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Comparison of the morphology and viability of gamma irradiated vegetative cells, wet cysts, and dry cysts of the soil ciliate *Colpoda cucullus*

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ABSTRACT
The soil ciliate, *Colpoda cucullus*, can tolerate various types of environmental stress, including 4,000 Gy gamma radiation, by forming resting cysts (encystment). In this study, we found that *C. cucullus* resting cycts also were able to tolerate 8,000 Gy gamma radiation. Irradiated wet cysts and dry cysts were morphologically indistinguishable from non-irradiated cysts and were able to successfully revert to vegetative cells (excystment). The viability (i.e., excystment) of dry cysts was higher than that of wet cysts after 8,000 Gy gamma irradiation. However, vegetative cells did not survive 8,000 Gy gamma radiation and decomposed.

Keywords: Resting cysts; *Colpoda*; Gamma radiation; Tolerance

INTRODUCTION
The soil ciliate *Colpoda cucullus* can survive in standing water and moist soil. In addition, *C. cucullus* vegetative cells can form resting cysts to enable them to tolerate various types of environmental stress; e.g., desiccation (Corliss and Esser, 1974), acid (Sogame et al., 2011), high and low temperature (Taylor and Stickland 1936), freezing (Uspenskaya and Lozina-Lozinski, 1979), UV (Uspenskaya and Lozina-Lozinski, 1979; Matsuoka et al., 2017), and gamma radiation (Saito et al., 2020).

In general, gamma radiation is harmful for biological organisms. Whole-body exposure to 10 Gy gamma radiation is lethal for most vertebrate animals (Thornley 1963; Daly 2009). Most bacteria also cannot survive a gamma radiation dose of 200 Gy (Thornley 1963; Daly 2009). However, invertebrates and some microorganisms, such as extremophiles, can tolerate exposure to a high dose of gamma radiation. For example, the fruit fly *Drosophila melanogaster* can survive for 2 days after exposure to 1,500 Gy gamma radiation (Parashar et al., 2008) and the nematode *Caenorhabditis elegans* show almost no harmful effects after exposure to 1,000 Gy gamma radiation (Jonson and Hartman, 1988). In addition, the hyperthermophilic archaeon *Pyrococcus furiosus* has been reported to survive more than 2,500 Gy gamma radiation (DiRuggiero et al., 1997), the halophilic archaeon *Halobacterium* sp. can survive exposure to 5,000 Gy gamma radiation (Kottemann et al., 2005), and the

radioresistant bacterium *Deinococcus radiodurans* survives without loss of viability after exposure to 5,000 Gy gamma radiation (Moseley and Mattingly, 1971). It is amazing that *D. radiodurans* can even survive a dose of 12,000 Gy gamma radiation (Misra et al., 2013) and even a greater dose (Daly et al., 1994; Daly et al., 2009).

We have previously shown that *C. cucullus* resting cysts tolerate 4,000 Gy gamma radiation: most cysts were able to revert to vegetative cells after irradiation and the viability of 4,000 Gy gamma irradiated wet cysts was higher than that of irradiated dry cysts (Saito et al. 2020). In the present study, we found that *C. cucullus* resting cysts have a more extreme tolerance to gamma irradiation. Therefore, we studied the viability (i.e., excystment) and morphology of gamma irradiated *C. cucullus* wet and dry cysts.

MATERIALS AND METHODS

Cell culture and induction of encystment and excystment

Colpoda cucullus R2TTYS (Sogame et al., 2019b) was cultured in a 0.05% (w/v) rice leaf infusion supplemented with 0.05% (w/v) Na_2HPO_4 (final concentration). Occasionally, bacteria (*Klebsiella pneumoniae* NBRC13277) were added to the culture medium as a food source for *C. cucullus*.

Encystment of *C. cucullus* vegetative cells was induced by suspending the cells at high cell density (> 10,000 cells/ml) in 1 mM Tris-HCl (pH = 7.2) supplemented with 0.1 mM $CaCl_2$ (final concentration).

