Journal of Bioscience and Bioengineering
VOL. 128 No. 1, 28–32, 2019

www.elsevier.com/locate/jbiosc

Ferrous chloride and ferrous sulfate improve the fungicidal efficacy of cold atmospheric argon plasma on melanized *Aureobasidium pullulans*

Shizu Fukuda,[1] Yuko Kawasaki,[1] and Shingo Izawa[2,*]

Department of Food Science and Nutrition, Graduate School of Human Life Science, Doshisha Women's College of Liberal Arts, Teramachi Imadegawa, Kyoto 602-0893, Japan[1] *and Department of Applied Biology, Graduate School of Science and Technology, Kyoto Institute of Technology, Matsugasaki Hashiuecho 1, Kyoto 606-8585, Japan*[2]

Received 31 July 2018; accepted 17 December 2018
Available online 22 January 2019

Since cold atmospheric pressure plasma (CAP) has not only bactericidal activity but also fungicidal activity without toxic residues and thermal damage, it is considered as an alternative method for sterilization of fungi on the surfaces of perishable foodstuffs and human bodies. *Aureobasidium pullulans* is a ubiquitous yeast-like fungus and called black yeast because it produces melanin, a dark biological pigment. It is well known that various melanized fungi show hyper-resistance to extreme stress conditions including high levels of radioactivity. Curiously, however, there is very little information about the fungicidal effects of CAP on melanized fungi. Therefore, we herein investigated the effects of CAP on *A. pullulans*, using cold atmospheric argon plasma (Ar plasma). We found that ammonium sulfate repressed the synthesis of melanin in *A. pullulans* as well as *Aureobasidium melanogenum*. Although the non-melanized *A. pullulans* cells were efficiently killed by the exposure of Ar plasma, the melanized cells showed the significant resistance to Ar plasma as well as to hydrogen peroxide and thermal stress. In order to improve the fungicidal efficacy of Ar plasma, we examined the combination of Ar plasma and Fenton reaction. We realized that $FeCl_2$ and $FeSO_4$ significantly improved the sterilization efficacy of Ar plasma on the melanized *A. pullulans*.

[**Key words:** *Aureobasidium pullulans*; Melanin; Cold atmospheric pressure plasma; Ar plasma; Fenton reaction]

Aureobasidium pullulans is a dematiaceous yeast-like fungus distributed widely throughout the environments including phylloplane of plants and human bodies (1–4). Although *A. pullulans* is biotechnologically important (3,5), its infection often causes various human diseases (4,6–11). Therefore, development of adequate fungicidal methods against *A. pullulans* is required.

As well as other dematiaceous fungi, *A. pullulans* produces melanin within the cell wall at the later stage of culture, although its exact mechanism of melanin synthesis remains to be clarified (3,8). Melanin is not essential for fungal growth but it has various biological functions including the scavenging activity of free radicals and antiradiation activity (12–14). Therefore, melanin provides dematiaceous fungi with tolerance to extreme environments, and various melanized fungal species are known to have resistance to very high levels of radioactivity (9,14–18). Additionally, fungal melanin often functions as a virulence factor and protects the fungus against the immune mechanisms of host cells (14,19,20).

Cold atmospheric pressure plasma (CAP) can be generated by dielectric barrier discharge, emitting UV photons and reactive species, and induces generation of various reactive oxygen species (ROS) such as hydroxyl radicals and hydrogen peroxide in the presence of moisture (21–25). Therefore, CAP shows effective bactericidal and fungicidal activities and has been recently utilized as a new sterilization tool in various fields including sanitation, medicine, and food processing (25–28). As a sterilization technique, exposure of CAP is advantageous because of the low-associated costs and minimal generation of thermal damage and residual toxicity (27,29). Hence, the CAP exposure is expected as a practical sterilization technique against *A. pullulans*.

In the presence of peroxide, transition-metal ions such as iron and copper cause Fenton reaction that generates highly toxic hydroxyl radicals (30). Fenton reaction and its modified reactions have been widely used for the degradation of a variety of refractory organics (31–33). Since CAP causes generation of hydrogen peroxide, it is expected that CAP exposure in the presence of transition-metal ions can induce Fenton reaction and, thus, oxidizing power of CAP could be enhanced. Indeed, Ravindran et al. (33) reported that iron chloride assisted the delignification efficiency of lignocellulosic biomass by CAP.

Curiously, there is almost no information about the fungicidal effect of CAP on the melanized fungi. Therefore, we herein examined susceptibility of melanized *A. pullulans* to cold atmospheric argon plasma (Ar plasma). Although the fungicidal efficacy of Ar plasma was insufficient against the melanized cells, it was improved by $FeCl_2$ and $FeSO_4$, suggesting that combination of CAP and Fenton reaction could be an effective sterilization method against dematiaceous fungi.

* Corresponding author. Tel./fax: +81 75 724 7764.
E-mail address: thioredoxin@kit.ac.jp (S. Izawa).

