water or DPIC solution (0.1 mM) containing *F. graminearum* (10^7 spores/ml) or *B. glumae* (10^4 CFU/ml) for 1 min and then were sealed individually in plastic bags for 2 days. For the controls, rice heads were treated with distilled water or DPIC solution without *F. graminearum* or *B. glumae*. The infected plants were placed in a green house, and the rice grains that exhibiting blight symptoms were counted 3 weeks after inoculation. The disease index was calculated as the number of diseased grains per rice head.

3. Results

3.1. Effect of DPIC on fungal spore germination

Spore germination was inhibited strongly by 0.1 mM DPIC for all eight of the fungal species tested in this study. Germination was determined by the length of the germ tube relative to the spore. The spore germination rate decreased to less than 50% after 12 h for *C. destructans*, *C. gloeosporioides*, and *Fusarium* spores treated with DPIC. The germination rate for *B. cinerea* and *M. oryzae* spores treated with DPIC was >50% after 12 h, but the germinated hyphae lengths were shorter with DPIC
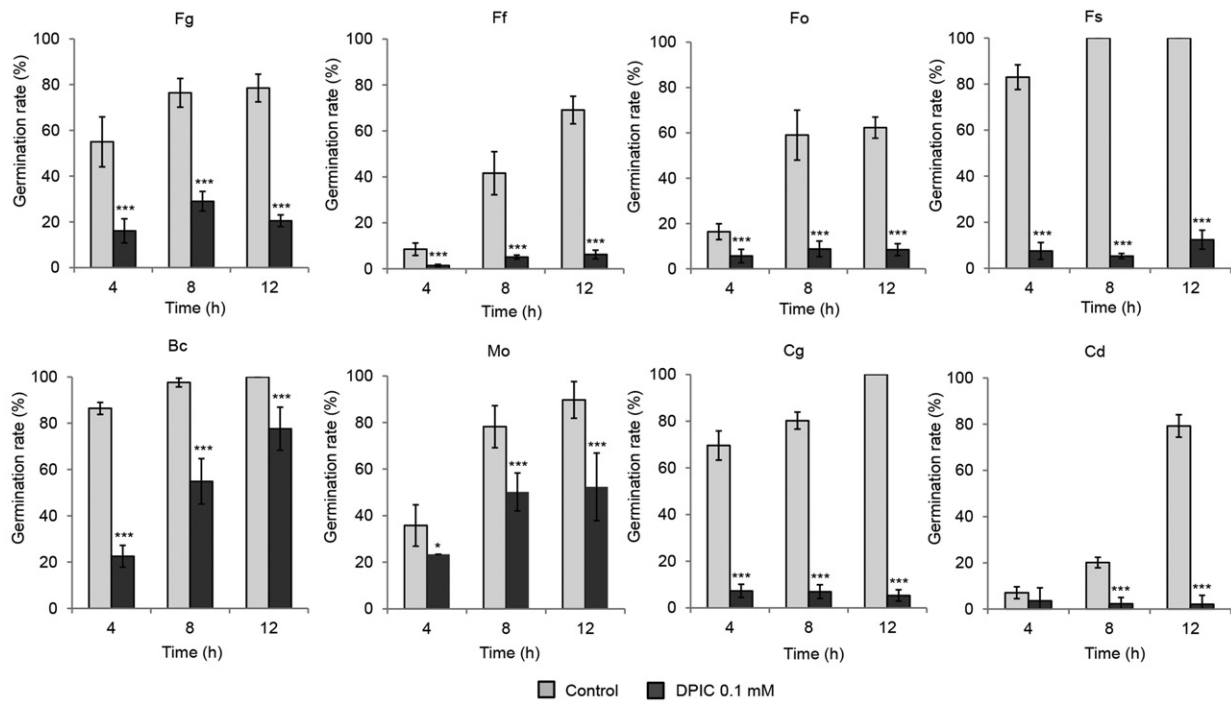
Figure 1. Effect of DPIC on fungal spore germination. Spores were treated with sterile distilled water (Control) or 0.1 mM DPIC (DPIC 0.1 mM). Fungal spore germination rates were determined after 4, 8, and 12 h of incubation at 25 °C. Asterisks indicate significant differences between Control and DPIC 0.1 mM samples (***$p < 0.001$). Fg: *F. graminearum*; Ff: *F. fujikuroi*; Fo: *F. oxysporum*; Fs: *F. solani*; Bc: *B. cinerea*; Mo: *M. oryzae*; Cg: *C. gloeosporioides*; Cd: *C. destructans*.