Excystment of *C. cucullus* cysts was induced by replacing the medium with a 0.2% (w/v) rice leaf infusion supplemented with 0.05% (w/v) Na_2HPO_4 (final concentration).

Sample preparation and gamma irradiation

For vegetative cell preparations, 1 ml of a cell suspension at low cell density (1,000 cells/ml) was put in Petri dishes. For cyst preparations, 1 ml of encystment-induced cells (10,000 cells/ml) was put in Petri dishes and incubated for 1 week. Dry cyst samples were prepared by air-drying wet cyst samples for 1 week. Six samples of each preparation were used for each irradiation experiment.

Samples of vegetative cells, wet cysts, and dry cysts were irradiated with 8,000 Gy gamma radiation (444 Gy/h, 18 h irradiation) using the RE2022 (Toshiba, Japan) in the National Agriculture and Food Research Organization (NARO). The radiation source was cobalt 60 (radioactivity, 42 TBq).

Cell viability and excystment assays

The viability of vegetative cells was determined by directly counting viable cells in 100 µl samples before and after gamma irradiation. The percent viability of vegetative cells was calculated as: Percent cell viability = (Number of viable cells after irradiation / Number of viable cells before irradiation) × 100. The excystment of wet and dry cysts was measured by directly counting excysted cells and un-excysted cells (> 100 cells in a randomly chosen field) by microscopy (Zeiss Stemi 305) before and after irradiation. The percent excystment was calculated as follows: Percent excystment = (Number of excysted cysts / Number of

excysted and un-excysted cysts) × 100. The percent viability of irradiated wet and dry cysts was calculated as follows: Percent cyst viability = (Percent excystment at 60 h after induction of excystment of irradiated samples / Percent excystment at 60 h after induction of excystment of non-irradiated samples) × 100. All values were shown as the mean ± SE of 6 identical samples. Statistical analysis was performed by the Mann-Whitney U test using the Bell Curve for Excel software (Social Survey Research Information Co., Ltd., Japan).

Microscopy

Vegetative cells, wet cysts, and dry cysts were observed with an Axio Vart.A1 optical microscope system (Zeiss, Japan). The vegetative cells were concentrated 10-fold by centrifugation (2,000 rpm for 1 min) and observed at low magnification (Fig. 1A). In addition, the cell samples were fixed in an equal volume of paraformaldehyde (PA) and observed at high magnification (Fig. 1A, upper right images).

For 4',6-diamidino-2-phenylindole (DAPI) staining, samples (wet cysts, irradiated wet cysts, and dry cysts) were suspended in a DAPI solution [1 mM Tris HCl (pH 7.2), 0.1 mM $CaCl_2$, 2 μg DAPI/ml] for 15 min. Wet cysts were also treated with PA and NP-40 and stained as follows: wet cysts were treated with a fixative solution [2% PA, 1 mM Tris HCl (pH 7.2), 0.1 mM $CaCl_2$] for 1 h, then treated with an NP-40 detergent solution [1% NP-40, 1 mM Tris HCl (pH 7.2), 0.1 mM $CaCl_2$] for 1 h, washed with wash buffer [1 mM Tris HCl (pH 7.2), 0.1 mM $CaCl_2$], and finally stained with the DAPI solution for 15 min. The samples were observed with a confocal laser microscope (Fluoview 10i, Olympus), using a DAPI filter (emission maximum 460 nm) with an excitation peak at 405 nm. Stained and unstained cells were counted (> 100 cells) and the data was expressed as the percent of stained cells as follows: Percent stained cysts = (Number of stained cysts / Total number of cysts) × 100.

