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Benzaldehyde as an insecticidal, antimicrobial, and antioxidant compound produced by *Photorhabdus temperata* M1021[§]

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The *Photorhabdus temperata* M1021 secretes toxic compounds that kill their insect hosts by arresting immune responses. Present study was aimed to purify the insecticidal and antimicrobial compound(s) from the culture extract of *P. temperata* M1021 through bioassay guided fractionation. An ethyl acetate (EtOAc) extract of the *P. temperata* M1021 exhibited 100% mortality in *Galleria mellonella* larvae within 72 h. In addition, EtOAc extract and bioactive compound 1 purified form the extract through to column chromatography, showed phenol oxidase inhibition up to 60% and 80% respectively. The analysis of ^{1}H and ^{13}C NMR spectra revealed the identity of pure compound as "benzaldehyde". The benzaldehyde showed insecticidal activity against *G. mellonella* in a dose-dependent manner and 100% insect mortality was observed at 108 h after injection of 8 mM benzaldehyde. In a PO inhibition assay, 4, 6, and 8 mM concentrations of benzaldehyde were found to inhibit PO activity about 15%, 42%, and 80% respectively. In addition, nodule formation was significantly ($P < 0.05$) inhibited by 4, 6, and 8 mM of benzaldehyde as compare to control. Moreover, benzaldehyde was found to have great antioxidant activity and maximum antioxidant activity was 52.9% at 8 mM benzaldehyde as compare to control. Antimicrobial activity was assessed by MIC values ranged from 6 mM 10 mM for bacterial strains and 8 mM to 10 mM for fungal strains. The results suggest that benzaldehyde could be applicable for developing novel insecticide for agriculture use.

Keywords: insecticidal compound, benzaldehyde, antimicrobial, antioxidant

Introduction

Exploration for new strategies and/or natural products to control destructive insects is desirable due to the increasing prevalence of resistance to existent insecticides and the problem of toxic non-biodegradable residues contaminating the environment (Jantan *et al.*, 2005). It has been investigated that the use of nematode symbiotic, entomopathogenic bacteria of the *Photorhabdus* genus as pest (insect) control agents can be effective substitution to chemical pesticides (Fridlender *et al.*, 1993). The *Photorhabdus* genus consists of 3 bacterial species: *P. luminescens*, *P. temperata*, and *P. asymbiotica* (Jang *et al.*, 2011). Molecular, biochemical and morphological data of the type strains *P. temperata*, i.e., *P. temperata* subsp. *temperata* strain XlNach^{T}, *P. temperata* subsp. *cinerea* strain 3107^{T}. *P. temperate* subsp. *Tasmaniensis* strain DSM 22387^{T}. *P. temperate* subsp. *Khanii* strain DSM 3369^{T} suggested that like *P. temperata* shares a common genus with *P. luminescens* and *P. asymbiotica* (Akhurst, 1980). The genus *Photorhabdus* consists of symbiotic nematode, gram-negative bacteria that belong to the γ-subdivision of the Proteobacteria, more specifically the family Enterobacteriaceae (Akhurst, 1980). *Photorhabdus* is a virulent pathogen, and complete genome analysis revealed that a vast majority of the genetic loci in *Photorhabdus* are involved in the synthesis of secondary metabolites that are active against a wide range of insects (Seo *et al.*, 2012; Park *et al.*, 2013) and microbes including bacteria and fungi (Eleftherianos *et al.*, 2007). The pathogenicity of the toxins is mainly due to their ability to suppress the immune systems of the insect hosts via hemolysis, antimicrobial peptide degradation, eicosanoid biosynthesis inhibition, and suppression of prophenoloxidase (proPO) activation (Eleftherianos *et al.*, 2007; Kim and Kim, 2011).

The phenoloxidase (PO) immune response is an immediate reaction against invading microbes in insects (Shrestha and Kim, 2008). Activated PO catalyzes the conversion of phenolic substrates to quinones, which are converted to melanin that is deposited at wound sites, forming nodules (Kanost *et al.*, 2004; Rodriguez-Andres *et al.*, 2012), and preventing microorganisms from entering and spreading throughout the insect body with factors that stimulate phagocytosis (Hu and Webster, 2000; Kang *et al.*, 2004). However, pathogens have evolved to interact with the protoporphyrinogen oxidase (PPO) system, which leads to abrogation of insect defense mechanisms or abnormal body softening, both of which could facilitate pest control (Eleftherianos *et al.*, 2007). Thus, PPO inhibitors may be potential insecticide candidates (Rodriguez-Andres *et al.*, 2012). Numerous compounds, extracted from *Photorhabdus* spp. have been reported to interfere with insect's PPO systems and overcome the immune barrier (Hu and Webster, 2000), which results a quick reduction in hemolymph PO activity and suppression of the host encapsulation response (Eleftherianos *et al.*, 2007; Rodriguez-Andres

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et al., 2012). Similarly, the metabolites produced by *Photorhabdus* bacteria exhibit antioxidant and antibiotic activities that prevent the growth of competing microorganisms and putrefaction of the nematode-infected insect cadavers (Clarke, 2008). This is important for the successful completion of the nematode parasite life cycle and for symbiont transmission to the nematode's progeny (Waterfield *et al.*, 2009).

