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Soil extract induces excystment and inhibits encystment in the terrestrial ciliate *Colpoda cucullus*

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ABSTRACT
The terrestrial ciliated protozoan *Colpoda cucullus* inhabits soil and survives water-limited condition by forming a resting cyst which is known to be highly resistant to harsh environments. Although encystment and excystment of this ciliate can be induced experimentally under laboratory conditions, little is known about the actual environmental factors that control such dynamic changes in cell morphology in nature. Therefore, in this study, we investigated the effects of soil extracts on *C. cucullus* and attempted to identify the active substances that may be present in the soil extract. The soil extract induced excystment of the resting cyst, and it inhibited encystment of the vegetative cell. These effects of the soil extract might be brought by fulvic acid, one of humic substances contained in soil, together with some soil inorganic elements.

Keywords: *Colpoda cucullus*, soil extract, excystment, encystment, fulvic acid

INTRODUCTION
In recent years, increasing attention has been paid to the soil microbes. Major groups of soil organisms include small flatworms, nematodes, rotifers, and tardigrades (Yarwood et al., 2020). The terrestrial ciliate *C. cucullus* inhabits soil and survives harsh environments with limited water by transforming to resting cysts (Watoh et al., 2005). This process is called "resting cyst formation" or "encystment", and the resulting cyst is known to be highly resistant to desiccation, high temperature or freezing, extreme pH and ultraviolet radiation (Taylor and Strickland, 1936; Maeda et al., 2005; Müller et al., 2010; Sogame et al., 2011; Matsuoka et al., 2017; Nakamura et al., 2020; Yamane et al., 2020). When the habitat restored again, the encysted *C. cucullus* cells rapidly transform into the vegetative state and break through the cyst wall to emerge from the cyst (Funadani et al., 2013). This process is called "excystment". *C. cucullus* culture has been established in our laboratory, in which en- and excystment can be experimentally induced routinely. For encystment induction, we add Ca^{2+}

to overpopulated vegetative cells (Yamaoka et al., 2004; Maeda et al., 2005; Matsuoka et al., 2009), since increase in extracellular Ca^{2+} and frequent mechanical stimulation by cell-to-cell contacting are known to induce encystment. For excystment induction, we add infusion of wheat leaves to the cyst, since chlorophyll-derived molecules such as porphyrin are known to induce excystment (Watoh et al., 2003; Tsutsumi et al., 2004; Akematsu and Matsuoka, 2007). Recently, we found that a rapid increase in temperature induces encystment in *C. cucullus* (Shimada et al., 2021). Vegetative cells of *C. cucullus* might receive the temperature signal from sunlight and encyst prior to water evaporation to avoid forthcoming dehydration in natural environments. However, very little is known about effective environmental factors that induce excystment in *C. cucullus* in nature. It has been reported that excystment could be triggered by adding soil extract in an oligotrich ciliate *Meseres corlissi* (Müller et al., 2006). However, water-soluble soil component(s) that induces excystment in *M. corlissi* was not identified in the study and the so-called "a soil factor" still remains unknown.

Therefore, the main objective of the present study was to investigate the effect of soil extract on resting cysts of *C. cucullus* and to identify water-soluble and excystment-inducible components in the soil extract, with a focus on organic and inorganic substances. On the other hand, it has been reported that excystment-inducible substances such as wheat leaf infusion and chlorophyllin showed inhibitory effect of encystment in *C. cucullus* (Tsutsumi et al., 2004). Therefore, in addition to its ability of excystment induction, we further investigated an ability of encystment inhibition of the soil extract by evaluating the effect of it or its components on vegetative cells after encystment induction.

MATERIALS AND METHODS

Culture

Culture of *C. cucullus* was performed according to our previous paper (Shimada et al., 2021). Briefly, vegetative cells of *C. cucullus* were cultured in autoclave-sterilized 0.05% (w/v) aqueous extract of dried wheat leaves (culture medium) in a flask and placed in a temperature-controlled room (25°C) for more than three weeks. During the incubation period, cells proliferated and spontaneously encysted on a bottom of the flask. Excystment was induced by washing cysts three times with deionized water (DW) and adding a sterile culture medium. Excysted vegetative cells cultured for two days were used in this study.