RESULTS AND DISCUSSION

Colpoda cucullus vegetative cells, wet cysts, and dry cysts were either not irradiated or irradiated with 8,000 Gy gamma radiation. They were then examined by optical microscopy and assayed for viability. After 8,000 Gy gamma irradiation, vegetative cells were decomposed and could not be seen by microscopy (Fig. 1A). However, both irradiated wet and dry cysts were morphologically indistinguishable from non-irradiated wet and dry cysts (Fig. 1). The percent viability of irradiated vegetative cells, wet cysts, and dry cysts was 0%, 12.0 ± 5.0 %, and 42.3 ± 12.0%, respectively (Fig. 2). These results indicated that *C. cucullus* wet and dry cysts could tolerate 8,000 Gy gamma radiation.

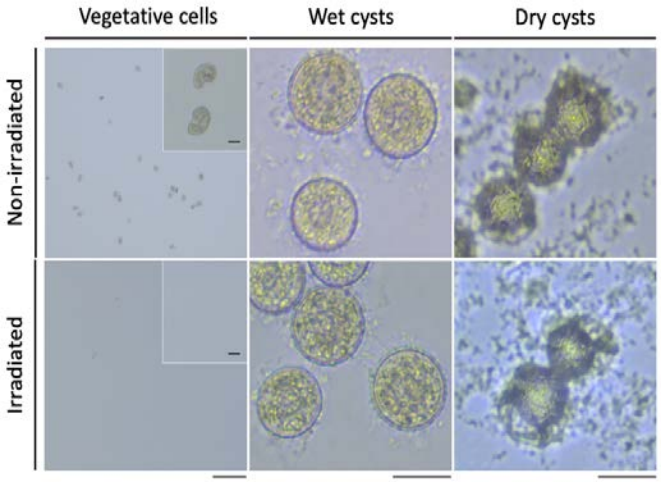

Fig. 1. Comparison of gamma irradiated and non-irradiated *C. cucullus* vegetative cells, wet cysts, and dry cysts by optical microscopy. Microscope images of non-irradiated (Top row) and 8,000 Gy gamma irradiated (Bottom row) cells and cysts. The upper right images in the vegetative cells in are higher magnifications of the cells in these samples. Scale bars mark: 250 μm and 20 μm (upper right images) for vegetative cells, and 20 μm for wet cysts and dry cysts.

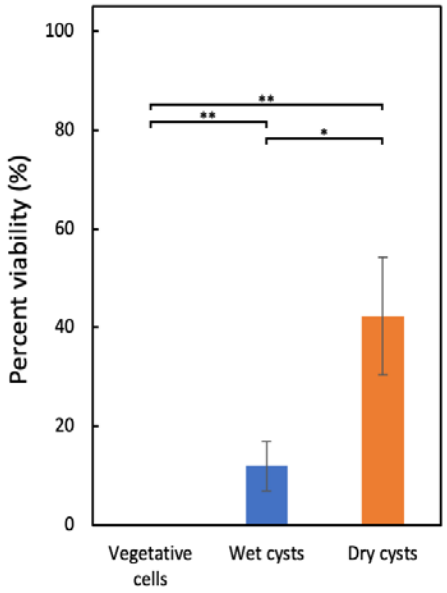

Fig. 2. Percent viability of 8,000 Gy gamma irradiated *C. cucullus* vegetative cells, wet cysts, and dry cysts relative to non-irradiated *C. cucullus* vegetative cells, wet cysts, and dry cysts, respectively. Each column shows the mean and standard error of six samples. Single asterisks and double asterisks indicate a significant difference at $p < 0.05$ and $p < 0.01$, respectively (Mann-Whitney U test).

