Title

Antimicrobial action of phenolic acids combined with violet 405-nm light for disinfecting pathogenic and spoilage fungi

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Highlights (85 characters, including spaces, per bullet point; 3 to 5 bullet points)

► Phenolic acids were screened for antifungal synergism with violet 405-nm light.

► Fungicidal action of combined phenolic acids with 405-nm light was investigated.

► The combination caused overproduction of reactive oxygen species in conidia.

► The combination produced no change in conidia membrane integrity.

► Intracellular ATP content and conidia growth rate were decreased by the combination.

Keywords: Synergistic antifungal activity, Fungicidal action, Phenolic acid, Violet 405-nm light, Oxidative stress, Growth delay

ABSTRACT

The aim of this study is to investigate the fungicidal spectrum of six phenolic-cinnamic and -benzoic acid derivatives using four fungi, *Aspergillus niger*, *Cladosporium cladosporioides*, *Trichophyton mentagrophytes* and *Candida albicans*, in a photocombination system with violet 405-nm light. This is the first study to examine the fungicidal mechanism involving oxidative damage using the conidium of *A. niger*, as well as an assessment of cellular function and chemical characteristics. The results of the screening assay indicated that ferulic acid (FA) and vanillic acid (VA), which possess 4-hydroxyl and 3-methoxy groups in their phenolic acid structures, produced synergistic activity with 405-nm light irradiation. FA and VA (5.0 mM) significantly decreased the viability of *A. niger* by 2.4 to 2.6-logs under 90-min irradiation. The synergistic effects were attenuated by the addition of the radical scavenger dimethyl sulfoxide. Generation of reactive oxygen species (ROS), such as hydrogen peroxide and hydroxyl radicals, were confirmed in the phenolic acid solutions tested after irradiation with colorimetric and electron spin resonance analyses. Adsorption of FA and VA to conidia was greater than other tested phenolic acids, and produced 1.55- and 1.85-fold elevation of intracellular ROS levels, as determined using an oxidant-sensitive probe with flow cytometry analysis. However, cell wall or membrane damage was not the main mechanism by which the combination-induced fungal death was mediated. Intracellular ATP was drastically diminished (5% of control levels) following combined treatment with FA and light exposure, even under a condition that produced negligible decreases in viability, thereby resulting in pronounced growth delay. These results suggest that the first stage in the photofungicidal mechanism is oxidative damage to mitochondria or the cellular catabolism system associated with ATP synthesis, which is a result of the photoreaction of phenolic acids adsorbed and internalized by conidia. This

photo-technology in combination with food-grade phenolic acids can aid in developing alternative approaches for disinfection of pathogenic and spoilage fungi in the fields of agriculture, food processing and medical care.

1. Introduction

Inactivation of pathogenic and toxinogenic fungi is an important problem for maintaining human and animal health and medical care, as well as ensuring continued agricultural productivity, crop storage, and food processing capabilities [1,2]. The therapeutic use of antifungal agents has expanded in order to eliminate threats posed by fungi, as exemplified by azole class antifungal agents becoming the mainstay for both the treatment and prevention of aspergillosis. However, azole resistance in the causal pathogen, *Aspergillus fumigatus*, has emerged among patients receiving chronic azole therapy [3]. In the field of agriculture, fungal resistance toward antifungal agents is a serious problem resulting in losses of fruit and vegetable yields. The grey fungus *Botrytis cinerea*, and the tomato leaf mold *Cladosporium fulvum*, are notorious as high-risk organisms for development of resistance against fungicides such as benzimidazole [4,5].

Violet-blue light, particularly 405-nm light, has significant antimicrobial properties against a wide range of bacteria and fungi [6], although the germicidal effect is lower than ultraviolet-C (UV-C) light [7]. Light emitting diode (LED) irradiation required a large fluence (1440 J/cm^2) to reduce *A. niger* conidia viability by only 50%, and was moderately effective toward *Trichophyton* sp. conidia and *Candida albicans*, reducing viability by 2.5-logs with a fluence of 360 J/cm^2 [6,8].

The number of antifungal agents available for therapy is considerably limited in comparison

with that of antibacterial agents [9]. Photodynamic antimicrobial chemotherapy (PACT) is a promising and effective approach for eliminating pathogenic microorganisms, which has been evaluated against bacteria, fungi and viruses in many studies [9,10]. PACT is a bactericidal system that combines visible light appropriate for photoreactions with a non-toxic photosensitizer. In the presence of oxygen, the activated photosensitizers produce reactive oxygen species (ROS) such as singlet oxygen and free radicals (superoxide radicals followed by hydroxyl radicals). Since these cytotoxic oxidants characteristically react with multiple targets, they typically produce irreversible cellular damage leading to cell death, and are unlikely to produce mutations that lead to resistance [11].

Effective photofungicidal activity has been demonstrated using various photosensitizers including phenothiazines (including methylene blue and toluidine blue), porphyrins (including those biosynthesized from the precursor 5-aminolevulinic acid), and phthalocyanine [10]. As an antifungal PACT, the application of the polyphenolic compound curcumin (diferuloyl methane; a non-toxic natural dye extracted from a plant) has been studied well. The photofungicidal spectrum was evaluated in five fungi, including *A. niger*, in combination with light over a range of 370-680 nm [12]. A low curcumin concentration (100 μM) under 420-nm light irradiation has been found to be highly effective for inactivation of *A. flavus*, one of the major aflatoxin producing fungi [13]. The possible fungicidal mechanism toward *B. cinerea* conidia is thought to be related to increased membrane permeability and mitochondrial damage associated with curcumin-mediated ROS generation [14]. Phenolic acids, which are naturally occurring compounds similar to curcumin, have been evaluated for photoantimicrobial activity as well as the mechanism by which ROS are generated under irradiation over a range from UV-A to violet light [15-21]. As a novel approach for disinfection technology, *Escherichia coli* O157:H7 and *Listeria innocua* on spinach leaves were decontaminated by using a fog to improve dispersion

of food-grade phenolic acids, ferulic acid (FA) or gallic acid (GA), followed by UV-A irradiation [22]. The data illustrates the potential of this combination to achieve decontamination of fresh produce in the fields of agriculture and food processing. However, this photosystem needs further optimization for industrial scale applications. While the antifungal efficiency of many kinds of phenolic acid in the absence of light was investigated in certain filamentous fungi, the photofungicidal spectrum and mode of action of phenolic acids remains unclear [23]. Investigation of the photofungicidal efficacy for pathogenic and spoilage fungi can aid in developing alternative approaches for the disinfection process using phenolic acids.

Therefore, the first aim of the present study is to assess synergistic fungicidal activity using six phenolic acids containing a basic structure of cinnamic or benzoic acid, namely coumaric acid (CA), FA, caffeic acid (CaA), chlorogenic acid (CnA), vanillic acid (VA) and GA, under 405-nm light irradiation applied for an appropriate time to four fungi. The 405 nm wavelength is in the violet-blue range and was used as a light source because this range of wavelengths elicits weak germicidal efficacy and is not harmful, which has been confirmed by *in vivo* experiments in mice [24]. The four fungi tested included *T. mentagrophytes* and *C. albicans*, which are the causative agents of the diseases dermatophytosis and candidiasis, respectively [8,24]. *A. niger* and its conidium exhibit low sensitivity to inactivation by treatment with 405-nm light [8]. This fungus genus also includes species that are common food contaminants that produce a number of mycotoxins and cause serious human mycoses [25]. Moreover, the genera *Cladosporium*, *Aspergillus* and *Candida* are important organisms in fungi-related fruit and vegetable deterioration during storage [26].

A second aim of this study is to investigate the mechanism responsible for synergistic fungicidal activity resulting from combined treatment with phenolic acids and 405-nm light irradiation. We previously demonstrated that the photobactericidal mechanism of UV-A light in

the presence of the cinnamic acid derivative FA caused increased oxidative modifications and subsequent disruption of the bacterial membrane in *E. coli* cells [20]. In the present study, the photofungicidal mechanism is explored using phenolic acids derived from cinnamic and benzoic acids on *A. niger* conidium. Various parameters related to cellular function and chemical characteristics were monitored along with cellular viability, adsorption to conidia, intracellular ROS generation, membrane permeability, ATP content and growth rates. Moreover, the generation of hydrogen peroxide and hydroxyl radicals was confirmed in phenolic acid solutions following irradiation.

2. Materials and methods

2.1. Chemistry

CA, FA, CaA, VA and GA were purchased from the Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). ChA was obtained from Sigma Aldrich (St. Louis, MO, USA). The chemical structures of these phenolic acids are shown in Fig. S1. In the photofungicidal screening assay, stock solutions for all phenolic acids were prepared at 200-fold of the evaluated concentration by dissolving the compounds in 80% (v/v) dimethylsulfoxide (DMSO). In the study of the photofungicidal mechanism, all phenolic acids were dissolved in pure water (Hikari Pharmaceutical Co., Ltd., Tokyo, Japan) to final concentrations of 2.5 mM and 5.0 mM prior to addition to the assay. In the mechanistic analysis, pure water was used to exclude chemical species potentially generated from DMSO during photocombination with phenol acids [18]. All other experimental materials were purchased from commercial sources.