Soil extract

The soil used in the experiments was obtained from a garden on the campus of Kochi University, Japan (33°32'57.4"N 133°29'12.3"E). A glass container containing 300 g of the garden soil was filled with DW to 500 mL and autoclaved at 121°C for 15 min. After centrifugation at 700 × *g* for 2 min, supernatant was filtered through a Stericup Quick Release-GP (Millipore, MA, USA) and stored at 4°C until use.

Encystment and excystment

Vegetative cells of *C. cucullus* were washed twice with 1 mM Tris-HCl (pH 7.2), and suspended at a high cell density (25,000 cells/mL) in an encystment-inducing medium (EnIM) consisting of 0.05 mM $CaCl_2$ and 0.5 mM Tris-HCl (pH 7.2). Soil extract or water-soluble components in the soil extract were added to EnIM to examine their encystment-inhibitory effect on the encystment-induced vegetative cells. In this study, two different types of cysts were prepared from vegetative cells: wet cysts were prepared by incubation in EnIM for 2 days, and dried cysts were prepared by incubation for 2 weeks in EnIM, followed by removal of EnIM and drying for one week at room temperature. For excystment induction, cysts were prepared on a Petri dish, washed with DW, and the excystment-inducing medium (ExIM, 0.2% (w/v) aqueous extract of dried wheat leaves) was added. To investigate excystment-inducible effect, soil extract or water-soluble components in the soil extract were added to wet and dried cysts instead of ExIM.

Encystment and excystment rates

For measurement of the encystment rate, numbers of cysts and swimming vegetative cells were counted among more than 200 cells under a stereomicroscope (SMZ-10, Nikon, Tokyo, Japan) at 3 h and 5 h after encystment induction. The encystment rate was calculated by the following formula.

Encystment rate (%) = $C / (C + V) \times 100$,

where C and V denote numbers of cysts and vegetative cells, respectively.

For measurement of the excystment rate, numbers of filled and empty cysts were counted among more than 1,000 cysts at 5 h (for wet cysts) or 24 h (for dried cysts) after excystment induction. The excystment rate was calculated by the following formula.

Excystment rate (%) = $Ce / (Ce + Cf) \times 100$,

where Ce and Cf denote numbers of empty and filled cysts, respectively.

Elemental analysis of soil extract

Inductively coupled plasma optical emission spectrometry (ICP-OES) was performed on ICPE-9000 (Shimadzu, Tokyo, Japan) to measure concentrations of elements contained in the soil extract.

Statistical analysis

All experiments were carried out five times. Data of en- and excystment rate (%) were expressed as mean values ± standard errors and analyzed by Welch's t-test. Asterisks in figures indicate significant differences at $0.01 < p < 0.05$ (*) or $p < 0.01$ (**), and "ns" indicates that the mean values are not significantly different ($p > 0.05$).

RESULTS

Effect of soil extract on wet and dried resting cysts

In the present study, aiming to identify the element that induces excystment in *C. cucullus* in natural environment, we investigated effect of soil extract on both wet and dried resting cysts. First, soil extracts of various dilutions were added to wet resting cysts, and the excystment rate after 5 h was measured. As shown in Fig. 1a, the positive control, ExIM, induced excystment at a significantly higher rate than no treatment (DW). Similarly, the soil extract (SE) induced excystment in a concentration-dependent manner. Next, the effect of the soil extract was similarly examined using dried resting cysts (Fig. 1b). Since the excystment rate was expected to be lower in dried resting cysts than in wet resting cysts, the excystment rate was measured 24 h after the addition of the soil extract at each dilution rate. As shown in Fig. 1b, the soil extract also showed a concentration-dependent excystment-inducing effect on the dried resting cysts.