Although both irradiated wet and dry cysts showed no apparent damage due to 8,000 Gy irradiation by optical microscopy (Fig. 1), their viability after irradiation was significantly reduced (Fig. 2). Therefore, 8,000 Gy gamma radiation produced considerable damage in wet and dry cysts that was not visible by optical microscopy. To investigate radiation damage to the cell membrane, ectocyst (the outermost layer of the cyst wall), and/or the endocyst (the inner layers of the cyst wall), wet cysts (Control), wet cysts treated with PA and NP-40 (PA+NP-40), irradiated wet cysts (Irradiated), and dry cysts (Dry cysts) were stained with DAPI (Fig. 3). Although DAPI is difficult to permeate through viable cell membrane, it passes through damaged cell membrane and preferentially binds dsDNA. Therefore, DAPI

staining can be used to investigate whether a cell membrane and/or cyst wall has been damaged. In this study, the nuclei of wet cysts were barely stained with DAPI, but the nuclei of wet cysts treated with PA and NP-40 were stained (Fig. 3A, 3B). The nuclei of 8,000 Gy gamma irradiated wet cysts were also stained with DAPI (Fig. 3A): the percent of DAPI-stained irradiated wet cysts was similar to that of DAPI-stained wet cysts after PA and NP-40 treatment (Fig. 3B). Non-irradiated dry cysts were also stained with DAPI (Fig. 3A): the percent of DAPI-stained non-irradiated dry cysts was similar to that of DAPI-stained wet cysts after PA and NP-40 treatment (Fig. 3B). These results indicated that both 8,000 Gy gamma irradiation and desiccation damaged both *C. cucullus* cell membranes and cyst walls.

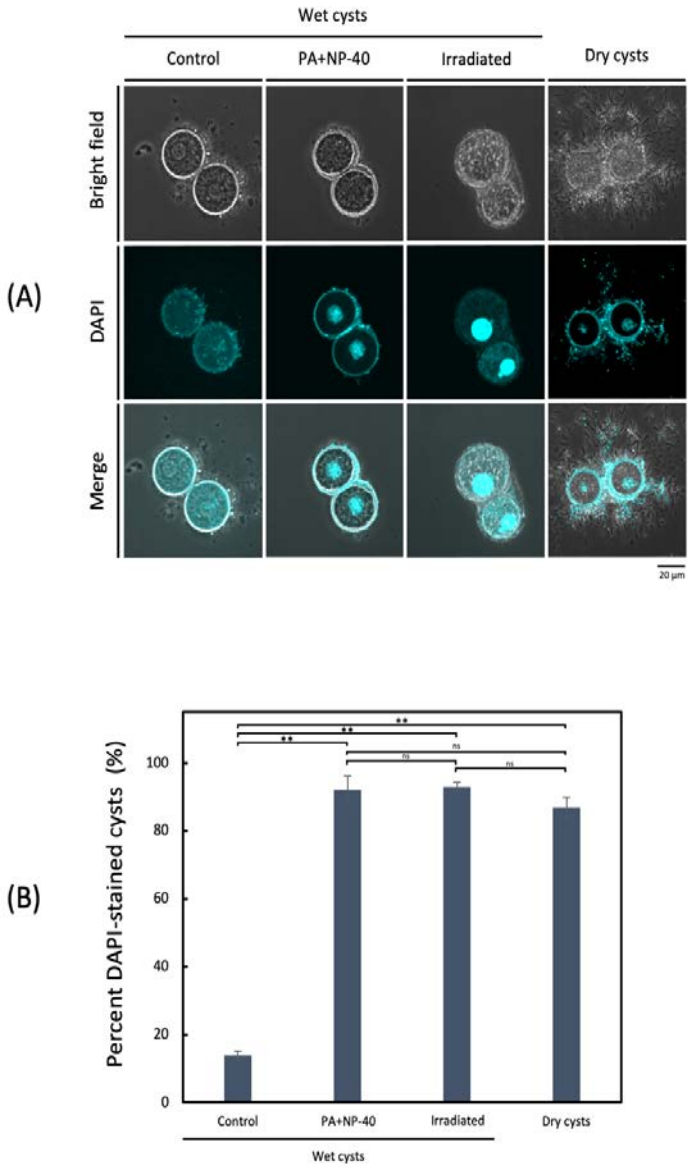

Fig. 3. DAPI stained *C. cucullus* cysts. (A) Confocal laser microscope images of DAPI-stained wet cysts (Control), wet cysts after PA and NP-40 treatment (PA+NP-40), irradiated wet cysts (Irradiated), and dry cysts (Dry cysts). Bright field images (Top row), images of DAPI-stained cysts (Middle row), and the merged images (Bottom Row) are shown. (B) Percent of DAPI-stained wet cysts (Control), wet cysts after PA and NP-40 treatment (PA+NP-40), irradiated wet cysts (Irradiated), and dry cysts (Dry cysts). Each column shows the mean and standard error of six samples. Double asterisks and ns indicate a significant difference at $p < 0.01$ and not significant among samples, respectively (Mann-Whitney U test).