2.2. Light source and irradiation

All photocombination assays were performed according to a previously described method with some modifications [19]. An irradiation device equipped with one LED element, 405-nm LED (NVSU333A, U405; Nichia Corp., Anan, Japan) was used for all experiments. The wavelength range and the peak were 400–410 nm and 405 nm, respectively. All experimental assays were performed using 24-well culture plates (AGC Techno Glass Co., Ltd., Tokyo, Japan) incubated at 25°C. The irradiation device was positioned 35 mm above the well-bottom of the microbial culture plates, which was placed on a magnetic stirrer to provide continuous stirring. The irradiance at the distance used was 84.5 mW/cm^2, which was measured using a laser power and energy meter (Nova II; Ophir Optronics Solutions, Ltd., Saitama, Japan) equipped with a photodiode sensor (PD-300-UV; Ophir Optronics Solutions, Ltd.). Total fluence was calculated by multiplying irradiance by the exposure time (sec) and expressed as J/cm^2; 5.1 J/cm^2 for 1-min, 76 J/cm^2 for 15-min, 152 J/cm^2 for 30-min, 203 J/cm^2 for 40-min, 304 J/cm^2 for 60-min and 456 J/cm^2 for 90-min exposures.

2.3. Microbial strains

Microorganisms used in the determination of photofungicidal activity were purchased from NITE Biological Resource Center (NBRC) and the Institute of Food Microbiology (IFM); *A. niger* NBRC 105649, *C. cladosporioides* IFM 63149, *T. mentagrophytes* IFM 59813 and *C. albicans* NBRC 1385.

2.4. Photofungicidal assay

Photofungicidal activity was determined by plating and counting the colony-forming units (CFU) remaining after treatment of a microbial suspension, as reported previously [19]. Conidial and *Candida* sp. yeast suspensions were prepared after incubation on potato dextrose agar (PDA, Nissui Pharmaceutical Co. Ltd.) for 14 days at 25°C and in Sabouraud broth for 24 h at 28°C, respectively, and cell densities were determined as previously described [19,27]. Colony formation to determine viable cell counts for *Candida* sp. and all other strains were performed using Sabouraud agar (Nissui Pharmaceutical Co. Ltd., Tokyo, Japan) and PDA, respectively.

The photofungicidal screening assay was performed using conidia or yeast suspensions, approximately 3-5×10^6 conidia/mL or 2-3×10^7 CFU/mL, respectively. An aliquot of the cell suspension (0.026 mL) was added to each well of a 24-well plate containing 2.624 mL of individual phenolic acids, which were added as a 200-fold dilution of the stock solutions (final tested volume, 2.65 mL). The final concentration of compounds in the assay was 2.5 mM, except for the experiment with *A. niger*, which was performed with a concentration of 5.0 mM, followed by exposure to 405-nm light. The survival of fungi was determined by the colony count method with visual observation after incubation for 72 h at 25°C for *A. niger*, 70 h at 25°C for *C. cladosporioides*, 96 h at 25°C for *T. mentagrophytes*, and 72 h at 28°C for *C. albicans*. When colonies were not detected or were below the detection limit (10 CFU/mL), the logarithm CFU value was set to 0.5. DMSO as a control for the absence of phenolic acids was added at a final concentration of 0.4% (v/v). To investigate whether the generation of hydroxyl radicals was involved in mediating the photofungicidal activity, the radical scavenger DMSO was added to the test samples at a concentration of 10% (v/v) according to a previously described method [15].

2.5. Adsorption of phenolic acid to A. niger conidia

A. niger conidia (approximately 2×10^8 conidia/mL) were treated with phenolic acids at a concentration of 2.5 mM for 1 min at room temperature in a total volume of 1 mL. The samples were then centrifuged (10000×*g*, 1 min) and the supernatant was collected and filtered using a membrane filter (0.22 μm pore size). The filtrate was diluted 50-fold with pure water and the compound concentration in the filtrate was determined by analysis with an HPLC system equipped with a COSMOSIL column (Cholester, 4.6×150 mm; Nacalai Tesque Inc., Kyoto, Japan) using a flow rate of 0.5 mL/min. The maximum absorbance wavelengths were used as the detection wavelengths for the phenolic acids FA (314 nm), CaA (315 nm), ChA (322 nm) and VA (255 nm). The mobile phase consisted of methanol (solvent A) and 0.05% acetic acid aqueous solution (solvent B). The composition of solvent A was maintained at 30% for 5 min and increased from 30 to 50% for 15 min and to 70% for 3 min and maintained at 70% for 2 min. Quantification was performed by establishing calibration curves for each phenolic acid, which were obtained over a concentration range from 2.5 to 70 or 100 μM. Control samples of each compound (without conidia) were prepared in the same manner described above. Concentrations based on the calibration curves corresponded to initial concentrations of the compounds. Adsorption of phenolic acids to conidia was determined based on the observed decrease in concentration in the supernatant (relative to the initial concentration in the control samples) after incubation with conidia, and was expressed as the molar amount per conidium. The number of conidia treated with the compounds was determined by the colony counting method described above.

2.6. Staining assay for flow cytometry

The reactive oxygen species (ROS)-sensitive probe 2',7'-dichlorodihydrofluorescein diacetate dye (DCFH-DA; AAT Bioquest, Inc., Sunnyvale, CA, USA) was used to evaluate intracellular ROS levels in conidia [28]. A suspension of *A. niger* conidia (approximately 2×10^5 conidia/mL in pure water) was treated with a combination of phenolic acids (5.0 mM) and a 90-min exposure at 405-nm light. Conidia were incubated for 1 h at 37°C in Dulbecco's phosphate-buffered saline (Nacalai Tesque, Inc.) containing 10 μM DCFH-DA. Assay samples included conidia treated only with 405-nm light for 90 min and with the compound alone in the dark for an equivalent duration.

Alterations in membrane permeability were determined using propidium iodide staining (PI; Live/Dead BacLight Bacterial Viability and Counting Kit, Thermo Fisher Scientific Inc., Waltham, MA, USA) [20]. *A. niger* conidia treated as described above (irradiation with 405-nm light after addition of FA or CaA) were subsequently stained with 10 μM PI for 20 min at 37°C.

The resulting cells were analyzed using a FACS Verse (Becton Dickinson, Franklin Lakes, NJ, USA) flow cytometer. In the analysis, non-conidia particles (background) were excluded by employing a selection gate for the conidial signal that was defined for particle size (FSC) and complexity (SSC) using an untreated conidial sample [7]. Simultaneously, fungal viability after each treatment was determined as described above.

2.7. Measurement of intracellular ATP content

Following treatment of *A. niger* conidia (approximately 5×10^5 conidia/mL) with the combination of FA or CaA (5.0 mM) and 405-nm light (304 J/cm^2 in a 60-min exposure), ATP

content in the conidia was measured by a luminescence method for determination of antifungal activity against conidia based on Japanese Industrial Standards (JIS L 1921:2015) [29], which modifies International Organization for Standardization (ISO 13629-1:2012) [30]. A luciferin/luciferase kit (Lucifell AT100; Kikkoman Biochemifa Company, Chiba, Japan) and luminometer (Lumitester C-110; Kikkoman Biochemifa Company) were used for the ATP measurements. According to the kit manufacturer's protocol, intracellular ATP was extracted with an ATP releasing reagent in a 15-min reaction after decomposition of extracellular ATP with an ATP eliminating reagent in a 20-min reaction. The treatment time of conidia with each reagent was performed in reference to JIS L 1921:2015 [29]. Simultaneously, samples prepared with FA or CaA alone (without light) and light exposure alone (without phenolic acids), and samples incubated for 60 min (without both phenolic acid and light) were also measured as described above. The fungal viability was determined by counting CFU. ATP quantification was performed by establishing calibration curves for each ATP concentration using an ATP standard reagent kit (Kikkoman Biochemifa Company).

2.8. Measurement of A. niger growth curves

Conidium samples of *A. niger* were assayed for their growth using the growth delay analysis method in a liquid broth with some modifications, which was established previously by growth analysis of *C. cladosporioides* conidia [31]. Conidia of *A. niger* were treated as described in section 2.7. A conidia aliquot (0.020 mL) was mixed with 0.155 mL of sterile water followed by 0.025 mL of potato dextrose broth (Becton Dickinson) containing bis(2-ethylhexyl) sulfosuccinate sodium salt (Tokyo Chemical Industry Co., Ltd.) at a concentration of 0.005% (w/v) in a 96-well culture plate (Corning Inc., New York, NY, USA). The culture plate was

subsequently sealed with sealing tape (Thermo Fisher Scientific Inc.). Fungal growth in each well was recorded as an optical density at 620 nm (OD_{620}) every 15 min at 25°C with a plate reader (Multiskan FC 3.1; Thermo Fisher Scientific, Inc.). During the measurement session, the plate reader was set to intermittently shake the plate (10 sec shaking followed by a 20-sec pause). A mixture prepared in the manner described above, but without phenolic acids and conidia, was used to obtain well blank values (OD_{620}), which were recorded with each measurement. Incubation times required to reach a value of 0.04 (OD_{620}) were derived from the regression line during the period of logarithmic growth phase for each sample.

2.9. Measurement of light-generated hydrogen peroxide

Measurement of H_2O_2 content of each phenolic acid solution was conducted with a colorimetric method using xylenol orange according to the protocol described by Nakamura et al. [16]. Individual 5.0 mM phenolic acid solutions in pure water (total volume 1 mL) were incubated at 25°C with or without an irradiation with 405-nm light (76 J/cm^2) for 15 min. After reaction with xylenol orange, the absorbance of the mixture was measured using a UV-Vis spectrophotometer (UV-1850; Shimadzu Corporation, Kyoto, Japan). The generated H_2O_2 concentration was calculated using a standard curve over a concentration range of 0.2 to 5.0 μM. The detection limit for the assay was 0.4 μM.