1389-1723/$ – see front matter
https://doi.org/10.1016/j.jbiosc.2018.12.008

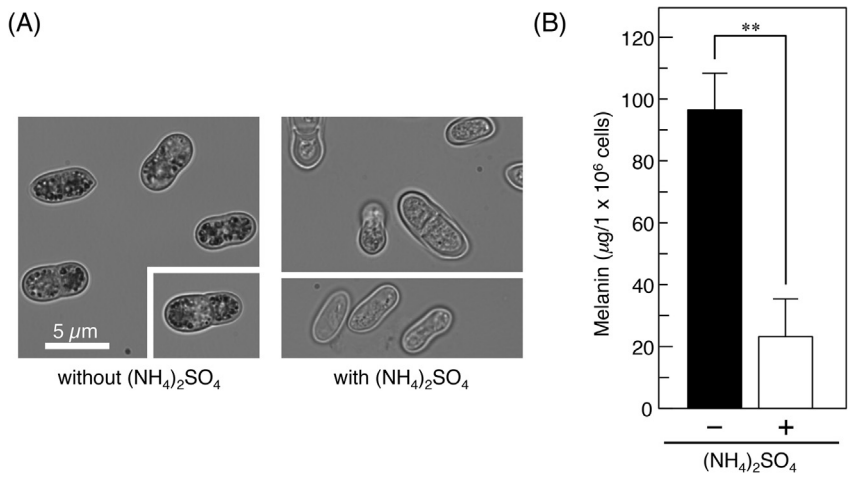

FIG. 1. Cells were cultivated in PDB liquid medium with or without 0.5% $(NH_4)_2SO_4$ at 25 °C for 144 h. (A) Representative microscopic images of *A. pulullans*. The bar represents 5 μm. (B) Cellular melanin levels. Data are expressed as means ± SD ($n = 3$). $^{**}P < 0.01$.

MATERIALS AND METHODS

Strain and medium A dematiaceous fungi *A. pullulans* NBRC 6353 was obtained from National Institute of Technology and Evaluation (NITE), Japan. Potato dextrose broth (PDB) medium (2.4% w/v potato dextrose broth, pH 5.2) was used for cell cultivation. PDB was purchased from Becton, Dickinson and Company (Sparks, MD, USA). *A. pullulans* cells from PDB agar plates were inoculated in 5 ml PDB liquid medium to prepare the seed culture. After 72 h cultivation at 25 °C, cells from the seed culture were diluted in 30 ml of PDB liquid medium and cultivated for 144 h (6 days) at 25 °C with reciprocal shaking (125 rpm) in Erlenmeyer flasks. The initial cell density was adjusted 2×10^6 cells/ml. In order to repress the synthesis of melanin, PDB medium was supplemented with $(NH_4)_2SO_4$ (0.5% w/v as the final concentration).

The CAP system We used the identical CAP system previously described (34). Briefly, the outside of the quartz tube (the outer diameter of 10 mm and inner diameter of 8 mm) was surrounded by an aluminum sheet, and a cylindrical-shaped wire mesh of stainless steel was contacted on the inside wall of the tube. In order to cause a barrier discharge using a dielectric of the quartz, a high voltage of 9.3 kV was applied at 11 kHz between the aluminum sheet and wire mesh. Since argon (Ar) gas is widely used as the feed gas in the CAP system due to its low ionization energy and economic efficiency (26,34,35), we also used Ar gas as the feed gas, which was introduced into the tube at a flow rate of 0.8 standard liters per minute (SLM). The distance between the edge of the radiation port and surface of the plate was adjusted to 3.0 cm. The CAP treatment was carried out in a room at 25 °C. The temperatures of Ar plasma plume (24.5 ± 0.5 °C) and Ar gas alone (24.0 ± 0.5 °C) were measured using a bar mercury thermometer (Toa Keiki Mfg. Co., Ltd., Tokyo, Japan). The sample temperature hardly changed before and after the exposure of Ar plasma.

Stress treatment For the stress treatment, cells were collected by centrifugation and diluted using 100 mM sodium acetate buffer (pH 5.2) to 1.0×10^6 cells/ml. In order to assess the survival of *A. pullulans* under Ar-plasma stress, approximately 500 cells were spread on a PDB agar plate (90 mm in diameter, Sansei Medical Co. Ltd., Kyoto, Japan), and then exposed to Ar plasma on the surface of the plate. To induce the Fenton reaction, cells were diluted in 100 mM sodium acetate buffer (pH 5.2) containing various concentrations of $FeCl_2$ or $FeSO_4$. The relative survival rate was calculated as colony-forming units (CFUs). In order to assess the susceptibility to H_2O_2 and heat shock, *A. pullulans* cells diluted in 100 mM potassium phosphate buffer (pH 6.8) were treated with H_2O_2 (80 mM and 100 mM) or heat shock at 46 °C for 60 min. After the stress treatment, cells were immediately plated on YPD agar plates (2.0% glucose, 2% peptone, 1% yeast extract, and 2% agar, pH 5.5) to calculate CFUs. The survival of cells without the plasma treatment or stress treatment was taken as 100 %.