After 8,000 Gy gamma irradiation and the induction of excystment, both wet cysts and dry cysts gradually reverted to vegetative cells (Fig. 4). At 60 h after the induction of excystment, 80% of the non-irradiated dry cysts had reverted to vegetative cells. However, only about 10% and 40% of the gamma irradiated wet and dry cysts, respectively, had reverted to vegetative cells at 60 h after the induction of excystment (Fig. 4). In contrast, the percent of DAPI-stained gamma irradiated wet cysts and non-irradiated dry cysts were very similar: both were about 90% (Fig. 3). Hence, 8000 Gy irradiation caused serious damage to *C. cucullus* cells in addition to direct damage to their cell membranes.

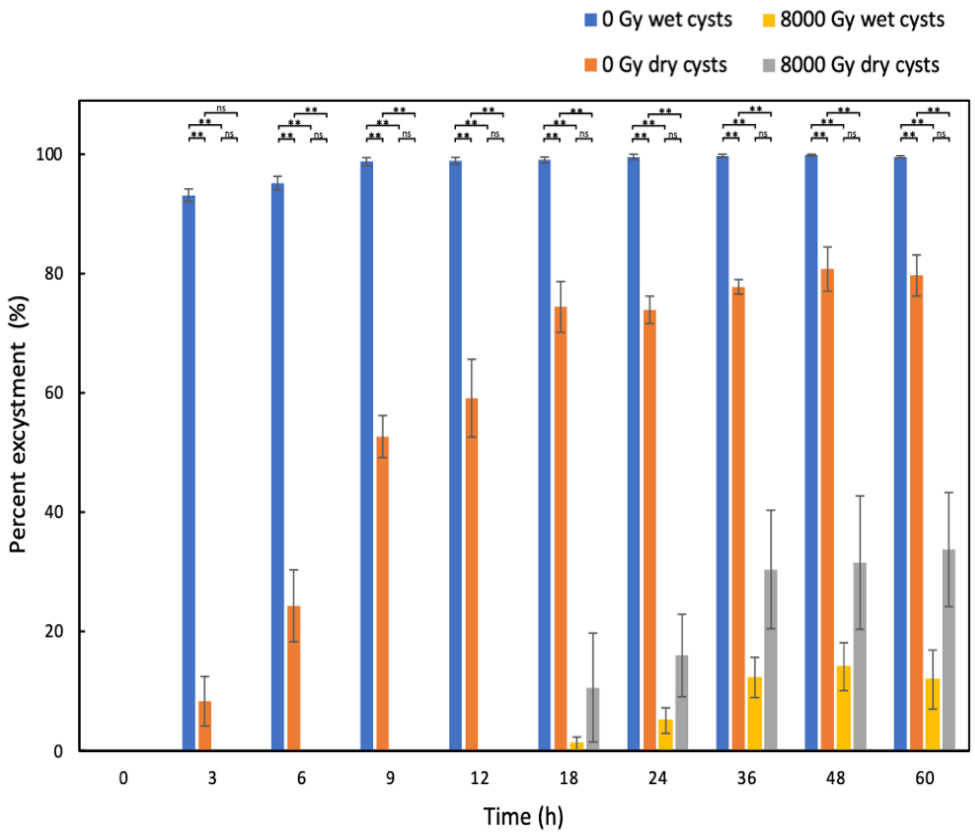

Fig. 4. Excystment of *Colpoda* non-irradiated and irradiated wet cysts and dry cysts as a function of time after the induction of excystment. Each column and bar shows the mean and standard error of six samples, respectively. Double asterisks and ns indicate a significant difference at $p < 0.01$ and not significant among samples, respectively (Mann-Whitney U test).