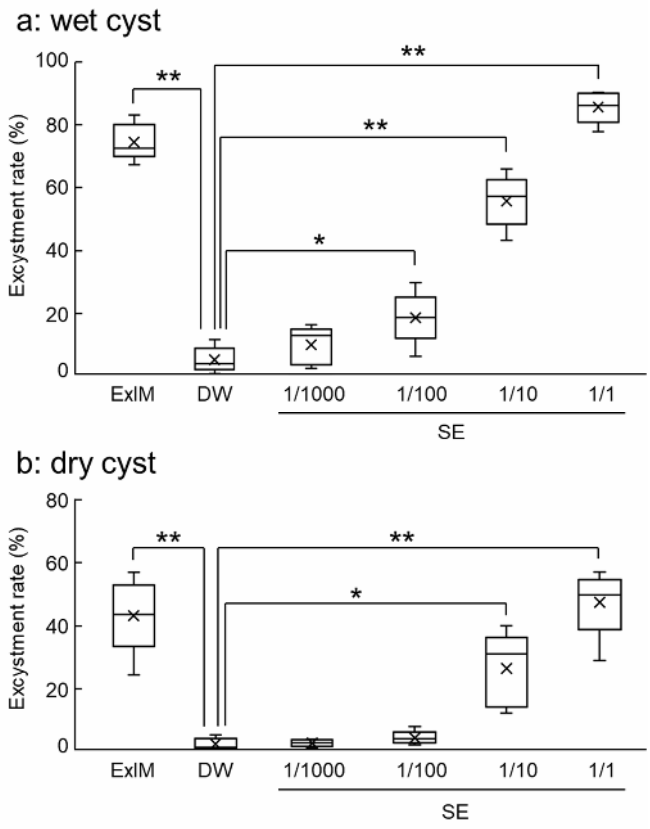

Fig. 1. Effect of soil extract prepared at various dilutions on wet (a) and dry (b) resting cysts. Boxes indicate the median and interquartile ranges, and whiskers extend to minimum and maximum values (n=5). Mean values are indicated by crossed-out symbols. In both types of resting cysts, excystment-inducing medium (ExIM, shown as the positive control) induced excystment at a significantly higher rate than the negative control, deionized water (DW). Similarly, soil extract (SE) also induced excystment in a concentration-dependent manner. *: $0.01 < p < 0.05$, **: $p < 0.01$.

Effect of inorganic elements and fulvic acid on excystment

To determine the type and content of inorganic elements in the soil extract, ICP-OES was performed (Fig. 2). Silica was the most abundant element among inorganic elements contained in the soil extract (0.95 mM). Since the concentrations of other inorganic elements detected in the soil extract were all less than half of silica, this result raised a possibility that silica might be the element that triggers excystment. To verify this hypothesis, various inorganic elements, including silica, were added to wet resting cysts and the excystment rate was examined. The concentrations of inorganic elements were adjusted to equal the concentrations detected in the soil extract. As shown in Fig. 3, the soil extract (1/1 dilution) induced excystment when compared to DW as control. On the other hand, the addition of any inorganic elements did not show the inducing effect of excystment. Moreover, excystment was not induced even when the mixture of all inorganic elements was added to the resting cyst (data not shown).

The effect of fulvic acid on wet resting cysts was examined, since fulvic acid is one of the major components of humic substances in the soil. Standard soil fulvic acid was obtained from Japanese Humic Substances Society (JHSS), and was added at a concentration of 100 µg/mL to the wet resting cysts, independently or together with the inorganic elements detected in the soil. As shown in Fig. 4, fulvic acid alone did not induce excystment. However, when added with Na_2SiO_3, excystment was induced, and the rate was significantly higher than with fulvic acid alone. Excystment did not occur with NaCl+fulvic acid, suggesting that the effect was due to the presence of SiO_3 anion. Other elements did not show any effect even when added together with fulvic acid.