Figure 2. Effect of washing with distilled water on spore germination. Spores exposed to DPIC were washed with distilled water, resuspended in fresh liquid MM, incubated for 12 h, and then spore germination was observed using an optical microscope. Spores that were continuously exposed to DPIC without washing were used as the control (Control). Scale bars, 10 μm. Fg: *F. graminearum*; Ff: *F. fujikuroi*; Fo: *F. oxysporum*; Fs: *F. solani*; Bc: *B. cinerea*; Mo: *M. oryzae*; Cg: *C. gloeosporioides*; Cd: *C. destructans*.
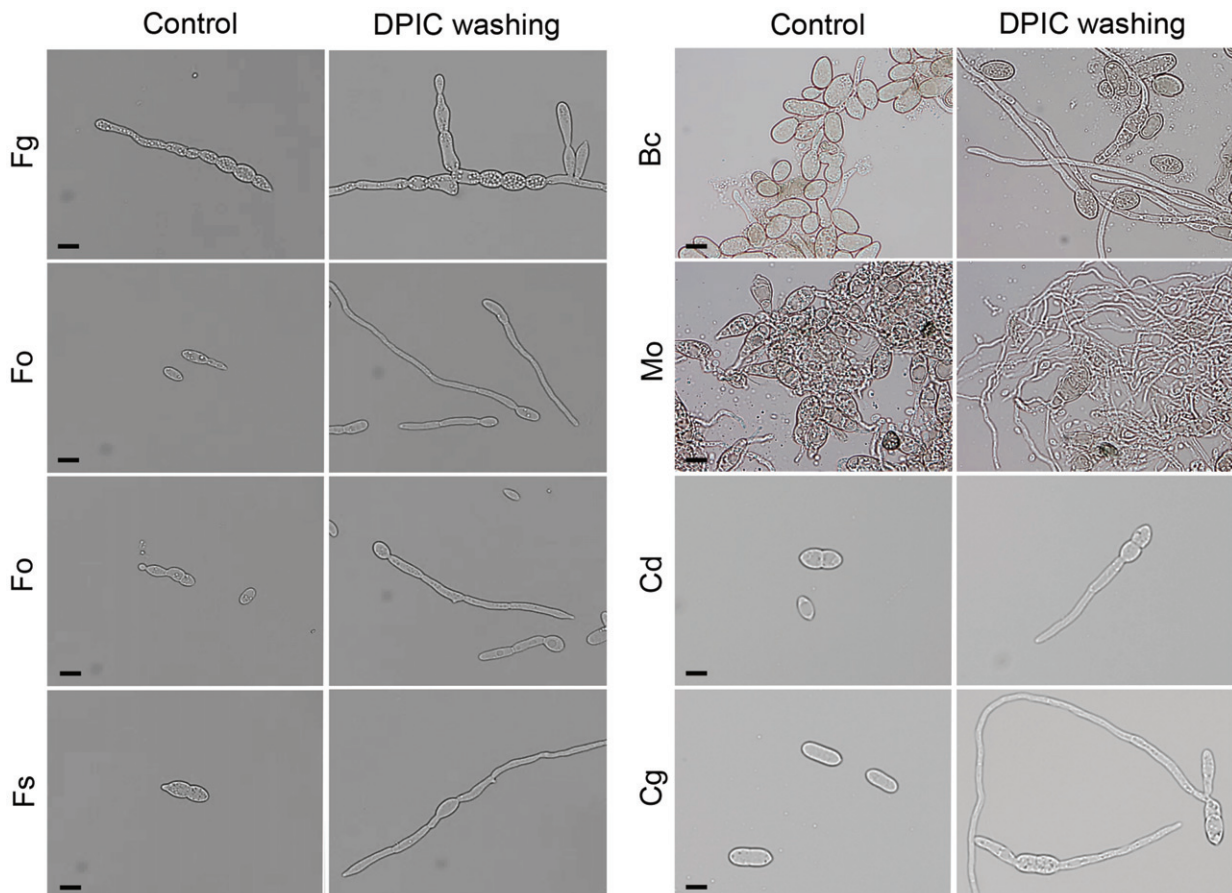
treatment than without DPIC treatment (Supplementary Figure S1; Figure 1). However, when the spores treated with DPIC were washed with water and transferred to fresh liquid MM, the germination rates were recovered (Supplementary Figure S2; Figure 2).