2.10. Statistical analysis

All experiments were performed as three or four independent experiments, and the results are presented as the mean with standard deviation. Statistical analyses were performed using

either a two-tailed, unpaired Student's *t*-test (Microsoft Excel 365; Microsoft Corporation, Redmond, WA, USA) or a one-way analysis of variance (ANOVA) followed by the Tukey-Kramer honestly significance difference test for multiple comparison using Excel Tokei ver. 7.0 software (Esumi Co., Ltd.; Tokyo, Japan). *P* values < 0.05 were considered significant.

3. Results

3.1. Photofungicidal screening assay

Photofungicidal activity against four fungi was investigated by treating with a combination of phenolic acids and 405-nm light. In Fig. 1A, a fluence at 456 J/cm^2 (90-min exposure) without phenolic acid (control sample) resulted in about 1-log unit reduction in viable *A. niger* cell counts, compared with viability observed in the absence of irradiation. Viability similar to control levels was observed in the presence of 5.0 mM concentrations of CA, CaA, ChA and GA and 405-nm light irradiation. In contrast, strong synergistic photofungicidal activity was observed in samples treated with FA and VA in combination with irradiation, leading to reductions in cell counts of 2.4 and 2.6-logs, respectively.

For *C. cladosporioides*, 60-min irradiation (304 J/cm^2) alone (control sample) and in combination with 2.5 mM CA, CaA, ChA or GA did not significantly reduce viability compared with samples receiving phenolic acid treatment in the dark (Fig. 1B). In contrast, when FA and VA were combined with light exposure, although high cell counts were observed in the absence of irradiation (≈ 4.3-logs), viabilities were reduced by 1.6 and 3.3-logs, respectively, an effect that is clearly different from the other phenolic acids tested.

Synergistic reductions in *T. mentagrophytes* viability were observed in the presence of FA

and VA (2.5 mM) followed by 30-min irradiation (152 J/cm^2) (Fig. 1C). Notably, colony formation was not observed after irradiation in the presence of FA. Similarly, *C. albicans* treated with FA, ChA and VA (2.5 mM) and irradiated at a fluence of 203 J/cm^2 (40-min exposure) exhibited significantly decreased viability relative to irradiated controls (Fig. 1D). In the dark condition (non-irradiated), experiments for each fungus treated with phenolic acid showed no significant decreases in viable cell counts relative to the initial counts (Fig. S2).

In the presence of 10% (v/v) DMSO, the photofungicidal activity in *A. niger* conidia in the absence of phenolic acids and in the presence of FA and VA was attenuated (Fig. 2). Thus, the effect of adding FA and VA on photofungicidal activity was lost and viability was consistent with that of irradiation alone.

3.2. Adsorption of phenolic acids to A. niger conidia

Treatment of *A. niger* conidia with phenolic acids for 1 min resulted in decreased concentrations of the phenolic acids in the supernatant of conidia suspension; this suggests that the compounds adsorbed to the conidia. The molar amounts of FA and VA adsorbing to the conidia were 2.35×10^{-3} and 2.94×10^{-3} pmol per conidium, respectively, which is much greater than the observed adsorption of CaA and ChA (0.51×10^{-3} and 0.90×10^{-3} pmol per conidium, respectively) (Fig. 3). These data suggest that FA and VA are more easily adsorbed to the surface of conidia than CaA and ChA.

3.3. Analyses of intracellular ROS level and membrane permeability of A. niger conidia

The combination of 5.0 mM FA or VA and a 456-J/cm^2 fluence (90-min irradiation) elicited

significant photofungicidal activity against *A. niger* conidia compared to samples treated with CaA, ChA and light exposure alone and initial counts (Fig. 4A); FA and VA yielded 98% or 97% reduction in the viability, respectively. The relative level of intracellular ROS generated was estimated by flow cytometry analysis of conidia. As shown in Figs. 4B and S2, irradiating conidia for 90 min without phenolic acid (control) produced only a minimal (1.12-fold) increase in ROS levels compared to samples incubated for 90 min in the dark. In the presence of CaA or ChA, irradiation induced 1.34- or 1.48-fold increases in ROS levels; however, these values were not significantly greater than ROS levels observed in the presence of light exposure alone. In contrast, FA or VA in combination with irradiation increased ROS level by 1.55- or 1.85-fold, respectively, which was significantly different from samples treated with light exposure alone. Only significant differences between CaA and VA were observed among the various phenolic acid treatment groups. Moreover, incubation in the dark in the presence of phenolic acids did not produce changes in ROS levels, compared to the ROS levels observed in the absence of phenolic acids (Fig. S4).

Additionally, Fig. 4C shows that combination treatments with FA or CaA did not induce significant alterations in PI fluorescence levels (1.12- or 1.17-fold increase, respectively) compared to the initial sample (without incubation, irradiation and phenolic acid treatment).

3.4. Intracellular ATP content of A. niger conidia

Fig. 5A shows that treatment of *A. niger* conidia with a combination of 5.0 mM FA or CaA and 405-nm light exposure (304 J/cm^2) and irradiation alone induced negligible changes in viable cell counts compared to untreated cells. Fig. 5B shows that light exposure, FA and CaA decreased ATP content to 12.8 ± 1.4, 19.0 ± 1.5 or 15.1 ± 1.6 pM, respectively, whereas levels

of 26.3 ± 3.5 pM were observed in the control sample incubated for 60 min in the absence of irradiation and phenolic acid (control). Combining phototreatment with FA or CaA yielded much lower ATP content: 1.4 ± 0.2 pM for FA or 6.5 ± 2.3 pM for CaA. Notably, the greatest reduction in ATP (to ≈ 5% of control levels) was observed in the sample receiving a combination of irradiation and FA.

3.5. Effect of combination treatment on A. niger growth

No differences in the growth profile (germination and mycelial extension) were observed among samples treated with FA, CaA and control following incubation in the dark or treatment with irradiation alone (Figs. S5 A and B). When treatment of conidia with FA and CaA was combined with 405-nm light, the increases in turbidity were remarkably different from those observed in the dark condition, light exposure only and control. The result was that delayed growth was observed in the presence of FA and CaA with irradiation compared to all other samples. As shown in Table 1, phototreatment in the presence of phenolic acids significantly delayed growth time (h): 30.21 ± 1.15 for FA and 26.86 ± 0.70 for CaA. Moreover, FA with irradiation delayed growth by nearly four hours longer than the CaA with irradiated. In contrast, individual treatments scarcely affected growth compared to sample incubation alone (see Figs. S5 C and D).

3.6. Light-generated hydrogen peroxide

Generation of H_2O_2 was observed in all sample solutions that were irradiated at a fluence of 76 J/cm^2 (Fig. 6). Significantly, the concentration of H_2O_2 generated in samples containing

phenolic acids (5.0 mM) was higher than that of the sample receiving irradiation alone (control). Irradiation of the solution containing VA produced the highest H_2O_2 yield (272 μM). Furthermore, H_2O_2 concentrations were generally below the detection limit (0.4 μM) in non-irradiated samples, with the exception of CA (0.56 μM).

4. Discussion

The six phenolic acids tested in this study are classified as either cinnamic acid derivatives (CA, FA, CaA and ChA) or benzoic acid derivatives (VA and GA). de Oliveira et al. [21] reported that the synergistic bactericidal activity of phenolic acids with 365-nm irradiation requires the presence of propionic acid as a side chain, which stabilizes free radicals by resonance. In a photobactericidal screening test of phenolic acid derivatives, the cinnamic acid derivatives CA, FA and CaA exhibited the greatest synergistic activity with light irradiation against *E. coli* O157:H7; whereas the benzoic acid derivatives VA and GA showed no photobactericidal activity. Furthermore, low levels or the absence of activity might be attributable to increased dissociation energy followed by a reduced rate of free radical formation due to carboxylic group substitution on the phenolic ring [32]. However, our present results indicated that the combination of FA and VA with 405-nm light clearly produced synergistic activity against all four fungi tested. This represents an improvement over antifungal techniques using violet light. Surprisingly, VA showed synergistic effects against *A. niger* and *C. albicans* that are comparable to FA under the same experimental condition. Synergistic effects might be dependent on a compound's ability to scavenge free radicals, which was enhanced by substitution of a methoxy group at the meta-position in cinnamic and benzoic acids [32]. In contrast, synergistic effects were scarcely observed with combinations of the cinnamic acid

derivatives CA or CaA, despite CaA exhibiting a higher scavenging activity than FA and VA [32]. Moreover, it was observed that the synergistic fungicidal characteristics of phenolic acids were different from their characteristics against bacteria described in previous studies [16,21].