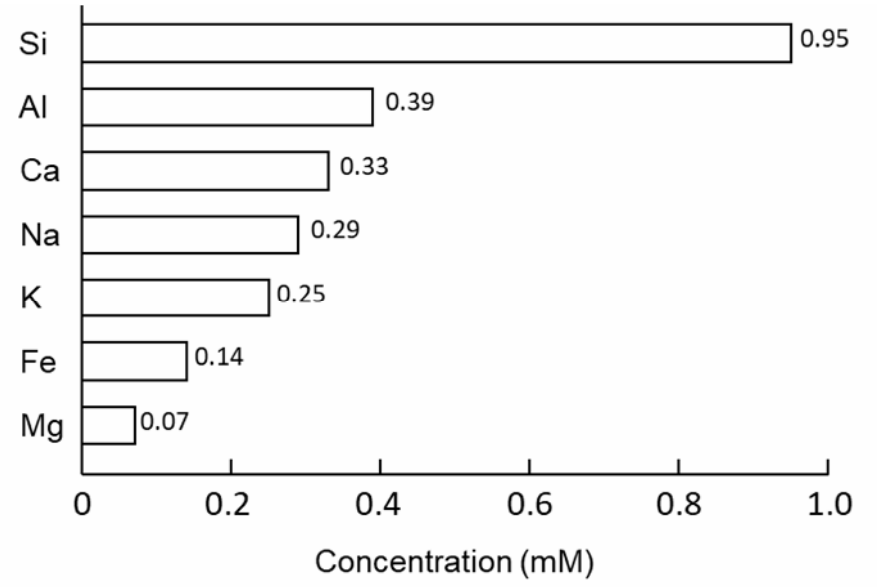

Fig. 2. Inorganic elements detected in the soil extract. Inductively coupled plasma optical emission spectrometry (ICP-OES) showed that silica (Si) is the most abundant element among inorganic elements contained in the soil extract. Data of other trace elements are not shown here.

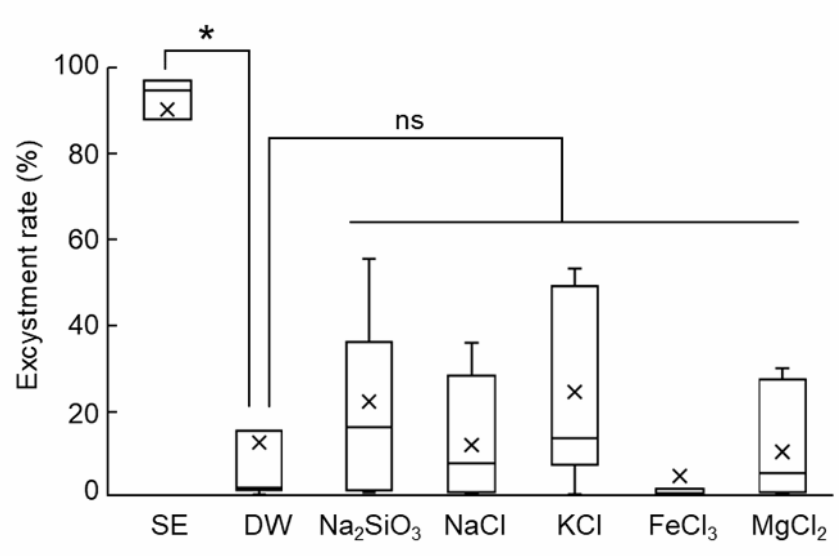

Fig. 3. Effect of inorganic elements on wet resting cysts. Compared to deionized water (DW), soil extract (SE) induced excystment. On the other hand, any inorganic reagents added did not show excystment-inducing effect. Boxes indicate the median and interquartile ranges, and whiskers extend to minimum and maximum values (n=7). Mean values are indicated by crossed-out symbols. The asterisk represents a significant difference from the DW control ($p < 0.01$), and ns represents no significant difference.