Melanin assay and microscopic analysis Cellular melanin levels were measured by the method of Fernandes et al. (36). Melanin and other reagents were purchased from Wako Pure Chemicals (Osaka, Japan). An Olympus microscope IX83 system (Olympus, Tokyo) was used for microscopic analysis.

Statistical analysis Data are presented as means ± SD. The significance of differences was assessed using the paired *t*-test. Compared results were considered to be significantly different when the $P < 0.05$.

RESULTS

Supplementation of $(NH_4)_2SO_4$ blocked the synthesis of melanin in *A. pullulans* Recently, it has been reported that supplementation of 2.0% w/v $(NH_4)_2SO_4$ in the medium blocks the biosynthesis of melanin in *Aureobasidium melanogenum* (37). Therefore, we first examined whether melanin synthesis in *A. pullulans* can also be repressed by the supplementation of $(NH_4)_2SO_4$. In the case of *A. pullulans*, cell growth was severely blocked by 1.0–2.0% $(NH_4)_2SO_4$ in the PDB liquid medium. However, 0.3–0.5% $(NH_4)_2SO_4$ had little effect on the cell growth, and the cell densities in the culture media reached to 9.0×10^5 cells/ml after 144 h of cultivation regardless of $(NH_4)_2SO_4$. On the other hand, notable differences were observed in their colors when cells were cultivated with or without 0.5% $(NH_4)_2SO_4$ (Fig. 1A), suggesting effective inhibition of melanization by the supplementation of 0.5% $(NH_4)_2SO_4$. Therefore, we compared the intracellular levels of melanin in *A. pullulans* grown in the PDB liquid medium with or without $(NH_4)_2SO_4$ (Fig. 1B). These results clearly demonstrated that supplementation of $(NH_4)_2SO_4$ in the PDB medium prevented melanization of *A. pullulans* as well as *A. melanogenum*. Herein, we prepared melanized and non-melanized cells of *A. pullulans* for the following analyses by the cultivation in PDB liquid medium with or without 0.5% $(NH_4)_2SO_4$ at 25 °C for 144 h.

Melanized *A. pullulans* showed the hyper resistance to hydrogen peroxide and heat shock We next examined the effects of melanization on the stress tolerance of *A. pullulans*. The melanized and non-melanized cells were treated with H_2O_2 or heat shock and their survival rates were compared. As shown in Fig. 2, the non-melanized cells were efficiently killed by the treatment with 80 or 100 mM H_2O_2 for 60 min. In contrast, the melanized cells were scarcely killed by H_2O_2 and showed hyper-tolerance to H_2O_2. The melanized cells also showed higher tolerance to heat shock. Although more than half of the melanized cells were killed by the treatment with an acute heat shock at 46 °C, their survival rates were higher than those of the non-melanized cells. These results clearly suggest that melanization is involved in the acquisition of stress tolerance in *A. pullulans*.

Melanin conferred resistance to CAP in *A. pullulans* We next examined whether melanin confers resistance to CAP in *A.*

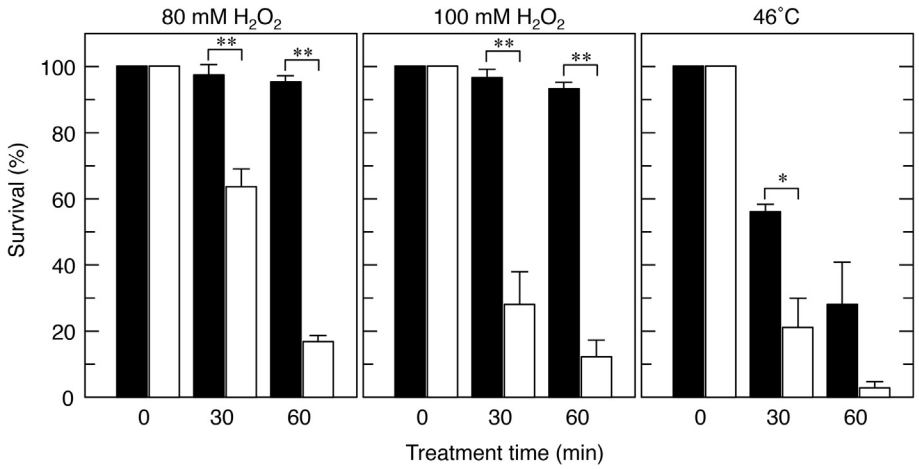

FIG. 2. Survival of the melanized cells (closed bars) and non-melanized cells (open bars) of *A. pullulans* under oxidative stress (80 or 100 mM H_2O_2) or heat shock at 46 °C. After the stress treatment for 0–60 min, cells were immediately spread on YPD agar plates. The relative survival rate was calculated as colony-forming units (CFU). The survival of cells without the stress treatment was taken as 100 %. Data are given as means ± SD ($n = 3$). $^*P < 0.05$. $^{**}P < 0.01$.

pullulans. The melanized and non-melanized cells were spread on PDB agar plates and then exposed to CAP (Ar plasma). Through the contact with the ambient air and moisture, Ar plasma generates various antimicrobial substances such as ozone, reactive nitrogen species (RNS), and reactive oxygen species (ROS) including hydroxyl radicals and hydrogen peroxide (24,29,38–40). As well as *Saccharomyces cerevisiae* cells (34), the non-melanized cells were efficiently killed by our CAP system (Fig. 3). On the other hand, the melanized cells maintained a high survival rate under the Ar-plasma stress. More than 60% of cells were still alive after the exposure of Ar plasma for 30 min. Exposure of Ar gas alone did not affect the survival of *A. pullulans*. This result clearly indicated that the melanized cells had higher tolerance to the exposure of Ar plasma than the non-melanized cells of *A. pullulans*.