The photobactericidal activity of phenolic acids is suggested to involve the oxidative action of ROS such as H_2O_2 and hydroxyl radicals [16-18]. A reaction between phenolic hydroxyl groups present on the compounds and light would lead to the transfer of a proton and electron from the hydroxyl group to dissolved oxygen and result in the generation of H_2O_2 followed by photolysis. These ROS can be detected with colorimetric and electron spin resonance spectrometry analyses in CaA, ChA and GA solutions following irradiation with 400-nm light over wavelengths from 380 to 420 nm [16]. Similar to the previous results, this study observed the generation of H_2O_2 and hydroxyl radicals in phenolic acid solutions following 405-nm irradiation, clearly indicating phenolic acid-mediated ROS production (Fig. S6). The concentration of H_2O_2 in the VA solution was markedly higher than in the solutions with the other compounds. The ability to generate ROS could be subject to an elimination reaction between the carboxyl group that is dependent on strong intermolecular H-bonds [33]. Hydroxyl radical is one of the primary agents capable of causing oxidative damage to bacterial cells [18]. The highly oxidative reaction is presumed to occur at the surface or the interior of bacterial cells because of its short half-life (estimated to be around 10^{-9} sec) and very small diffusion distance (estimated to be around 6 nm) [34,35]. As a result of radical generation, oxidative damage to the cytoplasmic membrane of *E. coli* cells is facilitated in the presence of FA upon UV-A irradiation [20]. Inactivation of *A. niger* conidia treated with FA or VA under irradiation was diminished dramatically in 10% (v/v) DMSO, which is well-known as a scavenger of hydroxyl radicals. Our results suggested that ROS are the primary mediators of the synergistic photofungicidal activity observed with our combination procedure.

Considering the role of hydroxyl radicals in fungicidal activity, the adsorption of phenolic acid and permeation across the cell wall are important factors due to the rapid reactivity of the radicals. Differences in the adsorption to *A. niger* conidia (1-min exposure) were observed among the tested compounds. FA and VA possessed suitable adsorption characteristics with conidia for all four fungi tested, yielding high photofungicidal activity against a wide variety of fungal species. Some published reports have proposed mechanisms for the antimicrobial activity of phenolic acids on bacterial membranes; undissociated phenolic acids cross the membrane by passive diffusion dependent on their lipophilicity, resulting in disturbance of the membrane structure, acidification of cytoplasm and protein denaturation [36,37]. A prior screening study showed that cinnamic acid derivatives increased efflux of intracellular ions such as potassium and phosphate more than benzoic acid derivatives, implying a superior effect on permeability across or association with the bacterial membrane [37]. Five of the phenolic acids tested in this study, excluding ChA, possess similar p*K*a values that are in the range of 4.34 to 4.57 [36]. In the case of fungi, the present study suggested that phenolic acid adsorption to conidia or permeability in conidia is affected by molecular lipophilicity, which is dependent on the substituents on the benzene ring rather than their basic structure (cinnamic or benzoic acid derivatives). The lipophilicity for FA and VA, which are substituted with a methoxy group at the 3-position on the benzene ring, would be suitable for adsorption to conidia and passive diffusion through the conidial cell wall.

The antimicrobial mechanism of light-activated curcumin involves increases in intracellular ROS levels and plays an important role in decreasing the viability of bacteria and fungus conidia [14,38]. When a significant decline in *A. niger* viability was observed in the presence of a combination of FA or VA and irradiation, intracellular ROS levels were observed to be increased, suggesting that high levels of ROS production following irradiation contributes to the

fungicidal effect in the presence of phenolic acids. In contrast, under dark conditions no decrease in viability and no change in ROS levels were observed. Moreover, major differences in ROS levels between the tested compounds were not observed. Because ROS probe fluorescence was determined after the end of light exposure, ROS remaining in conidia may be diminished by intracellular defenses against oxidative damage.

In an investigation of the action of photo-activated curcumin on *Botrytis* conidia, more than 50% of PI-stained the cells showed the loss of cell membrane integrity under conditions producing a 96% reduction in viability, as well as increases in intracellular ROS [14]. However, we assumed that the photofungicidal action of phenolic acids mainly involved intracellular effects of phenolic acids following permeation of the cell wall and the cytoplasmic membrane. This hypothesis is based on the observation that PI staining did not reveal damage to membrane permeability, even under conditions producing a 98% reduction in *A. niger* viability. The relationship between membrane integrity and cell viability has been also confirmed by microscopy analysis of *Penicillium* sp. conidia [28]. Subjecting the conidia suspension to exogenous H_2O_2 at a concentration of 30 mM resulted in 50-60% decrease in viability, despite the maintenance of membrane integrity (impermeable to PI) in > 99% of the conidia. In our study, the concentrations of H_2O_2 generated by 405-nm irradiation of dissolved phenolic acids were detected as 272 μM or less; notably, the concentration generated in the presence of FA was only 2.7 μM. It is unlikely that the exogenous ROS generated under these conditions causes disruption of the cell wall and cytoplasmic membrane. It has been reported that treatment of *Fusarium* sp. conidia with ChA in the dark increased membrane permeability and induced cell lysis; however, this treatment was conducted with a very high concentration at 14.1 mM [39]. At the concentrations of phenolic acids used in the present experiments (up to 5.0 mM), the viability of *A. niger* was not appreciably suppressed.

Curcumin was reported to decrease mitochondrial membrane potential and elevate intracellular ROS in *Botrytis* conidia, despite experiments being conducted under dark conditions [14]. Qin et al. revealed that treatment of *Penicillium* sp. conidia with H_2O_2 decreased complex III protein (associated with the respiratory chain) localization to mitochondria, and that the consequent ROS accumulation induced oxidation of mitochondria proteins, leading to collapse of the membrane potential [28]. In addition, disruption of membrane potential homeostasis decreased intracellular ATP content. In our study, the ATP content in *A. niger* conidia after photocombination with FA decreased to approximately 5% of control levels, despite no significant reduction in viability. ATP is a vital molecule required for many biological functions, including survival, growth and replication [40]. To verify the effect of reduced ATP levels on biological functions, the growth of conidia was evaluated. The greatest reduction in conidia growth was observed with the combination FA and 405-nm light. These results indicate that internally generated ROS resulting from the photoreaction of phenolic acids could trigger growth delay of conidia. It is suggested that oxidative damage to the mitochondria or cytosolic proteins that function in ATP generation occurring before dysregulation of membrane permeability is the first stage in the fungicidal mechanism, which is a consequence of phenolic acids adsorbing on the conidia surface and permeating across the cytoplasmic membrane. Subsequently, damage to the cell wall, cytoplasmic membrane, and organelles accounts for great reduction in viability, events that are thought to be produced by the accumulation of intracellular ROS. This is supported by the observation that the photofungicidal action of curcumin is accompanied by loss of membrane integrity when fungal viability is reduced [14].

In future studies, it is necessary to assess the localization of oxidative stress induced by combinations of phenolic acids and light exposure with respect to subcellular organelles, which

can be evaluated using co-localization of fluorescent staining. Moreover, data from practical applications would advance photo-disinfection technology by demonstrating the utility of food-grade phenolic acids activated with violet 405-nm light in the fields of agriculture, food processing and medical care.

5. Conclusion

In conclusion, the results of this study demonstrated the photofungicidal synergy with FA or VA and violet 405-nm light against four fungi. Moreover, the photofungicidal mechanism was tested by investigating various aspects of cellular function and chemical characteristics. These data demonstrated that 405-nm disinfection technology in combination with phenolic acids is effective for disinfection of pathogenic and spoilage fungi in medical applications and food industries. The fungicidal mechanism was associated with the formation of H_2O_2 and hydroxyl radicals following 405-nm radiation of the phenolic acid solutions. We found that intracellular ROS levels increased upon irradiation of phenolic acids, and that the increased ROS levels correspond to significantly reduced viability, but not changes in membrane permeability. Furthermore, ATP content was remarkably decreased, even under conditions that do not reduce viability, which is associated with vastly reduced growth. These results suggest that the first stage in the photofungicidal mechanism is oxidative damage to mitochondria or the cellular catabolism system associated with ATP synthesis, which is a result of the photoreaction of phenolic acids adsorbed and internalized by conidia.

Author contributions

A. Shirai designed the study and the three authors including K. Kawasaka and K. Tsuchiya collected the data; A. Shirai wrote the manuscript. All authors have approved the manuscript.

Declaration of competing interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

Acknowledgments

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Appendix A. Supplementary material

The following is the Supplementary data to this article:

Figures S1 to S6 are provided

Fig. S1. Chemical structures of tested phenolic acids.

Fig. S2. Antifungal activity of phenolic acids against four fungal species; (A) *A. niger*, (B) *C. cladosporioides*, (C) *T. mentagrophytes* and (D) *C. albicans*. Each cell suspension (initial density, 5×10^4 conidia/mL for conidia or 2×10^5 CFU/mL for yeast) was incubated in the dark for (A) 90 min, (B) 60 min, (C) 30 min and (D) 40 min in the presence of phenolic acids at concentrations of 5.0 mM (A) and 2.5 mM (B-D). Shaded and open bars indicate viable cell

counts of each sample before and after incubation, respectively. Data are presented as means ± SD (n=3). Significant differences were calculated based on comparison of pre- and post-incubation viabilities (two-tailed, unpaired *t*-test). Cont., sample without phenolic acid; ns, not significant.

Fig. S3. Dual-parameter dot plots of dichlorofluorescein (DCF)-stained conidia evaluated by flow cytometry after incubation for 90 min in the dark (A) or with light exposure at 456 J/cm^2 (B) in the absence or presence of 5.0 mM phenolic acids. The lower panels are histograms showing the distribution of DCF fluorescence intensity for conidia subjected to each of the treatments (C): dotted line, dark condition; solid line, with light exposure.

Fig. S4. Changes in intracellular ROS fluorescence levels for each treatment following incubation for 90 min in the dark. The fluorescence levels are expressed as arbitrary units (a.u.) of mean fluorescence relative to the fluorescence values of samples without phenolic acid. Data are presented as means ± SD (n=3). Different letters above the columns refer to significant differences between different groups ($P < 0.05$; one-way ANOVA). Cont., sample without phenolic acid.