However, it is now known that secondary metabolite production is common to *Photorhabdus* spp. when cultured *in vitro*, and several secondary metabolites, such as stilbene derivatives, anthraquinone derivatives, genistine, a furan derivative, and a phenol derivative have been identified and found to exhibit insecticidal, antioxidant, and antibiotic activities (Eleftherianos *et al.*, 2007; Chalabaev *et al.*, 2008). *P. temperata* culture broth also possesses immunosuppressive activity that inhibits hemocyte spreading behavior and nodule formation (Seo *et al.*, 2012). It was previously shown that the organic extract of *P. temperata* culture broth contains PO inhibitory factor(s) (Seo *et al.*, 2012). Collectively, these studies suggest the possibility of additional identification of PO inhibitory compounds originating from bacterial culture broth. The present study was conducted to purify and identify chemical constituent(s) responsible for PO inhibition activities present in the extract of *Photorhabdus temperata* M1021. The extract was then subjected to column chromatography and bioassay-guided isolation, which yielded a single purified compound that exerted inhibitory activities against cellular immune responses (PO inhibition). The compound was identified using gas chromatography-mass spectrometry (GC-MS) and nuclear magnet resonance (NMR) spectral analyses. The identified compound was analyzed for PO and nodule formation inhibition activities against *Galleria mellonella* larvae. The antimicrobial and antioxidant activities of the compound were also determined.

Materials and Methods

Maintenance of bacterial culture

P. temperata M1021 was identified and characterized from soil entomopathogenic nematodes collected from the South Korean locations reported in our previous study (KACC accession number 91627P) (Jang *et al.*, 2012). The bacterial culture was routinely maintained in Luria-Bertani (LB) broth (0.5% yeast extract, 1% NaCl, 1% tryptone) and incubated at 28 ± 2°C for 48 h to prepare a pre-culture with an initial optical density of 0.6 at 600 nm. The strain was cultured on LB agar plant and in broth for both short- and long-term storage and subsequent use in experiments.

Chemicals

Dimethyl sulfoxide (DMSO), L-3,4-dihydroxyphenylalanine (L-DOPA), 2,3,5-triphenyltetrazolium chloride (TTC), potato dextrose broth (PDB), 2,2-diphenyl-1-picrylhydrazyl (DPPH), vitamin E, benzaldehyde, chloroform, ethanol, ether, ethyl acetate, hexane, and methanol were purchased from Sigma-Aldrich Korea.

Chromatographic fractionation and bioassay of the culture extract

P. temperata M1021 was cultured in LB broth for 7 days at 28 ± 2°C in a shaking incubator at 200 ± 20 rpm. The culture broth was then centrifuged at 12,000 × *g* for 10 min at 4°C, the supernatant was filtered through a 0.2-μm cellulose acetate filter, and the culture filtrate (CF) was used for subsequent fractionation. A bioactivity-guided fractionation procedure was followed for metabolite purification. The CF was acidified to pH 2.8 ± 0.2 with 1 N HCl/NaOH, and subsequently 2 volumes of hexane was mixed with the CF and separated into organic and aqueous fractions. The aqueous phase was treated with EtOAc three times (1:1 v/v), and the organic layers were removed. The organic layers extracted from hexane and EtOAc were combined and dried under reduced pressure on a rotary evaporator (Korea) at 40°C. Nearly dry residues were re-suspended in 1 ml 100% methanol, which was used for initial bioassay against the *G. mellonella* larvae. The insecticidal bioassay was performed by injecting 10 μl EtOAc extract into the hemocoel of five instar *G. mellonella* larvae using a 10 μl Hamilton syringe. The EtOAc extract of *Escherichia coli* DH5α was processed using the same conditions as control. The resulting EtOAc extract was absorbed onto silica and subjected to fractionation by flash chromatography on Luna C_{18} 100A column (250 mm × 4.60 mm, 5 μm; Phenomenex), filled with silica gel (70 to 230 mesh; Merck), using a EtOAc-hexane (20:90, 50:50, 100:0, v/v) and MeOH-EtOAc (2:98, 5:95, 10:90, 20:80, 50:50, and 100:0, v/v) gradient. The bio-active fractions were confirmed with respect to significant PO inhibition activity.