3.2. Effect of DPIC on bacterial cell growth

DPIC inhibited *B. glumae*, *B. megaterium*, *E. coli*, *P. putida*, and *R. solanacearum* cell growth. Bacteria at an OD_{600} of 0.1 were inoculated into 20 ml of fresh liquid LB with 0.1 mM DPIC and incubated for 24 h at 30 °C with shaking. Bacterial growth was completely arrested in 0.1 mM DPIC and OD values did not increase in 0.1 mM DPIC (Figure 3).

3.3. Effect of DPIC on rice seed germination and seedling growth

There was no difference in the germination rates of rice seeds that were soaked in 0.1 mM DPIC and rice seeds that were not treated with 0.1 mM DPIC. The germination rate of DPIC-treated and untreated rice seeds was 80–90%. Although the root length was significantly shorter in rice treated with 0.1 mM DPIC than in untreated rice, the shoot length did not differ significantly in the DPIC-treated and untreated rice. However, after 7 days, the final shoot and root lengths did not differ in DPIC-treated and untreated rice (Figure 4).

3.4. Efficacy of DPIC against FHB and BPB on rice

The rice heads that were dipped in *F. graminearum* or *B. glumae* showed typical FHB or BPB symptoms after 3 weeks, but disease symptoms were not observed in the rice heads treated with either DPIC or water (Supplementary Figure S3 and Table 1). Rice heads that were treated with DPIC in the initial-anthesis stage showed reduced BPB severity compared to untreated rice heads (Supplementary Figure S3 and Table 1). However, DPIC treatment did not affect the disease severity of FHB caused by *F. graminearum*, and DPIC treatment did not reduce FHB or BPB severity when rice heads were treated with DPIC in the mid-anthesis stage (Supplementary Figure S3 and Table 1).