Fig. S5. Effect of the combination of phenolic acids and 405-nm light on *A. niger* growth curves. Growth in each sample was monitored by measuring optical density at 620 nm (OD_{620}) in panels A and B. Since the OD_{620} values were greater than 0.001, the values were plotted on a logarithm scale in panels C and D. The graphs show growth curves for each sample in the absence (A and C) or presence (B and D) of light exposure. Lines in all figures indicate samples without phenolic acid (solid lines), with FA (dashed dotted lines) and CaA (dotted lines). Values are

presented as means (n=3).

Fig. S6. Formation of DMPO/•OH adduct in solutions containing phenolic acids before and after irradiation. ESR spectra of mixtures of 4.5 mM FA, CaA or VA and 450 mM DMPO were recorded before and after an irradiation with 405-nm light for 1 min (5.1 J/cm^2). The control sample was the 450 mM DMPO solution prepared with pure water alone. Each figure shows the ESR spectrum for pure water (A and E), FA (B and F), CaA (C and G) and VA (D and H) before (A-D) and after irradiation (E-H). Values in the figure indicate signal intensity of the second line of the DMPO/•OH adduct.

Research data

The following is the Research data S1 to this article:

Detection of light-generated hydroxyl radicals

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Figure captions

Fig. 1. Photofungicidal activity of phenolic acids with 405-nm light irradiation (open bars) against four fungal species: (A) *A. niger*, (B) *C. cladosporioides*, (C) *T. mentagrophytes* and (D) *C. albicans*. Each cell suspension (initial density, 5×10^4 conidia/mL for conidia or 2×10^5 CFU/mL for yeast) was irradiated at fluences of (A) 456 J/cm^2 (for 90 min), (B) 304 J/cm^2 (for 60 min), (C) 152 J/cm^2 (for 30 min) and (D) 203 J/cm^2 (for 40 min) in the presence of phenolic acids at concentrations of 5.0 mM (A) or 2.5 mM (B-D). Shaded bars indicate viable cell counts of each sample incubated under a dark condition corresponding to each irradiation time. Data are presented as means ± SD (n=3). Significant differences (*$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$; two-tailed, unpaired *t*-test) were calculated based on comparisons of viability between the dark and irradiation conditions. Different letters above the columns refer to significant differences between different groups in irradiated samples ($P < 0.05$; one-way ANOVA). Cont., sample without phenolic acid; ns, not significant.

Fig. 2. Effect of DMSO on fungicidal activity of photoirradiated FA and VA against *A. niger*

conidia. Light exposure at a fluence of 456 J/cm^2 (for 90 min) was performed after addition of phenolic acids at a concentration of 5.0 mM followed by addition of DMSO to conidia suspension (initial density, 5×10^4 conidia/mL). Data are presented as means ± SD (n=3). Different letters above the columns refer to significant differences between different groups in samples treated with the same DMSO concentration ($P < 0.01$; one-way ANOVA). **$P < 0.01$ and ***$P < 0.001$; two-tailed, unpaired *t*-test. Cont., sample without phenolic acid.

Fig. 3. Amount of phenolic acid adsorption to *A. niger* conidia. Approximately 2×10^8 conidia/mL was treated with FA, CaA, ChA or VA at a concentration of 2.5 mM for 1 min. Data are presented as means ± SD (n=3). Different letters above the columns refer to significant differences between different groups ($P < 0.05$; one-way ANOVA).

Fig. 4. Changes in ROS levels and PI permeability in *A. niger* conidia after treatment with phenolic acids and 405-nm irradiation. Figs. A, B and C show viable cell counts of *A. niger* (initial density, 2×10^5 conidia/mL), the intracellular ROS levels (fluorescence levels) and membrane permeability (PI fluorescence) after phototreatment, respectively. The ROS levels were expressed as arbitrary units (a.u.) of mean fluorescence relative to the fluorescence levels of samples incubated for 90 min in the dark. PI fluorescence levels (a.u.) for the combination treatments were compared to the values obtained from initial sample (without incubation, exposure, and phenolic acid). Data are presented as means ± SD (n=3). Different letters above the columns refer to significant differences between different groups ($P < 0.01$ in Fig. A and $P < 0.05$ in Fig. B; one-way ANOVA). Cont., sample without phenolic acid; nt, not tested.

Fig. 5. Effect of combinations of phenolic acids and 405-nm light irradiation on ATP content in

A. niger conidia. Viable cell counts after each treatment are shown in panel A. ATP contents correlating to each treatment are shown in panel B. Shaded bars, 60-min incubation in the dark; open bars, 60-min light exposure (304 J/cm^2). Data are presented as means ± SD (n=3 for Fig. A or n=4 for Fig. B). Different letters above the columns refer to significant differences between different groups ($P < 0.05$; one-way ANOVA). Cont., sample without phenolic acid; nt, not tested.

Fig. 6. Concentrations of H_2O_2 generated in each phenolic acid solution with or without 405-nm light irradiation. Left (shaded) and right (open) bars for each treatment indicate H_2O_2 concentration after incubation in the dark and irradiation (76 J/cm^2), respectively. Data are presented as means ± SD (n=3). Significant differences (***$P < 0.001$; two-tailed, unpaired *t*-test) were calculated based on comparison of irradiated control and phenolic acid samples. Cont., sample without phenolic acid; nd, not detected.

Table 1. Effect of treatment with phenolic acid and 405-nm light irradiation on time required to reach 0.04 OD_{620}.

Incubation time (h)*					
Incubation alone	Light exposure alone	FA alone	FA + light exposure	CaA alone	CaA + light exposure
21.53 ± 0.89 [a]	23.40 ± 1.42 [a]	23.02 ± 0.56 [a]	30.21 ± 1.15 [b]	22.55 ± 0.48 [a]	26.85 ± 0.70 [c]

* Incubation time was derived from the regression line during the logarithmic growth phase for each sample, and is indicated as time required to reach 0.04 OD_{620} (see Fig. S5). Data are presented as means ± SD (n=3). Different letters refer to significant differences between different groups ($P < 0.01$, $P < 0.05$ only between a and c; one-way ANOVA).

Author statement

Akihiro Shirai: Conceptualization, Methodology, Investigation, Writing - Original Draft, Writing - Review & Editing, Visualization, Supervision, Funding acquisition.

Kaito Kawasaka: Methodology, Investigation.

Koichiro Tsuchiya: Methodology, Investigation, Writing - Original Draft.

Title

Antimicrobial action of phenolic acids combined with violet 405-nm light for disinfecting pathogenic and spoilage fungi

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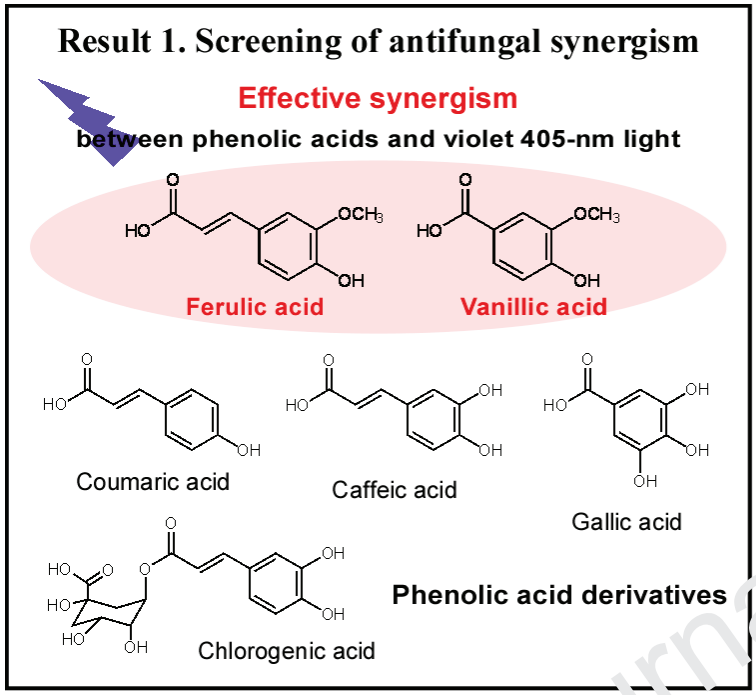
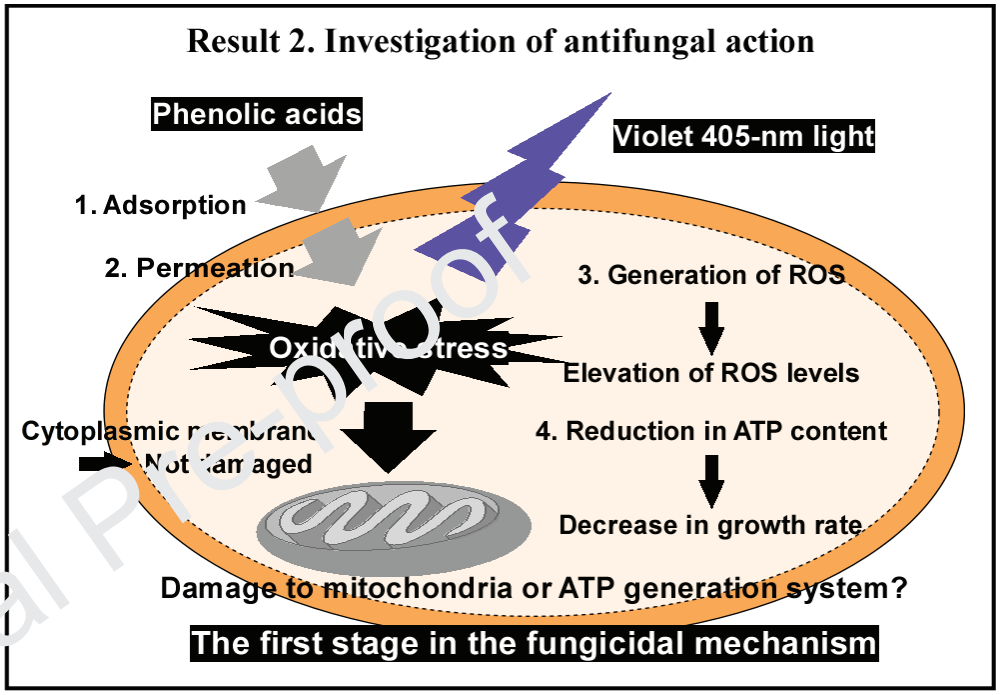
Declaration of Competing Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.