Radiation damage in cells (Richer et al., 2016) is mediated by reactive oxygen species (ROS) stress (Jung et al., 2017; Close et al., 2013; Imlay and Linn, 1988). In gamma irradiated cells, DNA is oxidized and proteins are carbonylated (Halliwell and Gutteridge 1999; Azzam et al., 2012). However, this damage can be repaired during the active cell phase of the hyperthermophile *P. furiosus* (Dirggiero et al., 1997). Repair of *C. cucullus* wet cysts

after 4,000 Gy gamma irradiation has been reported, but not for dry cysts due to its desiccation (Sogame et al., 2019a).

A graphical summary of our previous study (Saito et al., 2020) and this study is shown in Fig. 5. Damage in irradiated wet cysts was repaired during and after gamma irradiation, but damage in irradiated dry cysts was repaired only after the induction of excystment (Saito et al., 2020; Fig. 5). Therefore, both gamma irradiated wet and dry cysts were able to gradually revert to vegetative cells, but the excystment of wet cysts started sooner after the induction of excystment and was greater than that of dry cysts after 4,000 Gy gamma irradiation (Saito et al., 2020; Fig. 5). The repair delay in dry cysts may be due to the lack of water in these cysts, although we cannot exclud the possibility that wet cysts may be affected by radiation attenuation due to water. In this study, both wet cysts and dry cysts gradually reverted to vegetative cells after 8,000 Gy gamma irradiation, but the excystment of dry cysts tended to be faster and greater than that of wet cysts (Fig. 4). This result indicated that damage due to 8,000 Gy gamma irradiation could not be repaired during irradiation, but was repaired after the induction of excystment in both wet and dry cysts (Fig. 5). The damage in dry cysts, mediated by ROS stress produced by radiolysis of water due to the radiation (Azzam et al., 2012), was less than in wet cysts because of the lack of water in dry cysts. Hence, the excystment of dry cysts tended to be greater than that of wet cysts after 8,000 Gy irradiation (Fig. 4, 5). For non-irradiated cysts, > 90% of wet cysts had excysted and about 10% of dry cysts had started to excyst by 3 h after the induction of excystment, and almost all wet cysts and about 80% of dry cysts had excysted by 60 h after the induction of excystment (Fig. 4). However, 8,000 Gy irradiated wet and dry cysts had started to excyst at 18 h after the induction of excystment: about 10% and 40% of the gamma irradiated wet and dry cysts, respectively, were excysted by 60 h after the induction of excystment (Fig. 4).