4. Discussion

In this study, we showed that DPIC has strong antifungal and antibacterial effects on various fungal
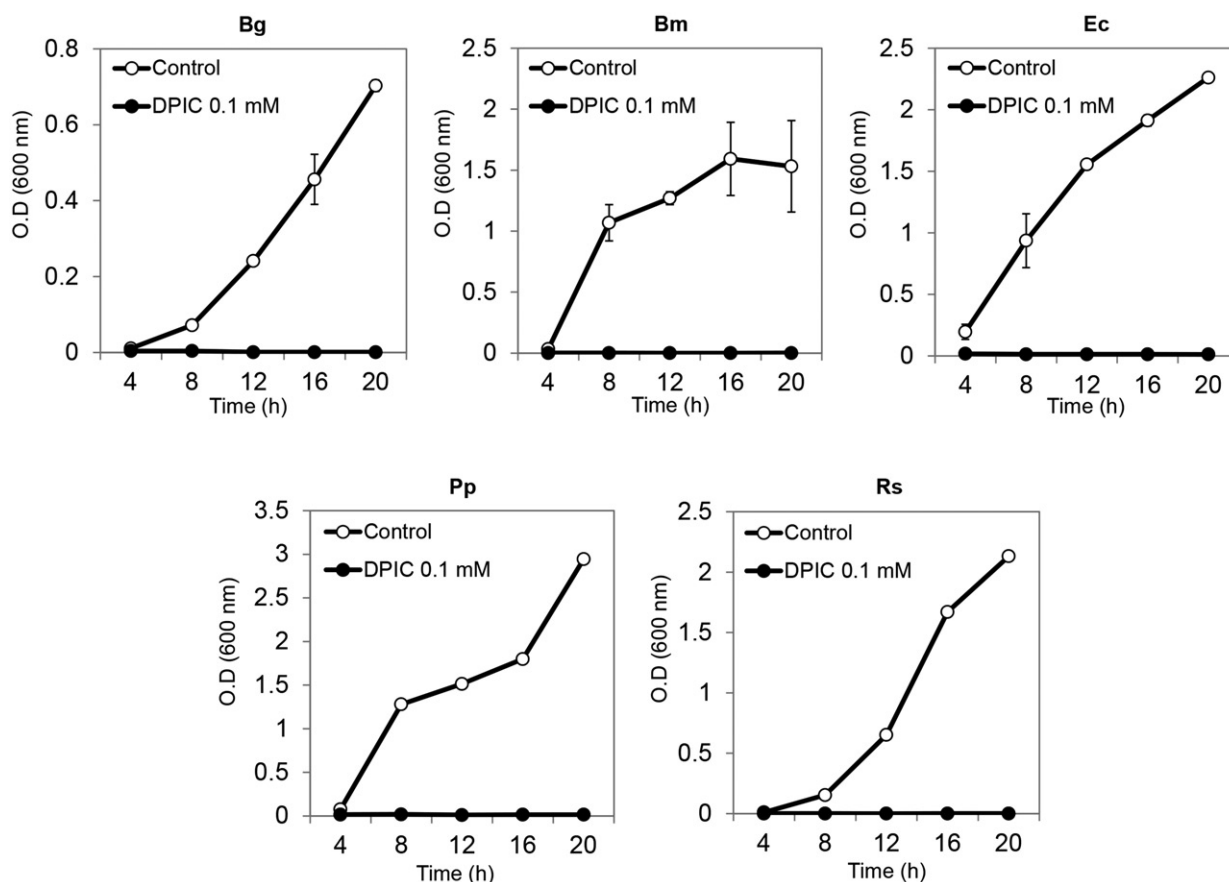