Thin-layer chromatography

Thin layer chromatography (TLC) was performed to investigate different frictions of the organic extracts of bacterial culture broth. Each fraction was spotted at base line, 3 cm above the bottom of a silica gel plate (20 × 20 cm) and subsequently placed in a shallow pool of the mixture of EtOAc/*n*-hexane and MeOH/EtOAc as an eluent in a development chamber, which was then allowed to run by capillary action until the solvent reached the top end of the plate. The plates were dried at room temperature, followed observation under ultraviolet (UV) light with 254 and 365 nm wavelengths (Vilber Lourmat).

Reverse-phase HPLC analysis

The bioactive fraction was subsequently subjected to high-performance liquid chromatography (HPLC; Waters), with the purpose of purification through C_{18} cartridge (Advantec, Inc.). Approximately 10 μl sample was injected into the HPLC equipped with a C18 column using the following conditions: (1) Shimadzu CBM-10 coupled with UV-VIS detector (SPD-10A) with pumps A and B (LC-10AD); (2) Solvent A-100% MeOH, Solvent B-Water with 5% acetic acid; (3) Solvent program: 0–20 min (50% = A; 50% = B), 20–40 min (80% A, 20% B), 40–60 min (100% A, 0% B); (4) flow rate was 1 min/ml; (5) C_{18} Column (Luna 5 μm, 100A, 250 × 4.60 mm); and (6) 20 μl single injection.

GC-MS and NMR analysis of the compound

The purified sample was analyzed via GC-MS SIM (6890N network GC system and a 5973 network mass selective detector [Agilent Technologies]), equipped with an HP-5MS column (30 m × 0.25 mm [i.d.], 0.25-µm film thickness). The carrier gas used helium at a constant flow rate of 1.0 ml/min. Two microliters of purified fraction was injected into the column in a split ratio of 1:20 for analysis with an ionization energy of 70 eV. Helium carrier gas was maintained at a head pressure of 30 kPa. The oven was programmed with a starting temperature of 60°C for 3 min and then held at 300°C for 48 min. The mass detector was operated in an electron impact mode with an ionization energy of 70 eV, a scanning range of 33 to 550 atomic mass units (amu), and a scan rate of 1.4 scans/s. Purified samples were respectively identified by comparing mass spectra and retention indices in the spectral database. The NMR spectra were obtained on an NMR spectrometer (Advance Digital) operating at 500 MHz (^{1}H, ^{13}C).

Rearing of *G. mellonella* larvae and insecticidal bioassay

G. mellonella were used as model insects for the bioassay. They were reared from their eggs on artificial diet according to the procedure followed by Ullah *et al.* (2014). Eggs laid by wax moths on butter paper were added into 150 g media and then incubated at 25 ± 2°C and relative humidity of 50 ± 5% to hatch the eggs. The small larvae were then transferred to a larger container containing 1,000 g of media.

The insecticidal activity of 10 µl EtOAc extract and/or benzaldehyde concentrations ranged from 2–8 mM were assayed via intra-hemocoel injection using a 10-µl Hamilton syringe. The injected larvae were transferred into a 90-mm Petri dish and incubated at 25 ± 2°C at a relative humidity of 50%, and the larval mortality rate was evaluated at 12-h intervals. Larvae injected with 10 µl methanol were used as a reference to assess death due to physical trauma from the injection. Ten larvae were used per treatment, three replicates were assayed, and all the experiments were repeated at least three times.

Analysis of PO and nodule inhibition of benzaldehyde

Hemolymph phenoloxidase activity was determined using L-DOPA as substrate as previously reported by Seo *et al.* (2012) with some modifications. The hemolymph was collected from *G. mellonella* larvae, placed in freezer for 10 min to stop the larval movement, followed by decapitation. Hemolymph was collected from 20 fifth instar larvae by incising the abdominal proleg and squeezing the hemolymph into 15-ml falcon tubes. The tubes were centrifuged at 10,000 × *g* for 5 min, and the hemolymph was collected in fresh tubes. PO inhibition activity was assayed in 96-well plates with 100 µl 50 mM phosphate buffered saline (PBS) containing 2 mM L-DOPA added to 20 µl compound 1 and/or benzaldehyde. PO activity was monitored by measuring absorbance at 495 nm using a plate reader (Dynatech MR5000, El Paso, TX, USA) over a period of 90 min. It should be noted that this approach predominantly detects dopachrome and/or dopaminechrome rather than melanin itself.