Ferrous chloride and ferrous sulfate improved the fungicidal efficacy of CAP Since the fungicidal efficacy of Ar-plasma exposure was insufficient against the melanized cells of *A. pullulans*, we attempted to improve it. Recently, Ravindran et al. (33) reported that iron chloride improved the efficiency of delignification of spent coffee waste by CAP through the induction of Fenton reaction and its following side reactions.

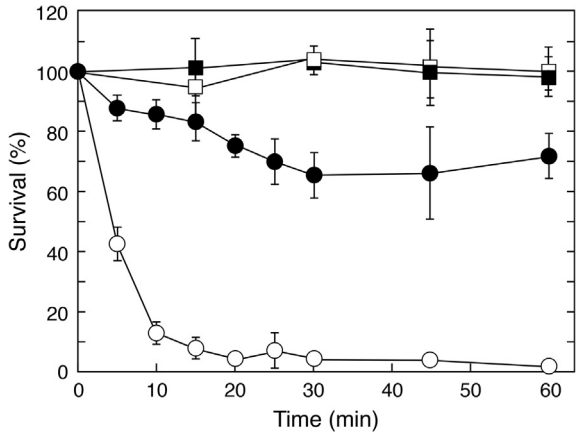

FIG. 3. Survival of melanized cells (closed symbols) and non-melanized cells (open symbols) of *A. pullulans* under Ar-plasma stress. Cells were spread on PDB agar plates and exposed to Ar plasma (circles) or Ar gas alone (squares) for the indicated period of time. The relative survival rate was calculated as CFU. The survival of cells without the treatments was taken as 100%. Data are given as mean ± SD ($n = 3$).

Since CAP exposure induces the generation of hydrogen peroxide (22–25), it seems quite plausible that Fenton reaction could be induced by Ar-plasma exposure in the presence of iron chloride. These backgrounds prompted us to expect that ferrous chloride ($FeCl_2$) also could improve the fungicidal effects of Ar plasma exposure on the melanized *A. pullulans*.

We first examined the concentration-dependent effects of $FeCl_2$. The melanized cells collected by centrifugation were resuspended in 100 mM sodium acetate buffer (pH 5.2) containing various concentrations of $FeCl_2$ and spread on the PDB agar plates. In the presence of 0.1–20 mM $FeCl_2$, survival rates of cells were significantly decreased by the exposure of Ar plasma for 30 min but not by the exposure of Ar gas alone (Fig. 4). Similar results were observed when cells were resuspended in the buffer containing 0.1–20 mM ferrous sulfate ($FeSO_4$) (Fig. 4). Since 2.5 mM ferrous compounds were most effective, we performed a time-course analysis of cell survival during the Ar-plasma exposure in the presence of 2.5 mM $FeCl_2$ or $FeSO_4$. We observed that 2.5 mM $FeCl_2$ and $FeSO_4$ significantly improved the fungicidal effects of Ar plasma on the melanized cells of *A. pullulans* (Fig. 5).

DISCUSSION

In this study, we provided several new findings on the physiology of *A. pullulans*. First, we herein found that the melanin biosynthesis in *A. pullulans* was blocked by the supplementation of $(NH_4)_2SO_4$, a representative inexpensive nitrogen source. Synthesis of melanin in *A. pullulans* was also blocked in a nutrient rich YPD medium (2% glucose, 1% yeast extract, 2% peptone) (data not shown). It is known that biosynthesis of melanin in *A. melanogenum* and *Verticillium dahlia* is repressed under conditions rich in nitrogen source (37,41). Currently, it is still unclear why the melanin biosynthesis is repressed by supplementation of nitrogen source in these fungi. In the case of *A. melanogenum*, it was clarified that $(NH_4)_2SO_4$ represses expression of the *PKS* gene, which encodes a polyketide synthetase and is responsible for the melanin biosynthesis (37). Since *A. pullulans* and *A. melanogenum* belong to the same genus, *A. pullulans* might have the similar regulatory mechanisms of melanin biosynthesis with *A. melanogenum*.