Highlights (85 characters, including spaces, per bullet point; 3 to 5 bullet points)

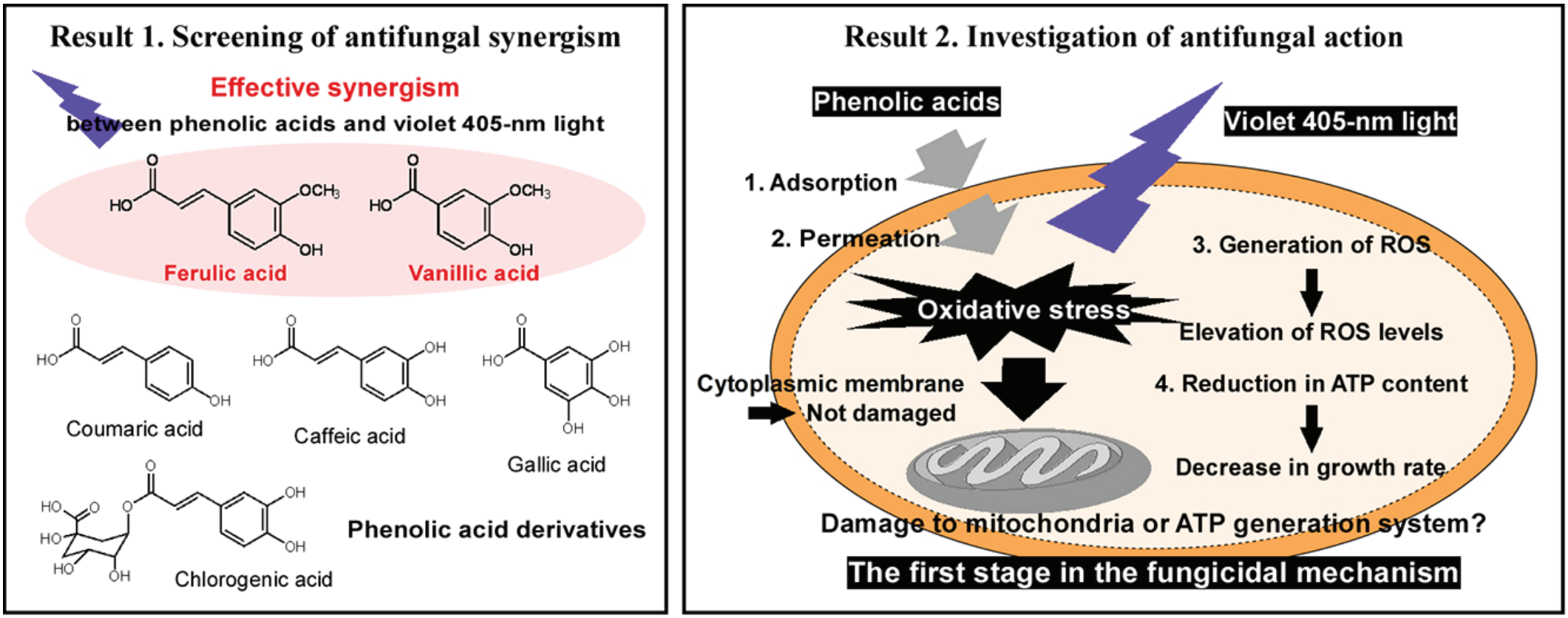
► Phenolic acids were screened for antifungal synergism with violet 405-nm light.

► Fungicidal action of combined phenolic acids with 405-nm light was investigated.

► The combination caused overproduction of reactive oxygen species in conidia.

► The combination produced no change in conidia membrane integrity.

► Intracellular ATP content and conidia growth rate were decreased by the combination.