Figure 3. Bacterial growth inhibition assays. Bacterial suspensions were inoculated into liquid MM with 0.1 mM DPIC (DPIC 0.1 mM) or without DPIC (Control) and incubated at 30 °C with shaking. OD_{600} measurements were taken every 4 h for 20 h and growth curves were generated. Bg: *B. glumae*; Bm: *B. megaterium*; Ec: *E. coli*; Pp: *P. putida*; Rs: *R. solanacearum*.
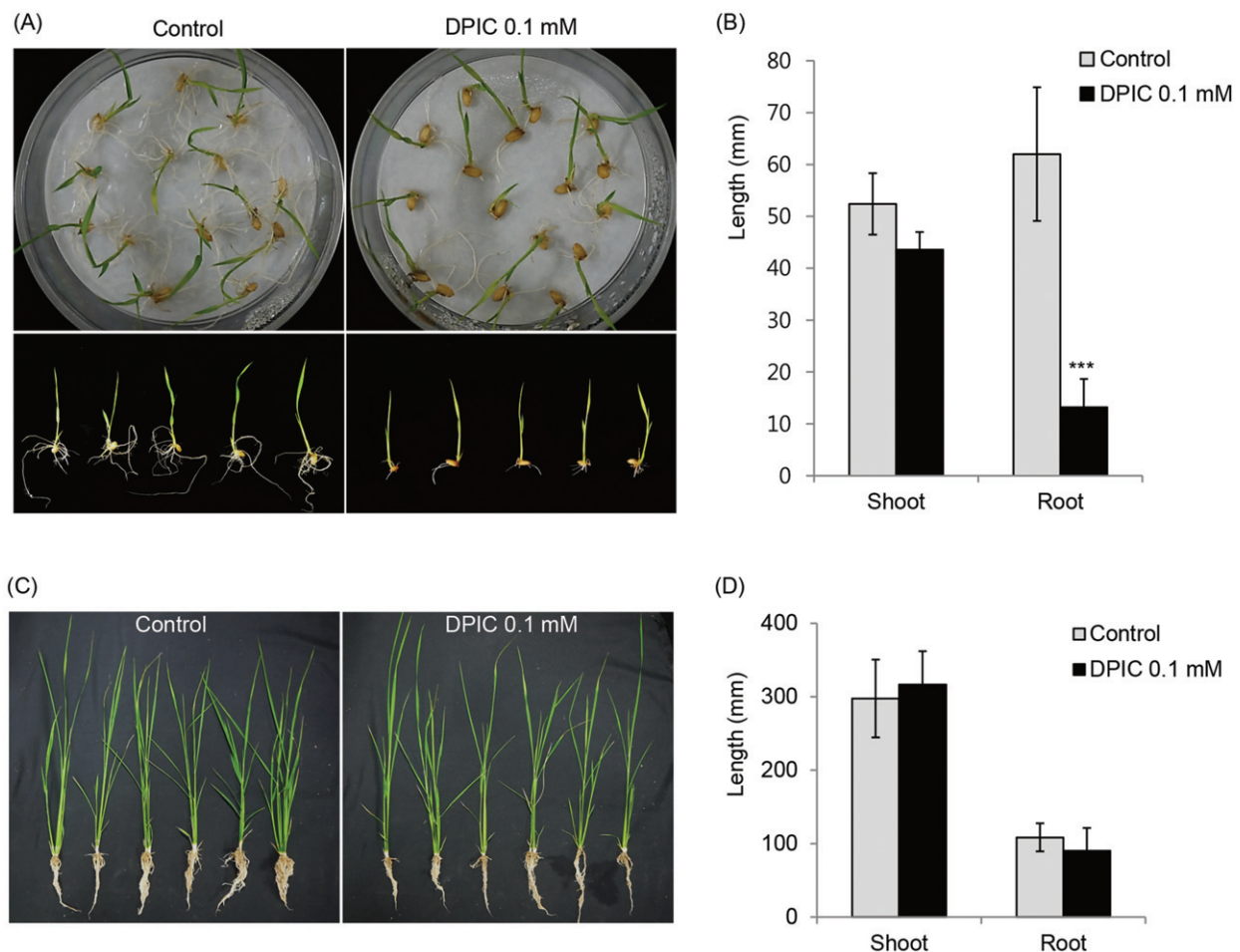
Figure 4. Effects of DPIC on rice seedling growth. Rice seeds were soaked in 0.1 mM DPIC or distilled water for 1 h, incubated for 7 days at 30 °C, and then the shoot and root lengths were measured (A,C). The germinated seeds were transferred to egg pots, were grown for another 7 days, and then the shoot and root lengths were measured (B,D). The plants were grown under a 14 h light and a 10 h dark cycle at 30 °C with 70% humidity. The shoot and root lengths of ten rice seedlings per condition (with or without DPIC) were measured and averaged. Asterisks indicate significant differences between the Control and DPIC 0.1 mM samples (***$p < 0.001$).