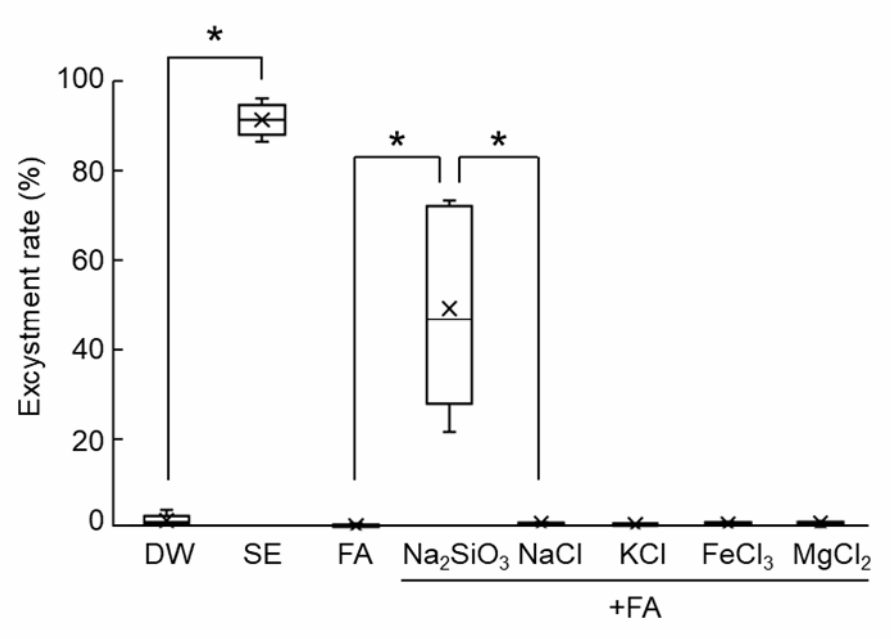

Fig. 4. Effect of fulvic acid with or without inorganic elements on wet resting cysts. Fulvic acid (FA) alone did not induce excystment. However, when added together with Na_2SiO_3, it induced excystment, the rate of which was significantly high than FA alone. The addition of NaCl with FA did not cause excystment. Boxes indicate the median and interquartile ranges, and whiskers extend to minimum and maximum values (n=5). Mean values are indicated by crossed-out symbols. Asterisks represent significant differences ($p < 0.01$).

Effect of soil extract and inorganic salts on encystment

This study also examined whether soil extracts have an inhibitory effect on encystment in *C. cucullus.* To evaluate encystment-inhibitory effect of the soil extract, vegetative cells of *C. cucullus* were treated with EnIM containing a 50% dilution of the soil extract or corresponding concentrations of various inorganic salt, and the encystment rate at 3 h and 5 h after induction of encystment was determined. As shown in Fig. 5a, treatment with EnIM with the soil extract strongly suppressed encystment already at the 3 h stage compared to EnIM alone. This inhibitory effect was weakened at 5 h, and more than half of the vegetative cells were encysted (Fig. 5b), suggesting that the soil extract has an effect of delaying the onset of encystment. This result, together with the results shown in Fig. 1, indicate that the soil extract not only has an excystment-inducing effect on both wet and dried cysts, but also encystment-inhibitory effect on the vegetative cells of *C. cucullus*. To identify substances present in the soil extract that inhibit encystment, vegetative cells were treated with EnIM containing half of each inorganic element detected in the soil extracts (Fig. 2), and encystment rates were examined. As shown in Fig. 5, all of the inorganic compounds tested did not inhibit encystment. In addition, the addition of a mixture of all inorganic salts to the encystment-induced vegetative cells did not inhibit encystment (data not shown). These results indicate that none of the inorganic elements in the soil extract alone have the ability to inhibit encystment in *C. cucullus*.

The involvement of fulvic acid in the inhibitory effect of the soil extract on encystment was investigated. Fulvic acid was added alone or with inorganic elements to encystment-induced vegetative cells, and the encystment rate was measured 5 h after the encystment induction. As shown in Fig. 6, fulvic acid alone showed no inhibitory effect, but when inorganic salts such as Na_2SiO_3 and NaCl were added together, encystment was suppressed to some extent. Other salts such as KCl, $FeCl_3$ and $MgCl_2$ did not show inhibitory effect on encystment. The addition of fulvic acid together with a mixture of all inorganic elements reduced the rate of encystment by about 20%.