Although our CAP system showed a sufficient fungicidal activity to the non-melanized cells of *A. pullulans* as well as *S. cerevisiae* (34,42), the melanized *A. pullulans* showed a higher resistance and more than 60% of cells survived after the exposure of Ar plasma for 60 min. It is well known that melanized microorganisms are

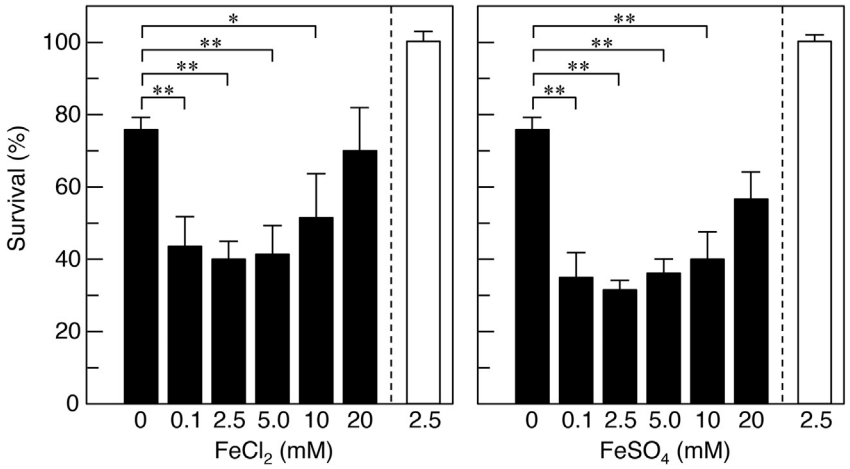

FIG. 4. Melanized cells suspended in 100 mM sodium acetate buffer (pH 5.2) containing 0–20 mM $FeCl_2$ or $FeSO_4$ were spread on PDB agar plates (pH 5.2) and exposed to Ar plasma (closed bars) or Ar gas alone (open bar) for 30 min. The relative survival rate was calculated as CFUs. The survival of cells without the plasma treatment was taken as 100 %. Data are expressed as means ± SD ($n = 3$). $^{*}P < 0.05$. $^{**}P < 0.01$.

generally hyper-resistant to various kinds of stress, and that some kinds of melanized microorganisms can inhabit extreme environments such as the polar regions and the damaged reactor at Chernobyl (43–45). Since melanin has antioxidant and free-radicals scavenging properties and functions as a UV absorbent (15,46–49), melanin presumably played a role as protective factors against ROS and UV photons generated by the Ar-plasma exposure. Therefore, our finding on the hyper-resistance against Ar plasma of the melanized *A. pullulans* seems quite reasonable, and it appears to be feasible that other melanized fungi are also hyper-resistance to ROS and UV photons generated by the exposure of CAP. Taking the antioxidant properties of melanin into consideration, our results imply that ROS and UV photons derived from Ar plasma might significantly contribute to the fungicidal activity of our CAP system. To see whether intracellular melanin can improve the tolerance to CAP and oxidative stress of *S. cerevisiae*, another project is now underway and we are constructing a strain of *S. cerevisiae* that synthesizes and accumulates melanin within the cell.

Although the exposure of Ar-plasma alone was not a satisfactory method to sterilize the melanized cells of *A. pullulans*, we found that presence of $FeCl_2$ or $FeSO_4$ significantly improved the fungicidal efficacy of Ar plasma. As a plausible explanation, it is considered that generation of highly toxic hydroxyl radicals was promoted through Fenton reaction in the presence of ferrous compounds and peroxides derived from CAP (22–25,30). The promoted production of hydroxyl radicals and other ROS presumably contributed to the improvement of the fungicidal activity of Ar-plasma exposure. Furthermore, it was reported that Fenton type reaction accelerates the degradation of melanin and that the degraded melanin can function as an efficient prooxidant agent (50). Degradation of melanin might also be one of primary causes for the improved fungicidal effects of Ar plasma with $FeCl_2$ and $FeSO_4$.

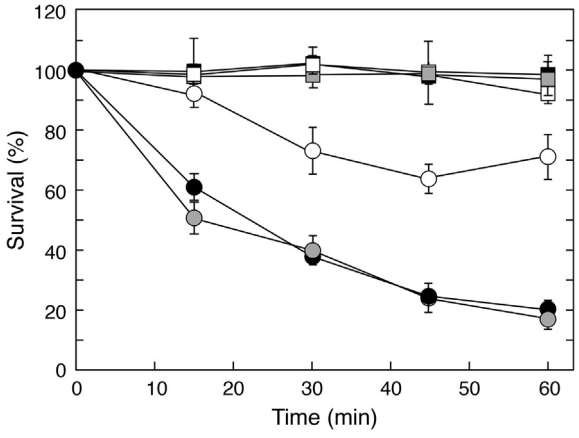

FIG. 5. $FeCl_2$ and $FeSO_4$ improved the fungicidal efficacy of Ar plasma against the melanized *A. pullulans*. Melanized cells suspended in 100 mM sodium acetate buffer (pH 5.2) with 2.5 mM $FeCl_2$ (closed symbols), with 2.5 mM $FeSO_4$ (shaded symbols), or without ferrous compounds (open symbols) were spread on PDB agar plates and exposed to Ar plasma (circles) or Ar gas alone (squares) for the indicated period of time. The relative survival rate was calculated as CFUs. The survival of cells without the treatments was taken as 100 %. Data are given as means ± SD ($n = 3$).