Graphics Abstract

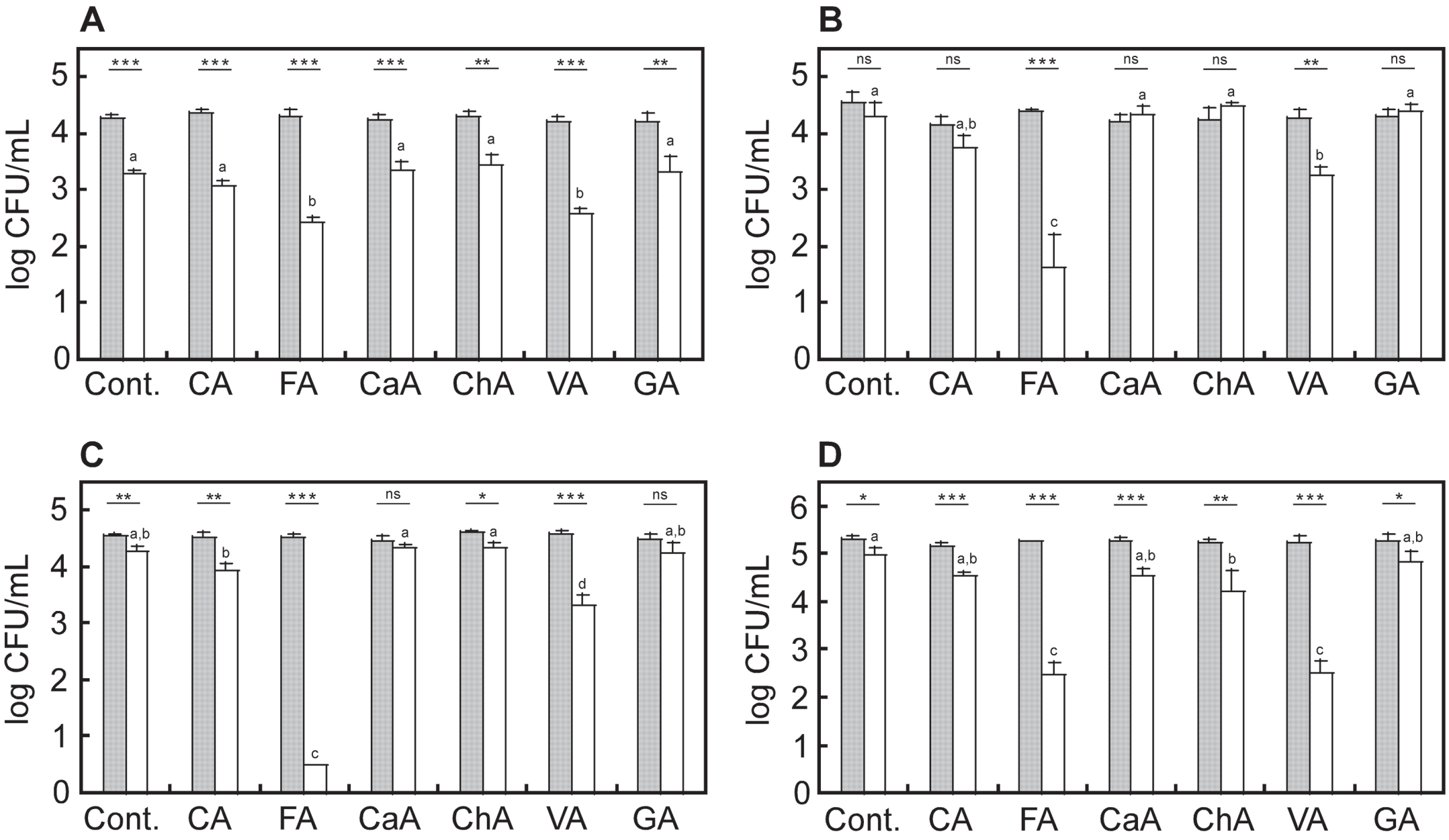


Figure 1

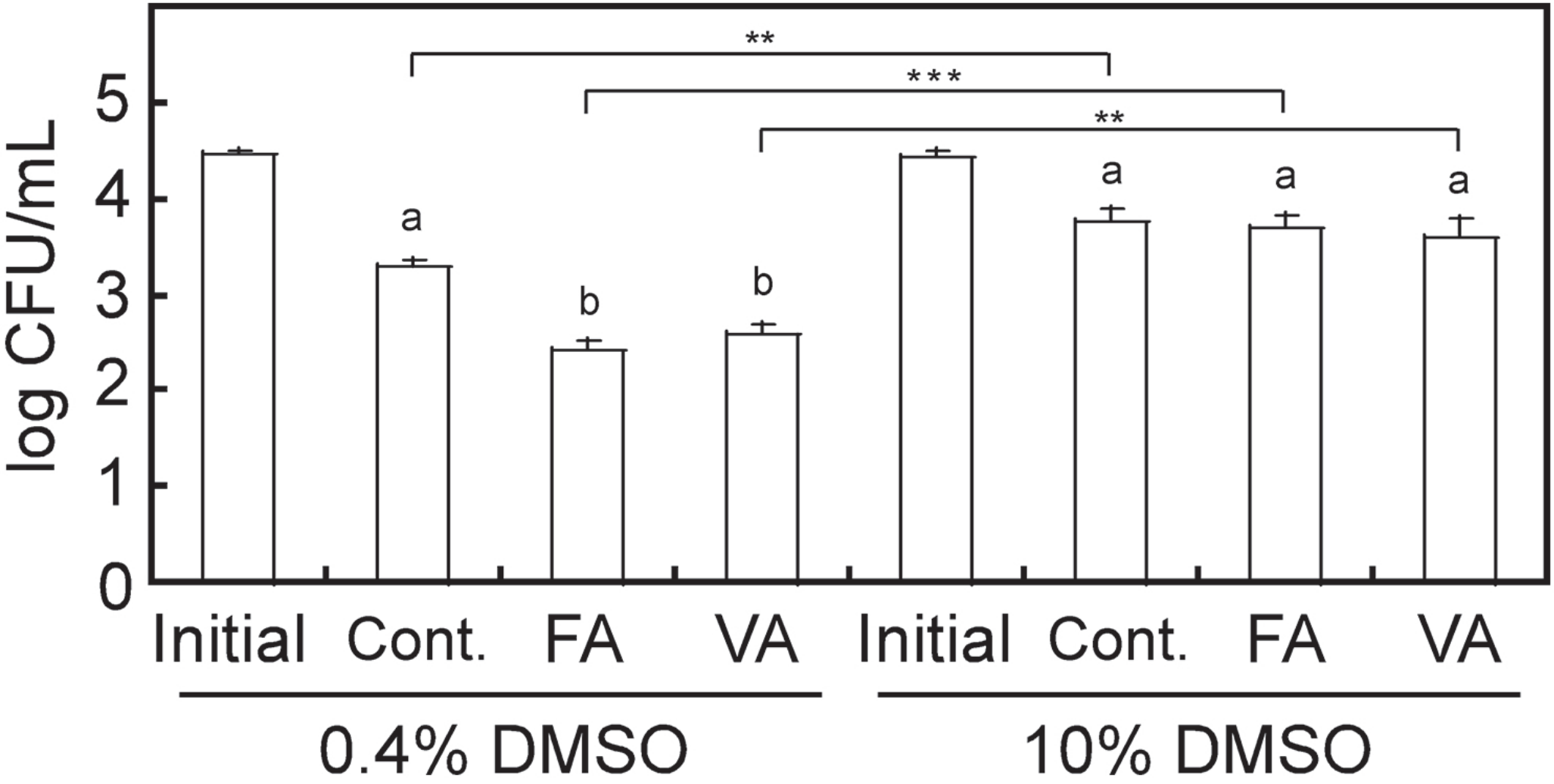

log CFU/mL
0
1
2
3
4
5
**
***
**
a
b
b
a
a
a
Initial
Cont.
FA
VA
Initial
Cont.
FA
VA
0.4% DMSO
10% DMSO

Figure 2

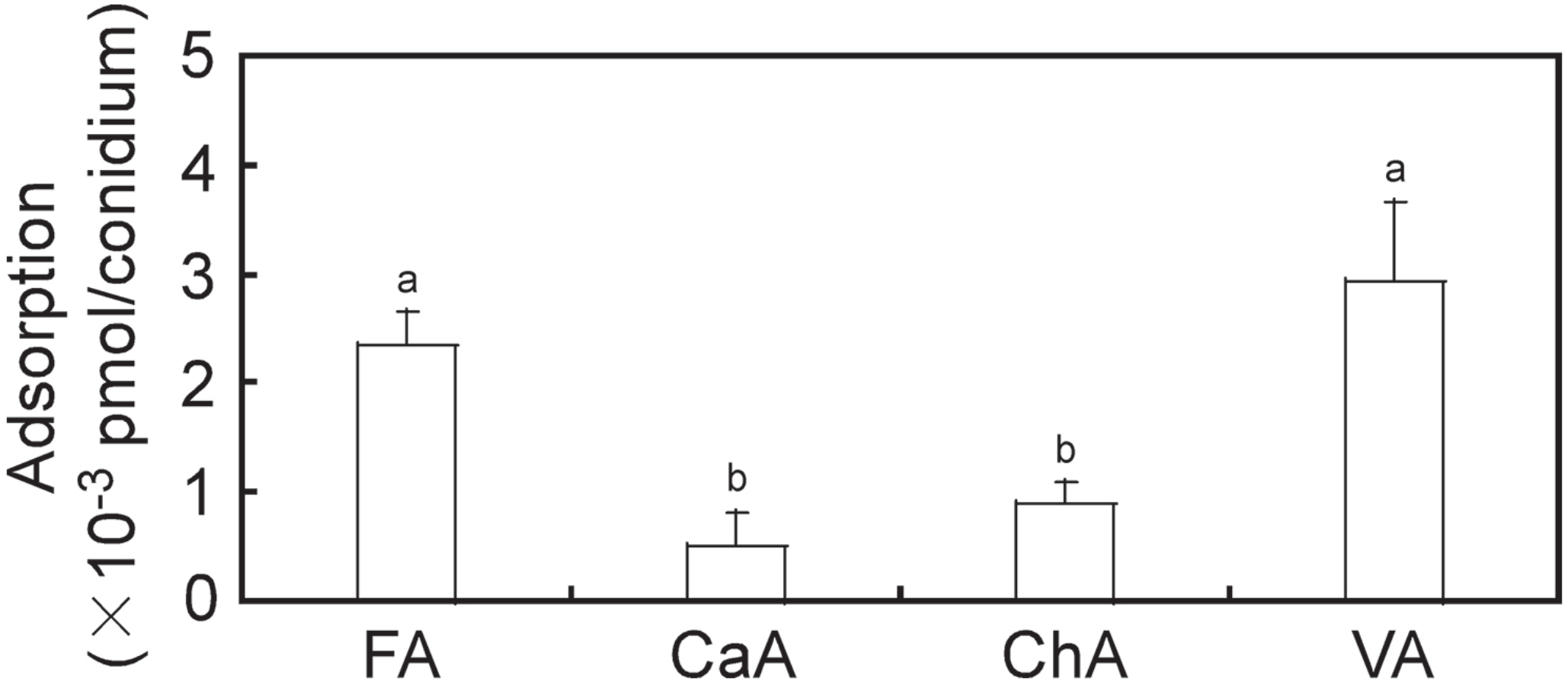


Figure 3

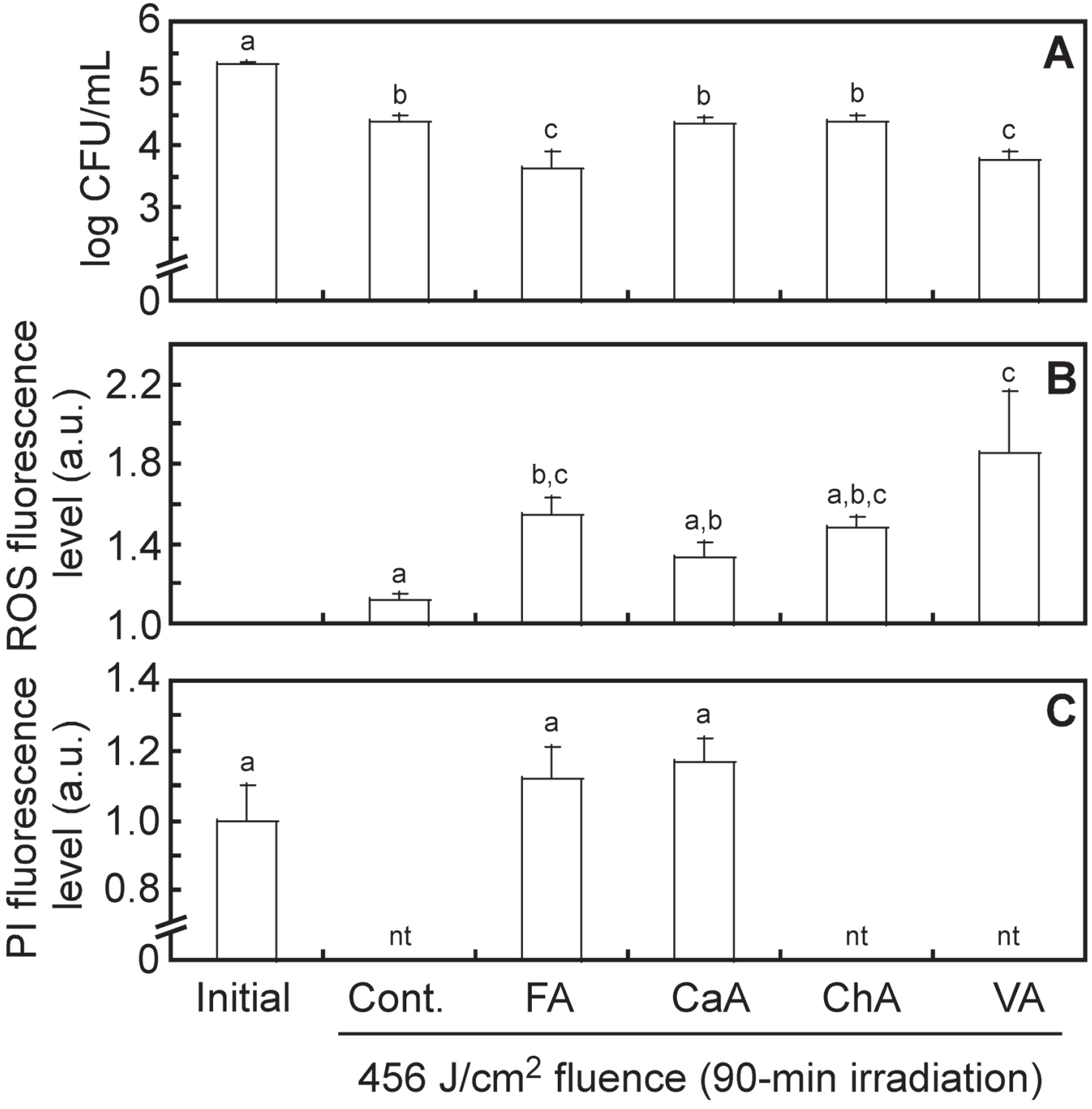

A
log CFU/mL
6
5
4
3
0
a
b
c
b
b
c
B
ROS fluorescence level (a.u.)
2.2
1.8
1.4
1.0
a
b,c
a,b
a,b,c
c
C
PI fluorescence level (a.u.)
1.4
1.2
1.0
0.8
0
a
nt
a
a
nt
nt
Initial
Cont.
FA
CaA
ChA
VA
456 J/cm2 fluence (90-min irradiation)