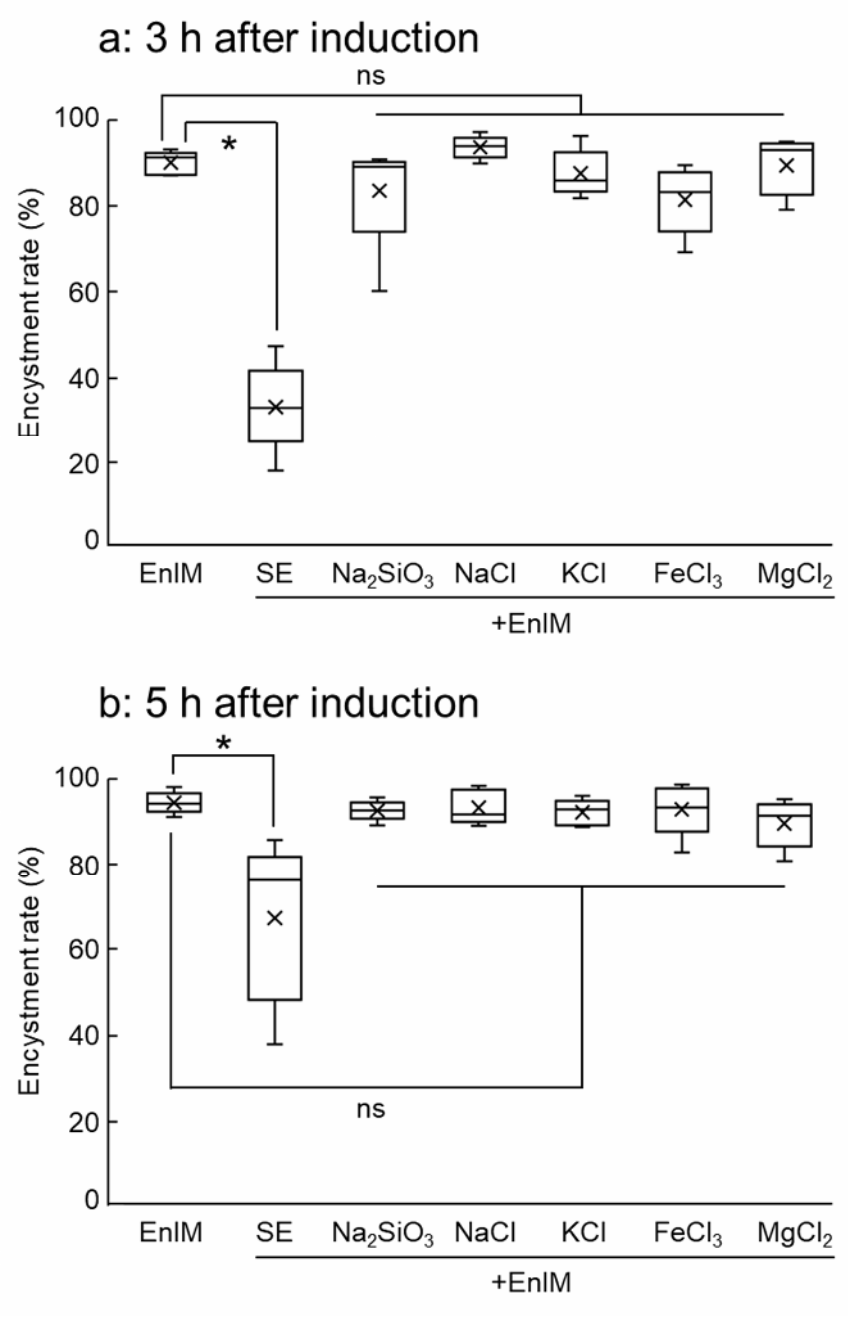

Fig. 5. Effects of inorganic elements on encystment. Encystment rates were determined at 3 h (a) and 5 h (b) after the vegetative cells were induced for encystment. The addition of soil extract (50% SE) to encystment-inducing medium (EnIM) significantly inhibited cyst formation compared to EnIM alone. On the other hand, all inorganic elements tested did not show the encystment-inhibitory effect. Boxes indicate the median and interquartile ranges, and whiskers extend to minimum and maximum values (n=5). Mean values are indicated by crossed-out symbols. Asterisk represents significant difference ($p < 0.01$), and ns represents no significant difference.