We examined the concentration-dependent effects of $FeCl_2$ and $FeSO_4$, and found that 2.5 mM ferrous compounds were the most effective for killing the melanized *A. pullulans*, while higher concentrations of $FeCl_2$ and $FeSO_4$ were less effective (Fig. 4). It is known that recycle of Fe(III) to Fe(II) can be blocked by the excess amount of $FeCl_2$ and the limited amount of H_2O_2 (51). It is likely that more than 2.5 mM $FeCl_2$ and $FeSO_4$ could be too much for the amount of H_2O_2 supplied by the exposure of Ar plasma.

Our results clearly demonstrated that the Ar-plasma exposure accompanied by Fenton reaction is a powerful method to sterilize dematiaceous fungi with hyper stress tolerance. Although other types of CAPs derived from different gases might have distinct antimicrobial efficacies (52–54), various types of CAPs also induce the generation of hydrogen peroxide (22–25). Therefore, it is presumable that Fenton reaction could be induced by other types of CAPs in the presence of ferrous compounds. Spraying transition-metal ions, such as iron and copper, on the sample in advance might be a simple and practical way to enhance the fungicidal and bactericidal effects of CAP exposure.

ACKNOWLEDGMENTS

This study was supported by the Japan Society for the Promotion of Science (grant number 17H03795 to S.I.). The authors declare that they have no conflict of interest.

References

1. **Prakitchaiwattana, C. J., Fleet, G. H., and Heard, G. M.:** Application and evaluation of denaturing gradient gel electrophoresis to analyse the yeast ecology of wine grapes, FEMS Yeast Res., **4**, 865–877 (2004).
2. **McGrath, M. J. and Andrews, J. H.:** Temporal changes in microscale colonization of the phylloplane by *Aureobasidium pullulans*, Appl. Environ. Microbiol., **72**, 6234–6241 (2006).