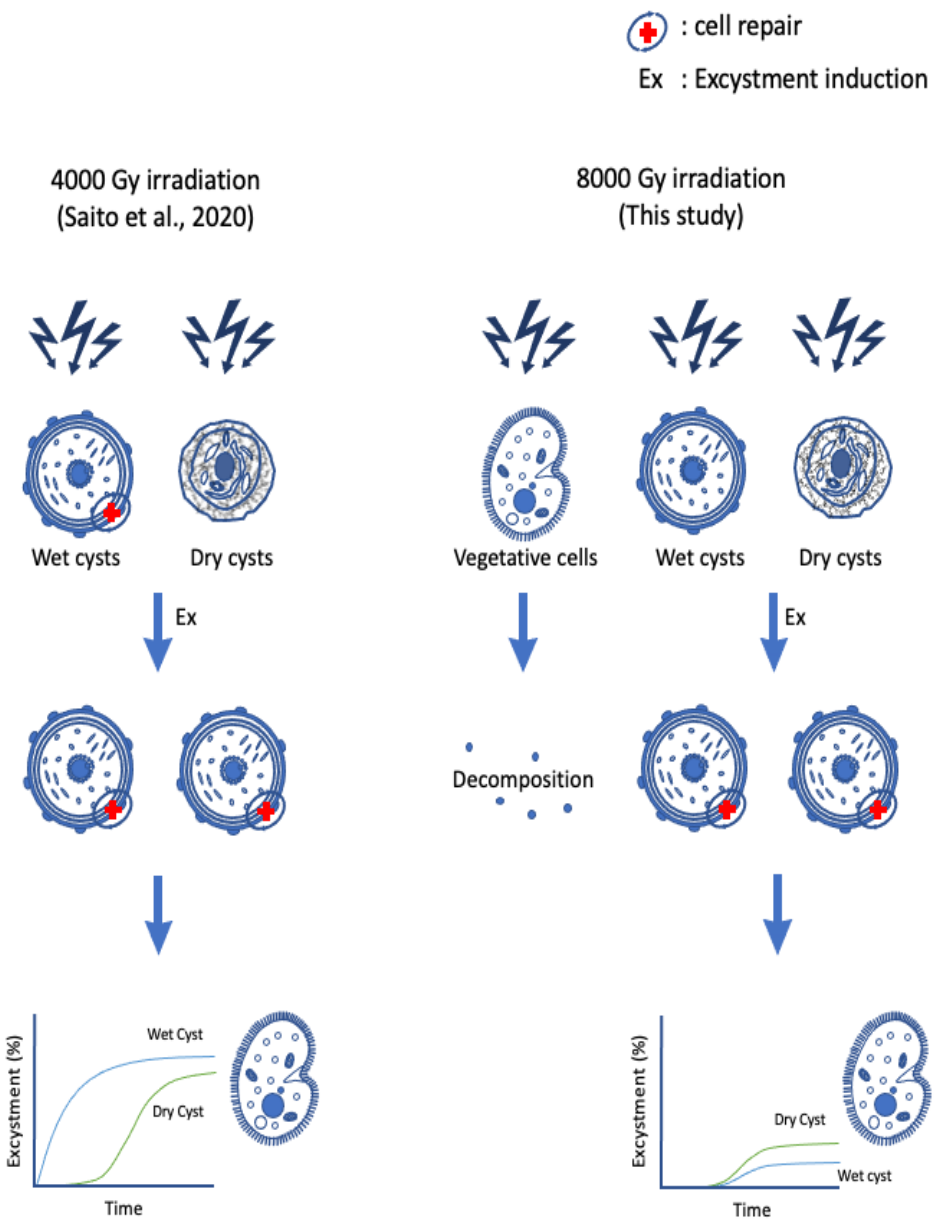

Fig. 5. Schematic summary of our current and previous results (Saito et al., 2020) on the events in the irradiation and induction of excystment of *C. cucullus* cells and cysts.

In this study, we found an extreme tolerance to gamma radiation in *C. cucullus* resting cysts. *Colpoda cucullus* vegetative cells do not have this tolerance and are decomposed by 8,000 Gy gamma radiation: the damage was lethal even at 500 Gy gamma radiation (Saito et al., 2020), similar to bacteria (Thornley 1963; Daly 2009). However, *C. cucullus* acquires

extreme tolerance to gamma radiation by forming resting cysts as cryptobiotic forms. Although *Colpoda* are not exposed to such high radiation in their habitats, they do have extreme gamma radiation tolerance. *Colpoda* may have evolved such tolerance as a strategy to adapt to terrestrial environmental stresses, such as desiccation, and to protect themselves from ROS stress (França et al., 2007). In resting cysts, the cyst wall is important to protect cysts from physical damage and for the maintenance of cell shape for cell repair (Sogame et al., 2019a). This may enable the excystment of resting cysts after environmental stress.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

SUBMISSION DECLARATION AND VERIFICATION

The authors declare that this manuscript is approved by all authors, has not been published before, and will not be published elsewhere in the same form without the written consent of the copyright-holder.

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