Nodule inhibition assay was conducted by injecting 2×10^6 *E. coli* cells as a positive control into hemocoel of *G. mellonella* larvae using a microinjector. After 12 h of incubation at room temperature, melanized nodules were counted using a microscope (Olympus) at 400× magnification. Furthermore, different concentrations of benzaldehyde (2, 4, 6, and 8 mM) were prepared and used as injection samples. From this solution, 25 µl was mixed with *E. coli* (2×10^6 cells) and injected into the fifth-instar *G. mellonella* larvae. After 12 h of incubation at room temperature, the formed nodules were counted under a microscope at 400× magnification. Each treatment was repeated three times.

Antioxidant activity of benzaldehyde

The antioxidant activity of benzaldehyde was assayed by measuring radical scavenging ability using DPPH as a stable radical and the microplate spectrophotometric method of Ono *et al.* (2008). Vitamin E was used as positive control, and the antioxidant activity of benzaldehyde was measured in reference to the positive control. Various benzaldehyde concentrations ranging from 2–10 mM were dissolved in methanol. The methanol solution of each sample (0.1 ml) was added to 3.9 ml freshly prepared DPPH methanol solution (0.1 mM). Afterwards, all samples were incubated for 90 min at room temperature in the dark, and then absorbance was measured at 517 nm using a spectrophotometer (Shimadzu). All tests were carried out in triplicate. The antioxidant index (AI) was calculated as ($[A_C - A_A] = A_C [0]$), where A_C (0) is the absorbance of the control at 0 min and A_A (t) is the absorbance of the antioxidant at 90 min. Tests were carried out in triplicate.

Antibacterial and antifungal assessment of benzaldehyde

Five bacterial and three fungal strains were used to investigate the antimicrobial activity of benzaldehyde. Five bacterial strains, including three Gram-negative (*Pantoea conspicua* RSC-6, *Enterobacter cowanii* RSC-3, and *Citrobacter youngae* RSC-5) and two Gram-positive bacteria (*Bacillus aryabhattai* RSC-7 and *Bacillus anthracis* RSC-9) were selected. Similarly fungal strains were consisted of *Phytophthora capsici*, *Rhizoctonia solani*, and *Corynespora cassiicola.* The bacterial strains were grown in liquid LB medium overnight at 28 ± 2°C, and fungal strains were grown in potato dextrose broth (PDB). A modified broth dilution-colorimetric assay using the chromogenic reagent TTC was used to detect the antibacterial and antifungal activity of benzaldehyde. The TTC powder was dissolved in sterile distilled water at a concentration of 5 mg/ml at room temperature then filtered through 0.22-µm filters and stored at -20°C until use. Benzaldehyde was dissolved in methanol at an initial concentration of 1 M and subsequently diluted to obtain concentrations ranging from 2 to 12 mM. To determine the antimicrobial activities, 100 µl of test solutions were added into 900 µl microbial suspensions (1×10^6 CFU/ml) and incubated at 25 ± 2°C for 4 h. The negative control consisted of 900 µl of the inoculum (1×10^6 CFU/ml) and 100 µl methanol. After incubation, 100 µl TTC was added into the mixture and incubated for 30 min. Only living microorganisms could convert TTC to formazan to produce a red color. Afterward, the mixture incubated with TCC was centrifuged at 10,000 × *g* for another 10 min, the pellet was added to 100 µl 50% ethanol, and the light