Table 1. Efficacy of DPIC against *F. graminearum* and *B. glumae.*

	Percentage of grains with disease symptoms[a]	
	Initial-anthesis inoculation	Mid-anthesis inoculation
DW	6.52A[b]	0.91A'
DPIC	1.94A	0.66A'
DW + Fg	26.10B	13.28B'
DW + Bg	52.34C	34.65C'
DPIC + Fg	17.08B	14.56B'
DPIC + Bg	25.10B	38.01C'

[a]Rice heads were inoculated by the dip inoculation method. DW: Distilled water; DPIC: Diphenyleneiodonium chloride; Fg: *F. graminearum*; Bg: *B. glumae.*
[b]Values with different letters within a column differ significantly according to Tukey's test ($p < 0.05$).

and bacterial plant pathogens. In fungi, reactive oxygen species (ROS) produced by NADPH oxidases have been shown to associate with various physiological processes and cellular differentiations, including the development of sexual fruiting bodies, ascospore germination, and hyphal growth [29]. Furthermore, it has been shown that the NADPH oxidase family of enzymes is also present in bacteria [30]. Appropriate levels of ROS produced by

NADPH oxidases are required for proper fungal and bacterial cell growth and proliferation [31,32]. DPIC inhibits NADPH oxidase activity, thus the DPIC-induced inhibition of spore germination and cell proliferation observed in this study may be related to a reduction in the cellular ROS produced.

In this study, we found that DPIC inhibits germination of all of the fungal spores tested and inhibits the growth of all of the bacteria tested (Figure 1). We focused on *F. graminearum* and *B. glumae* in this study because there has been an increase in FHB and BPB in rice fields in Korea due to climate change [33,34]. In addition, we showed previously that *F. graminearum* plays an important role in the disease dispersal and survival of *B. glumae* [9]. Therefore, alternative compounds that have broad-spectrum activity against both fungi and bacteria, such as DPIC, are needed to control plant diseases, such as *F. graminearum* and *B. glumae.*

Although DPIC displayed strong inhibitory effects on various plant pathogens *in vitro*, application of DPIC on rice heads was not enough to

control the diseases caused by *F. graminearum* and *B. glumae*. DPIC slightly reduced the incidence of disease caused by *B. glumae* when it was applied during initial-anthesis but not during mid-anthesis (Supplementary Figure S3 and Table 1) suggesting that DPIC can protect rice plants from *B. glumae* when applied during initial flowering.

Recent studies have shown that *Bacillus amyloliquefaciens* JCK-12 extracts and chemical fungicides reduce the incidence of FHB and that the combination of biological control agents and chemical fungicides reduces the incidence of fungicide resistance and lowers the effective dose of chemical fungicides, thereby reducing environmental damage and improving the efficacy of antifungal agents [35]. Therefore, we hypothesize that treatment with DPIC in combination with existing chemical fungicides will be more effective in controlling pathogens.

The indiscriminate use of fungicides and antibiotics in agriculture has led to the rapid emergence of drug-resistant pathogens, which are difficult to control [36,37]. For example, the application of tebuconazole, guazatine, or iminoctadine significantly reduces *F. graminearum* mycelial growth but increases production of the *F. graminearum* mycotoxin 3-acetyldeoxynivalenol four-fold as has been previously described for fungicide-resistant *F. graminearum* [15]. Furthermore, the application of tebuconazole has resulted in an increase in the extent of grain colonized by *Microdochium nivale*, which causes pink snow mold in cool season turf grass species [15,38]. Therefore, we need to develop alternative compounds with different modes of action than the chemicals currently used.

Importantly, we showed that fungal spores exposed to DPIC germinated normally when washed with distilled water and transferred to fresh medium (Figure 2) indicating that although DPIC can arrest germination of fungal spores and proliferation of bacterial cells, it does not have long-term toxicity to microorganisms. This characteristic of DPIC can be useful for controlling pathogens that infect plants during specific and short growth stages, such as flowering. Therefore, we expect that proper use of DPIC during pathogen infection can prevent pathogens from invading and weaken the appearance of resistant mutations. DPIC has potential as a safe and broad-spectrum antifungal and antibacterial agent to combat the increased resistance of plant pathogens to the currently available antimicrobial agents and to remove toxic chemicals from agriculture.

Disclosure statement

No potential conflict of interest was reported by the authors.

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