Figure 4

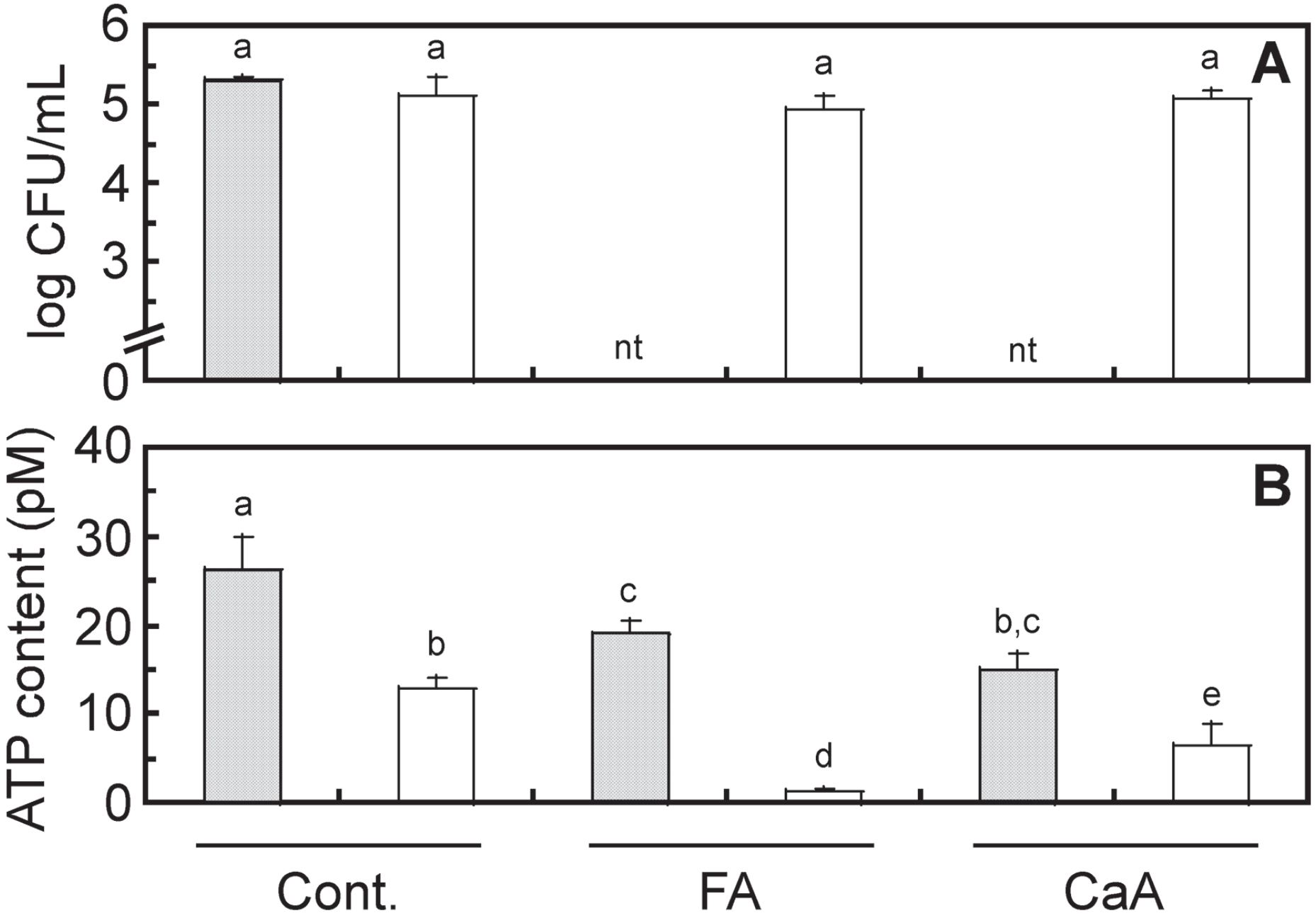

A
log CFU/mL
6
5
4
3
0
a
a
nt
a
nt
a
B
ATP content (pM)
40
30
20
10
0
a
b
c
d
b,c
e
Cont.
FA
CaA

Figure 5

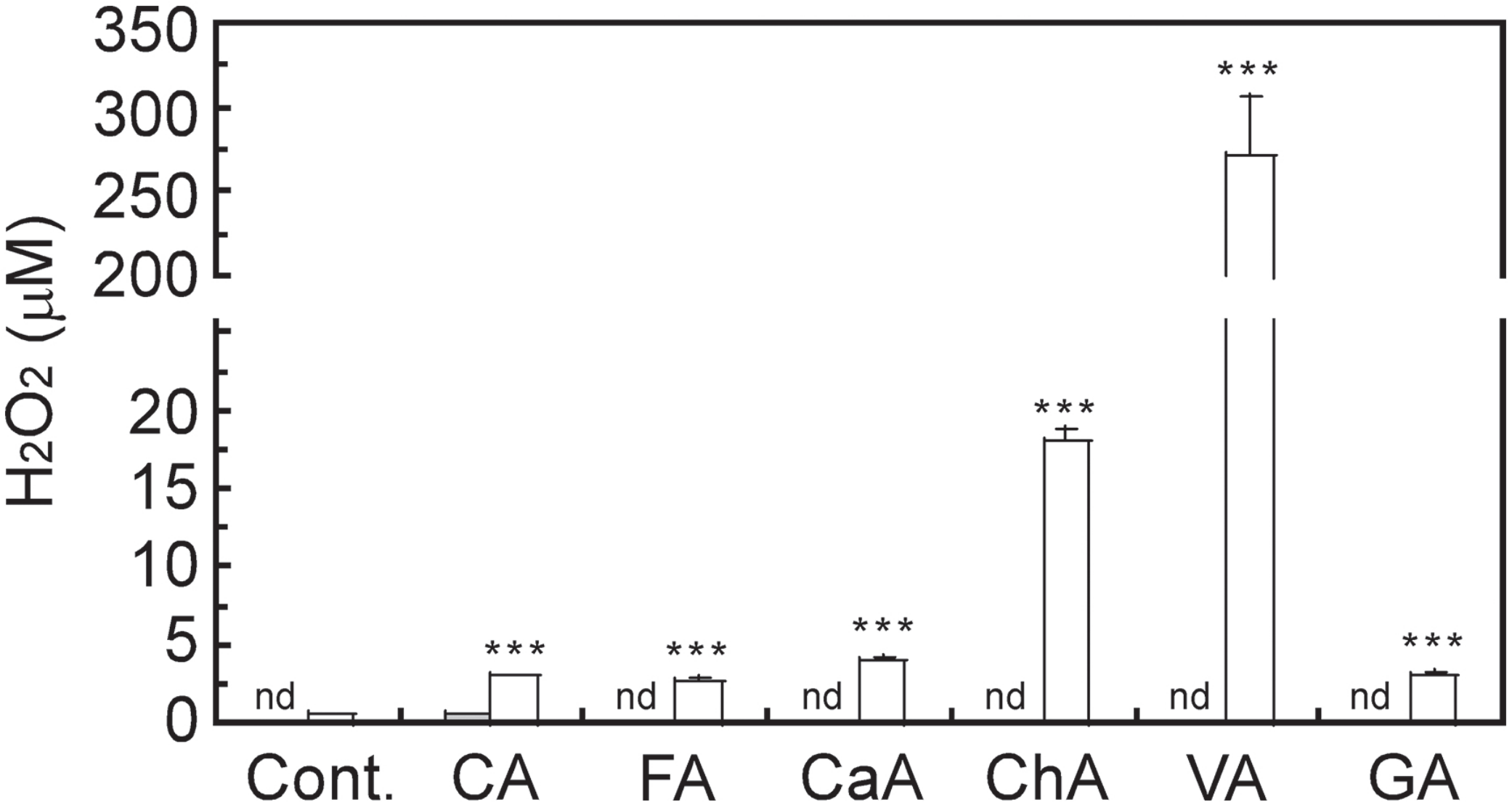

350
300
250
200
20
15
10
5
0
H2O2 (μM)
nd
***
nd
***
nd
***
nd
***
nd
***
nd
***
Cont.
CA
FA
CaA
ChA
VA
GA

Figure 6