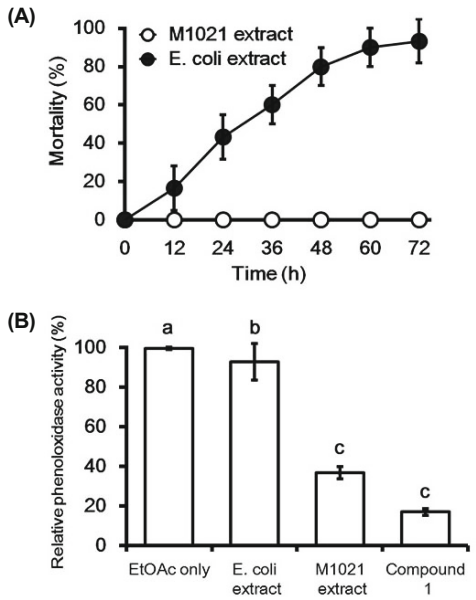

Fig. 1. Insecticidal activity due to immune suppression caused by bioactive extracts of *P. temperata* M1021. The resultant values are means of three replications, and error bars represent standard deviations. (A) Rate of mortality caused by intrahemocoel injection of the EtOAc extract of *P. temperata* M1021 in *G. mellonella* larvae in comparison EtOAc extract of *E. coli* DH5α, used as control. (B) Phenol oxidase inhibition induced by the EtOAc extract and purified bioactive compound 1 from the extract of *P. temperata* M1021. The PO inhibition activities of the EtOAc extract and compound were significantly different from controls (only EtOAc) and control 2 (*E. coli*, DH5α processed in the same way). Different letters over the error bars indicate significant differences at $P < 0.05$ levels as estimated by Duncan's multiple range test (DMRT).

absorption values for the formazan products were measured at 510 nm using a spectrophotometer. The percentage (%) of the microbial growth inhibition was determined as $([Ac - At]/Ac) \times 100$, where Ac was an average of three replicates of light absorption values at 510 nm of the negative controls, and At was an average of three replicates of light absorption values at 510 nm of the samples.

Statistical analysis

Standard deviations were determined using Microsoft EXCEL software (version 2013; Redmond). Mean values were compared with Duncan's multiple range test (DMRT), and differences were considered significant at a P value of 0.05 (analysis of variance; SAS release 9.1; SAS).

Results

Insecticidal effects of EtOAc extract of *P. temperata* M1021

Photorhabdus spp. are excellent entomopathogenic bacteria that have been extensively studied for their insecticidal potential and production of a wide range of toxin metabolites against insects. In the present study, the initial insecticidal bioassay was performed by injecting 10 µl EtOAc extract into the hemocoel of fully matured *G. mellonella* larvae. The result showed that intra-hemocoelic injections caused 40% mortality in *G. mellonella* larvae in the first 24 h, and 80%, 90%, and 100% mortality after 48, 60, and 72 h, respectively, compared to control injections (Fig. 1A). Larvae were considered dead if they were unable to move in a coordinated manner when prodded with a blunt probe.

PO inhibitory activities of the bioactive fractions

The bioactive fraction was fractionated on a silica column with a gradient of hydrophobic eluents using different mixtures of EtOAc and methanol. One sub-fraction of *P. temperata* M1021 broth showed significant toxicity against the larvae. The molecules in the active fraction were further fractionated on a silica column with different mixtures of hexane and EtOAc (Supplementary data Fig. S1). A single bioactive sub-fraction was further fractionated on an HPLC C_{18} column.

Eventually a highly purified single compound was assayed for its ability to inhibit PO in *G. mellonella* larvae, and it was found to exert significant ($P<0.05$) PO-inhibitory activities compared to controls (Fig. 1B). As described above, two control systems were used for the comparison (EtOAc and the EtOAc extract of *E. coli*). PO activity was inhibited up to 60% and 80% by the EtOAc extract and purified compound 1 treatments, respectively, compared with both controls.

Moreover, the bioactive purified fraction was identified and characterized through GC-MS and NMR techniques. The molecular mass and corresponding molecular formula of the compound was determined by MS studies. The presence

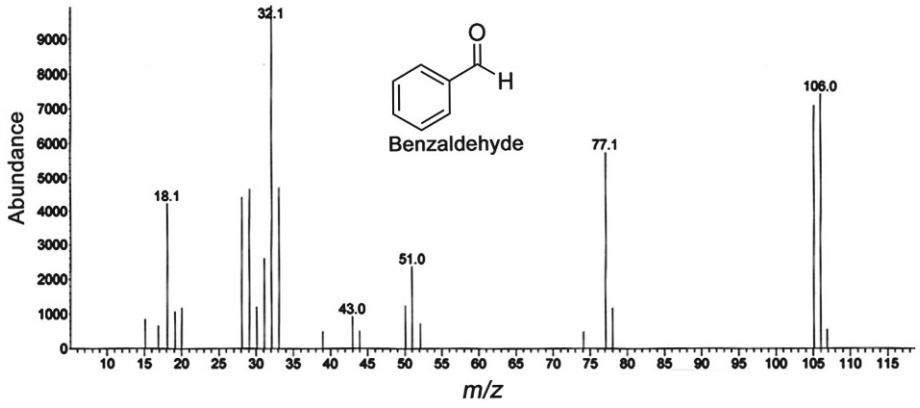

Fig. 2. MS spectrum of compound 1 from *P. temperata* M1021. The GC-MS chromatogram was obtained with 100 µl of a purified sample that was injected into the column using a 10:1 split injection mode. The oven temperature was initially held at 100°C for 3 min, then raised to 300°C for 5 min, and finally held at 300°C for 48 min.