3. **Chi, Z., Wang, F., Chi, Z., Yue, L., Liu, G., and Zhang, T.:** Bioproducts from *Aureobasidium pullulans*, a biotechnologically important yeast, Appl. Microbiol. Biotechnol., **82**, 793–804 (2009).
4. **Chan, G. F., Puad, M. S., Chin, C. F., and Rashid, N. A.:** Emergence of *Aureobasidium pullulans* as human fungal pathogen and molecular assay for future medical diagnosis, Folia Microbiol., **56**, 459–467 (2011).
5. **Prasongsuk, S., Lotrakull, P., Ali, I., Bankeeree, W., and Punnapayak, H.:** The current status of *Aureobasidium pullulans* in biotechnology, Folia Microbiol., **63**, 129–140 (2018).
6. **Hawkes, M., Rennie, R., Sand, C., and Vaudry, W.:** *Aureobasidium pullulans* infection: fungemia in an infant and a review of human cases, Diagn. Microbiol. Infect. Dis., **51**, 209–213 (2005).
7. **Hofman, V., Butori, C., Long, E., Le Fichoux, Y., and Hofman, P.:** *Aureobasidium pullulans* contamination in bronchial aspirates mimicking cryptococcosis: a rare diagnostic pitfall, Pathology, **40**, 729–732 (2008).
8. **Huang, Y. T., Liaw, S. J., Liao, C. H., Yang, J. L., Lai, D. M., Lee, Y. C., and Hsueh, P. R.:** Catheter-related septicemia due to *Aureobasidium pullulans*, Int. J. Infect. Dis., **12**, e137–e139 (2008).
9. **Revankar, S. G. and Sutton, D. A.:** Melanized fungi in human disease, Clin. Microbiol. Rev., **23**, 884–928 (2010).
10. **Morais, O. O., Porto, C., Coutinho, A. S., Reis, C. M., Teixeira, M. M., and Gomes, C. M.:** Infection of the lymphatic system by *Aureobasidium pullulans* in a patient with erythema nodosum leprosum, Braz. J. Infect. Dis., **15**, 288–292 (2011).
11. **Mittal, J., Szymczak, W. A., Pirofski, L., and Galen, B. T.:** Fungemia caused by *Aureobasidium pullulans* in a patient with advanced AIDS: a case report and review of the medical literature, JMM Case Rep., **5**, e005144 (2018).
12. **Eisenman, H. C. and Casadevall, A.:** Synthesis and assembly of fungal melanin, Appl. Microbiol. Biotechnol., **93**, 931–940 (2012).
13. **Lu, Y., Ye, M., Song, S., Li, L., Shaikh, F., and Li, J.:** Isolation, purification, and anti-aging activity of melanin from *Lachnum singerianum*, Appl. Biochem. Biotechnol., **174**, 762–771 (2014).
14. **Cordero, R. J. B. and Casadevall, A.:** Functions of fungal melanin beyond virulence, Fungal Biol. Rev., **31**, 99–112 (2017).
15. **Dadachova, K., Bryan, R. A., Howell, R. C., Schweitzer, A. D., Aisen, P., Nosanchuk, J. D., and Casadevall, A.:** The radioprotective properties of fungal melanin are a function of its chemical composition, stable radical presence and spatial arrangement, Pigment Cell Melanoma Res., **21**, 192–199 (2008).
16. **Dadachova, K. and Casadevall, A.:** Ionizing radiation: how fungi cope, adapt, and exploit with the help of melanin, Curr. Opin. Microbiol., **11**, 525–531 (2008).
17. **Cordero, R. J. B., Vij, R., and Casadevall, A.:** Microbial melanins for radioprotection and bioremediation, Microb. Biotechnol., **10**, 1186–1190 (2017).
18. **Pacelli, C., Bryan, R. A., Onofri, S., Selbmann, L., Shuryak, I., and Dadachova, E.:** Melanin is effective in protecting fast and slow growing fungi from various types of ionizing radiation, Environ. Microbiol., **19**, 1612–1624 (2017).
19. **Nosanchuk, J. D. and Casadevall, A.:** The contribution of melanin to microbial pathogenesis, Cell Microbiol., **5**, 203–223 (2003).
20. **Liu, G. Y. and Nizet, V.:** Color me bad: microbial pigments as virulence factors, Trends Microbiol., **17**, 406–413 (2009).
21. **Gaunt, L., Beggs, C., and Georghiou, G.:** Bactericidal action of the reactive species produced by gas-discharge nonthermal plasma at atmospheric pressure: a review, IEEE Trans. Plasma Sci., **34**, 1257–1269 (2006).
22. **Locke, B. R. and Shih, K.:** Review of the methods to form hydrogen peroxide in electrical discharge plasma with liquid water, Plasma Sources Sci. Technol., **20**, 34006 (2011).
23. **Wu, H., Sun, P., Feng, H., Zhou, H., Wang, R., Liang, Y., Lu, J., Zhu, W., Zhang, J., and Fang, J.:** Reactive oxygen species in a non-thermal plasma microjet and water system: generation, conversion, and contributions to bacteria inactivation—an analysis by electron spin resonance spectroscopy, Plasma Process. Polym., **9**, 417–424 (2012).
24. **Tresp, H., Hammer, M. U., Winter, J., Weltmann, K., and Reuter, S.:** Quantitative detection of plasma-generated radicals in liquids by electron paramagnetic resonance spectroscopy, J. Phys. D Appl. Phys., **46**, 435401 (2013).
25. **Ohshima, T., Ikawa, S., Kitano, K., and Maeda, N.:** A proposal of remedies for oral diseases caused by *Candida*: a mini review, Front. Microbiol., **9**, 1522 (2018).
26. **Heinlin, J., Morfill, G., Landthaler, M., Stolz, W., Isbary, G., Zimmermann, J. L., Shimizu, T., and Karrer, S.:** Plasma medicine: possible applications in dermatology, J. Dtsch. Dermatol. Ges., **8**, 968–976 (2010).
27. **Hoffmann, C., Berganza, C., and Zhang, J.:** Cold atmospheric plasma, methods of production and application in dentistry and oncology, Med. Gas Res., **3**, 21 (2013).
28. **Mir, S. A., Shah, M. A., and Mir, M. M.:** Understanding the role of plasma technology in food industry, Food Bioprocess Technol., **9**, 734–750 (2016).
29. **Lackmann, J. W. and Bandow, J. E.:** Inactivation of microbes and macromolecules by atmospheric-pressure plasma jets, Appl. Microbiol. Biotechnol., **98**, 6205–6213 (2014).