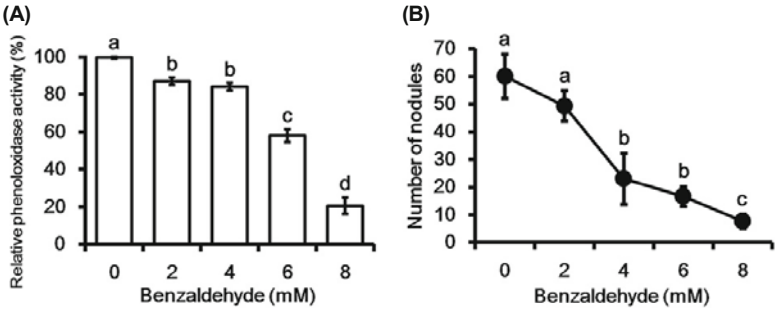

Fig. 3. Insect mortality due to immune suppression characteristics of benzaldehyde, identified from *P. temperata* M1021. The resultant values are the averages of three replications, and the error bars represent standard deviations. Different letters over the error bars indicate significant differences at $P < 0.05$ levels as estimated by Duncan's multiple range test (DMRT). (A) Phenol oxidase inhibition by benzaldehyde in a dose dependent manner in a comparison with treatment of MeOH only, used as negative control. (B) Inhibition of nodule formation in the larvae of *G. mellonella* in the result of benzaldehyde treatment in a concentration dependent manner as compare to control. *E. coli* DH5α cell (2×10^6) were used as a positive control.

of a molecular ion peak M^+ at *m/z* 106 along with the major fragments at *m/z* 105 (M^+-H) and 77 (M^+-COH) indicated the presence a benzene ring and an aldehyde group, respectively. The structure of benzaldehyde was further confirmed by analyzing the ^{1}H and ^{13}C NMR spectra (Supplementary data Fig. S2). The ^{1}H NMR spectrum exhibited the characteristic signals for the mono-substituted benzene ring in the region δ 7.88–7.46, which was supported by three sp^2 methine signals in the region δ 135.2–117.1 in ^{13}C NMR spectrum (Supplementary data Fig. S3). The quaternary carbon (C-1) appeared at δ 135.7, whereas the aldehyde carbonyl carbon (C-7) appeared at δ 190.4. All of the above spectroscopic observations were in complete agreement with the published data for benzaldehyde (Fig. 2).

Inhibitory activities of benzaldehyde on PO and nodule formation

Benzaldehyde was assessed for its inhibitory activities on immune-associated characteristics, such as inhibition of PO activity and hemocyte nodule formation. The inhibitory effects were assayed by the PO-mediated oxidation of L-DOPA in fifth instar larvae of *G. mellonella*. The PO inhibitory patterns revealed that PO activity was markedly inhibited by benzaldehyde in a concentration-dependent manner (Fig. 3A). The half-maximal inhibitory concentration (IC_{50}) value of PO inhibition for benzaldehyde was calculated as 5.5 mM.

PO also has a crucial role in melanogenesis; it converts phenols to quinones, which subsequently polymerize to form melanin. Hemocyte nodule formation is a form of insect cellular immunity that is accompanied by melanization reactions. In the present study, different concentrations of benzaldehyde were assayed to determine nodule inhibition in *G. mellonella* larvae. The results revealed that benzaldehyde significantly inhibited hemocyte nodulation in a dose-dependent manner in response to a bacterial challenge compared to control. Results revealed that 4, 6, and 8 mM concentrations of benzaldehyde significantly reduced nodule formation compared with *E. coli* cells used as a control (Fig. 3B). The IC_{50} values of nodule inhibition due to benzaldehyde treatment was calculated to be 5.8 mM.

Insecticidal activities of benzaldehyde

Extracellular metabolites produced by *Photorhabdus* spp. cause toxicity in a diverse range of insects. These toxins mainly consist of extracellular secondary metabolites that are released into the culture broth during the growth cycle. Benzaldehyde concentrations ranging from 0–8 mM were bioassayed against *G. mellonella* larvae through intra-hemocoel injection to assess insecticidal activity. The results revealed that benzaldehyde toxicity was significantly increased in a dose-dependent fashion. The mortality rate for the 4 mM concentration was approximately 45% after 108 h, whereas the 6 and 8 mM concentrations of benzaldehyde caused up to 73% and 100% mortalities within 108 h, respectively (Fig. 4A). The results suggest that apart from high-molecular-weight protein toxins, the insecticidal potential of *Photorhabdus* can be largely attributed to the secondary metabolites, which are equally effective as protein toxins.

Antioxidant assay of benzaldehyde

The antioxidant activity of benzaldehyde was measured in terms of radical scavenging ability using DPPH as a stable radical. Vitamin E was used as positive control. Benzaldehyde exhibited concentration-dependent DPPH scavenging activity, and the maximum antioxidant value was 52.9% observed

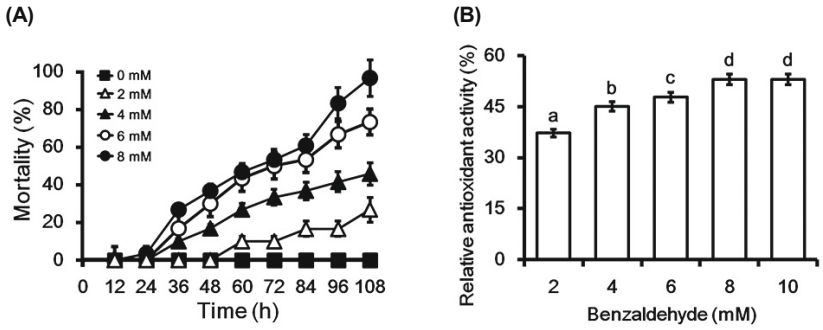

Fig. 4. Benzaldehyde behavior towards the *G. mellonella* larvae in a Dose-dependent manner. (A) Mortality caused by intra-hemocoel injection of benzaldehyde in a Dose-dependent manner (2–8 mM) in a compression with larvae injected with MeOH only, used as control. (B) Relative antioxidant activity of benzaldehyde in a way of concentration gradient (2–10 mM) in the reference of Vitamin E, used as positive control. Different letters over the error bars indicate significant differences at $P < 0.05$ levels as estimated by Duncan's multiple range test (DMRT). The antioxidant activity was increased with successively higher doses of benzaldehyde; however, the activity remained the same at 8 and 10 mM of benzaldehyde (as indicated by the same letters).

Table 1. Antibacterial activity of benzaldehyde assay by using the chromogenic reagent 2,3,5-triphenyltetrazolium chloride (TTC). The potency was assessed by MIC and IC_{50} values of benzaldehyde in a comparison with control. The resultant values of IC_{50} are mean ± SD of three repeats (n=3).

S.No.	Bacterial strains	Benzaldehyde concentration (mM)	
		MIC	IC_{50}
1	*Bacillus anthracis* RSC-9	8.0	5.0 ± 0.4
2	*Pantoea conspicua* RSC-6	10.0	6.1 ± 0.3
3	*Enterobacter cowanii* RSC-3	8.0	4.5 ± 0.23
4	*Citrobacter youngae* RSC-5	10.0	7.0 ± 0.2
5	*Bacillus aryabhattai* RSC-7	6.0	4.0 ± 0.28

for 8 mM benzaldehyde. However, there was no increase in antioxidant activity despite higher benzaldehyde concentrations (Fig. 4B). The IC_{50} value was calculated as 5.6 mM. Certain compounds react rapidly with DPPH depending on the number of available hydroxyl groups, like the positive control vitamin E, whereas some compounds require longer reaction times. Thus, the absorbance values were measured every 30 min during the testing of DPPH radical-scavenging activity. The percentage inhibition of DPPH was increased with all benzaldehyde doses (except at the highest dose) up to 1.5 h later, when no further inhibition was observed.

Antibacterial and antifungal activities of benzaldehyde

Photorhabdus spp. secrete a versatile armory of antimicrobial molecules that exert a wide range of antimicrobial activities against Gram-negative and -positive bacteria. The present study was concerned with the antimicrobial activity of benzaldehyde identified from *P. temperata* M1021. The antimicrobial activities of benzaldehyde were evaluated against bacteria and fungi. The potency was assessed by the percent inhibition of bacterial and fungal growth in comparison with control. Zero concentration was considered as a negative control. Generally, benzaldehyde killed 72–80% at the minimum inhibitory concentration (MIC), but the inhibitory effect varied according to the type of tested microorganism (Table 2).