30. **Fenton, H. J. H.:** Oxidation of tartaric acid in presence of iron, J. Chem. Soc. Trans., **65**, 899–911 (1894).
31. **Wang, N., Zheng, T., Zhang, G., and Wang, P.:** A review on Fenton-like processes for organic wastewater treatment, J. Environ. Chem. Eng., **4**, 762–787 (2016).
32. **Mirzaei, A., Chen, Z., Haghighat, F., and Yerushalmi, L.:** Removal of pharmaceuticals from water by homo/heterogonous Fenton-type processes - a review, Chemosphere, **174**, 665–688 (2017).
33. **Ravindran, R., Sarangapani, C., Jaiswal, S., Cullen, P. J., and Jaiswal, A. K.:** Ferric chloride assisted plasma pretreatment of lignocellulose, Bioresour. Technol., **243**, 327–334 (2017).
34. **Itooka, K., Takahashi, K., and Izawa, S.:** Fluorescence microscopic analysis of antifungal effects of cold atmospheric pressure plasma in *Saccharomyces cerevisiae*, Appl. Microbiol. Biotechnol., **100**, 9295–9304 (2017).
35. **Shaw, A., Shama, G., and Iza, F.:** Emerging applications of low temperature gas plasmas in the food industry, Biointerphases, **10**, 029402 (2015).
36. **Fernandes, B., Matama, T., Guimarães, D., Gomes, A., and Cavaco-Paulo, A.:** Fluorescent quantification of melanin, Pigment Cell Melanoma Res., **29**, 707–712 (2016).
37. **Jiang, H., Liu, N. N., Liu, G. L., Chi, Z., Wang, J. M., Zhang, L. L., and Chi, Z. M.:** Melanin production by a yeast strain XJ-51 of *Aureobasidium melanogenum* isolated from the Taklimakan desert and its role in the yeast survival in stress environments, Extremophiles, **20**, 567–577 (2016).
38. **Isbary, G., Shimizu, T., Li, Y. F., Stolz, W., Thomas, H. M., Morfill, G. E., and Zimmermann, J. L.:** Cold atmospheric plasma devices for medical issues, Expert Rev. Med. Devices, **10**, 367–377 (2013).
39. **Uchiyama, H., Zhao, Q. L., Hassan, M. A., Andocs, G., Nojima, N., Takeda, K., Ishikawa, K., Hori, M., and Kondo, T.:** EPR-spin trapping and flow cytometric studies of free radicals generated using cold atmospheric argon plasma and X-ray irradiation in aqueous solutions and intracellular milieu, PLoS One, **10**, e0136956 (2015).
40. **Ouf, S. A., Basher, A. H., and Mohamed, A. H.:** Inhibitory effect of double atmospheric pressure argon cold plasma on spores and mycotoxin production of *Aspergillus niger* contaminating date palm fruits, J. Sci. Food Agric., **95**, 3204–3210 (2015).
41. **Xiong, D., Wang, Y., and Tian, C.:** Transcriptomic profiles of the smoke tree wilt fungus *Verticillium dahliae* under nutrient starvation stresses, Mol. Genet. Genom., **290**, 1963–1977 (2015).
42. **Itooka, K., Takahashi, K., Kimata, Y., and Izawa, S.:** Cold atmospheric pressure plasma causes protein denaturation and endoplasmic reticulux, Appl. Microbiol. Biotechnol., **102**, 2279–2288 (2018).
43. **Mironenko, N. V., Alekhina, I. A., Zhdanova, N. N., and Bulat, S. A.:** Intraspecific variation in gamma-radiation resistance and genomic structure in the filamentous fungus *Alternaria alternata*: a case study of strains inhabiting Chernobyl reactor no. 4, Ecotoxicol. Environ. Saf., **45**, 177–187 (2000).
44. **Zhdanova, N. N., Tugay, T., Dighton, J., Zheltonozhsky, V., and McDermott, P.:** Ionizing radiation attracts soil fungi, Mycol. Res., **108**, 1089–1096 (2004).
45. **Robertson, K. L., Mostaghim, A., Cuomo, C. A., Soto, C. M., Lebedev, N., Bailey, R. F., and Wang, Z.:** Adaptation of the black yeast *Wangiella dermatitidis* to ionizing radiation: molecular and cellular mechanisms, PLoS One, **7**, e48674 (2012).
46. **Rózanowska, M., Sarna, T., Land, E. J., and Truscott, T. G.:** Free radical scavenging properties of melanin interaction of eu- and pheo-melanin models with reducing and oxidising radicals, Free Radic. Biol. Med., **26**, 518–525 (1999).
47. **Herrling, T., Jung, K., and Fuchs, J.:** The role of melanin as protector against free radicals in skin and its role as free radical indicator in hair, Spectrochim. Acta A Mol. Biomol. Spectrosc., **69**, 1429–1435 (2008).
48. **Brenner, M. and Hearing, V. J.:** The protective role of melanin against UV damage in human skin, Photochem. Photobiol., **84**, 539–549 (2008).
49. **Liu, Y., Ai, K., Ji, X., Askhatova, D., Du, R., Lu, L., and Shi, J.:** Comprehensive insights into the multi-antioxidative mechanisms of melanin nanoparticles and their application to protect brain from injury in ischemic stroke, J. Am. Chem. Soc., **139**, 856–862 (2017).
50. **Zadlo, A., Pilat, A., Sarna, M., Pawlak, A., and Sarna, T.:** Redox active transition metal ions make melanin susceptible to chemical degradation induced by organic peroxide, Cell Biochem. Biophys., **75**, 319–333 (2017).
51. **Deguillaume, L., Leriche, M., and Chaumerliac, N.:** Impact of radical versus non-radical pathway in the Fenton chemistry on the iron redox cycle in clouds, Chemosphere, **60**, 718–724 (2005).
52. **Shintani, H., Sakudo, A., Burke, P., and McDonnell, G.:** Gas plasma sterilization of microorganisms and mechanisms of action (review), Exp. Ther. Med., **1**, 731–738 (2010).
53. **Shintani, H.:** Inactivation of bacterial spore, endotoxin, lipid A, normal prion and abnormal prion by exposures to several sorts of gases plasma, Biocontrol Sci., **21**, 1–12 (2016).
54. **Zhou, R., Zhang, X., Bi, Z., Zong, Z., Niu, J., Song, Y., Liu, D., and Yang, S.:** Inactivation of *Escherichia coli* cells in aqueous solution by atmospheric-pressure N_2, He, air, and O_2 microplasmas, Appl. Environ. Microbiol., **81**, 5257–5265 (2015).