Discussion

The entomopathogenic bacterium *P. temperata* is known to be a potent biological agent that can kill insects by overcoming their immune responses. The bacterium also secretes a range of antibiotics and antioxidants to suppress the growth of other invading microorganisms and to support nematode survival. *Photorhabdus* spp. genomes have been sequenced, and analyses revealed that nearly 6% of the genomes are predicted to be involved in the production of secondary metabolites (Duchaud *et al.*, 2003). This proportion is greater than the 3.8% observed in *Streptomyces*, the model organism for secondary metabolite production (and the source of >90% of clinically important antibiotics) (Waterfield *et al.*, 2009). Therefore, there is significant potential in *Photorhabdus* for the production of novel bioactive molecules. EtOAc extracts of culture broth exerted significantly inhibitory effects against the PO activities of *G. mellonella* hemocytes. A previous study predicted that a PO inhibitory compound(s) originated from entomopathogenic bacteria because an organic extract of *Photorhabdus* culture broth could be derived from a wide range of organisms, including prokaryotes, insects, reptiles, and mammals (Eleftherianos *et al.*, 2007, Seo *et al.*, 2012).

Table 2. Antifungal activity of benzaldehyde determined by using 2,3,5-triphenyltetrazolium chloride (TTC). The antifungal potency was measured in the term of MIC and IC_{50} values of benzaldehyde. The IC_{50} values are mean ± SD of three replicates (n=3).

S.No.	Fungal strains	Benzaldehyde concentration (mM)	
		MIC	IC_{50}
1	*Phytophthora capsici*	8.0	5.7 ± 0.43
2	*Rhizoctonia solani*	10.0	6.0 ± 0.26
3	*Corynespora cassiicola*	10.0	8.1 ± 0.34

The present study revealed that *P. temperata* M1021 synthesized and released these PO inhibitors to the culture medium. By fractionating the culture broth, this study identified a single PO inhibitor that was chemically identified and analyzed for its abilities to inhibit insect immune responses, PO activity, and hemocyte nodule formation. The results of the present study are supported by previous findings Seo *et al.* (2012) and Salvadori *et al.* (2012) that extracellular secondary metabolites were released into the culture broth by *Photorhabdus* bacteria (i.e., 1,3-dihydroxy-2-[isopropyl]-5-[2-phenylethenyl] benzene, benzylideneacetone, proline-tyrosine, acetylated phenylalanine-glycine-valine indole, oxindole, cis-cyclo-PY, and p-hydroxyphenyl propionic acid). These compounds were bio-assayed against different larvae (e.g., *Manduca sexta*, *Plutella xylostella*, *Salix exigua*, and *G. mellonella*) and were determined to be involved in suppressing insect host immune system activities (Eleftherianos *et al.*, 2007, Seo *et al.*, 2012), including hemolysis, degrading antimicrobial peptides, inhibiting eicosanoid biosynthesis and PO activity, ultimately inducing septicemia and killing the insect host (Shrestha and Kim, 2009; Kim and Kim, 2011). Later, chemical fractionation of *Photorhabdus* culture broth led to identification of a single PO-inhibitory compound, benzaldehyde.

The results of the present study reveal that benzaldehyde inhibited nodule formation in a dose-dependent manner. Upon closer inspection of the insect immune system, we determined that the two host defenses targeted by benzaldehyde–the enzyme PO and nodule formation–are functionally linked, and PO activation is a necessary step for melanotic nodule production (Rodriguez-Andres *et al.*, 2012). A previous study conducted by Wang *et al.* (2007), in which four derivatives of benzaldehyde from *Pieris rapae* larvae were evaluated for their PO inhibitory activity. Their results demonstrated that benzaldehyde and its derivatives significantly inhibited PO activity. Antibiotic activity has been known to be a very common characteristic of *Photorhabdus* and *Xenorhabdus* spp., and the compounds deployed are quite diverse. Benzaldehyde and its derivatives are widely used as environmentally safe antimicrobial compounds (Wang *et al.*, 2010; Velika and Kron, 2012). Considering their broad spectrum inhibitory effects, they are employed as bactericides, fungicides, and algaecides. Benzaldehyde interacts with the cell surface and induces cell death by causing disintegration of the cell membrane and release of the intracellular constituents (Cheng *et al.*, 2009).

Phenols and/or hydroxyl-benzaldehydes also cause intracel-

lular coagulation of cytoplasmic constituents, leading to cell death or inhibition of cell growth (Cheng *et al*., 2009; Velika and Kron, 2012). Collectively, our results indicate that the bioactive compound extracted from *P. temperata* M1021 inhibited PO in *G. mellonella*. GC/MS and NMR techniques were employed to identify the compound as benzaldehyde, which exhibited significant toxicity toward the *G. mellonella* larvae, as well as inhibitory activity against immune responses as demonstrated by reduced hemocyte nodulation in the larval gut. Benzaldehyde also exerted antibacterial and antioxidant activities, which suggest that it could be developed as a novel agricultural insecticide.

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