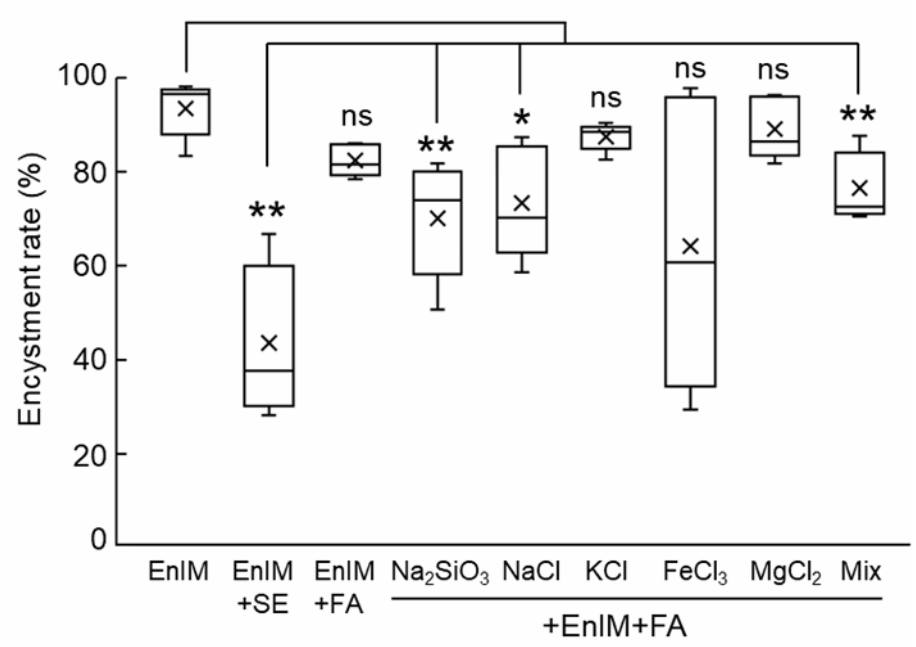

Fig. 6. Effect of fulvic acid with and without inorganic elements on encystment-induced vegetative cells. Encystment rates were determined at 5 h after the vegetative cells were induced for encystment. Compared to encystment-inducing medium (EnIM) as a positive control, addition of the soil extract (SE) inhibited encystment. Fulvic acid (FA), on the other hand, showed no inhibitory effect, but when added together with inorganic salts such as Na_2SiO_3 and NaCl, encystment rate was suppressed to some extent. Boxes indicate the median and interquartile ranges, and whiskers extend to minimum and maximum values (n=7). Mean values are indicated by crossed-out symbols. The asterisks represent significant differences from the EnIM control (*: $0.01 < p < 0.05$, **: $p < 0.01$), and ns represents no significant difference.

DISCUSSION

Much work has been conducted with various kinds of pathogenic protozoa to identify the key factors responsible for the induction of excystment. It has been demonstrated that, for example, exposure to sequential low-pH and subsequent exposure of proteases such as trypsin, chymotrypsin, and human pancreatic fluid stimulated excystation of in vitro-derived cysts in a parasitic protist *Giardia lamblia* (Boucher and Gillin, 1990; Rice and Schaefer, 1981) and metacercariae cysts of *Paragonimus ohira* or *P. heterotremus* (Ikeda, 2004; Intapan and Maleewong, 2001). The experimental conditions in these studies simulated a passage of cysts through a stomach and an intestine of their hosts, implying importance of mimicking natural environments in a life cycle of microorganisms in vitro. Similar studies have been conducted in free-living protists. It has been reported that chlorophyllin, polypeptone, yeast extract and aqueous extract of grain leaves induced excystment in a terrestrial ciliate *C. cucullus* (Akematsu and Matsuoka, 2007; Tsutsumi et al., 2004; Watoh et al., 2003). However, the natural factors actually inducing excystment remains unknown, since these substances are not always abundant around encysted cells in the soil, even when

habitat conditions become favorable again due to rainfall and other factors. Therefore, this study examined the effects of soil extracts on wet and dried cysts of *C. cucullus*, assuming a near-natural situation in which encysted cells are exposed to rainwater spread in the soil. The results showed that the soil extract had an excystment-inducing effect on both wet and dried cysts.

In the present study, as shown in Fig. 2, concentrations of inorganic elements contained in the soil extract were determined by ICP-OES. When added alone or in a mixture, none of these inorganic elements showed either excystment-inducing or encystment-inhibiting effects. Fulvic acid likewise showed neither induction of excystment nor inhibition of encystment alone. However, interestingly, strong excystment-inducing activity was detected when Na_2SiO_3 coexisted with fulvic acid (Fig. 4). Likewise, some degree of inhibition against encystment was also detected in the presence of fulvic acid and some inorganic salts including Na_2SiO_3 (Fig. 6). In Japan, agricultural soils typically contain fulvic acid at concentrations of 0.5 - 30 mg/mL (Kuwatsuka et al., 1992; Watanabe et al., 2001). On the other hand, concentrations of silica were measured to be approximately 0.2 mM in the middle river water and 0.6 mM in the groundwater, respectively (Kubo and Yamahira, 2020; Miyashita, 2004). In this study, a mixture of 0.1 mg/mL fulvic acid and 0.5 mM Si showed a strong excystment-inducing effect. Since these concentrations are within the range of the environment of wet soils in common agricultural lands in Japan, they are definitely the main factors for inducing natural excystment, and maybe for suppressing encystment, of *C. cucullus*.

ICP-OES showed that Al and Ca exist in the soil extract at concentration of 0.39 and 0.33 mM, respectively. Aluminum metal is insoluble in water and $AlCl_3$ is unsuitable for use in experiments because when dissolved in water it produces hydrochloric acid, which is toxic to vegetative cells of *C. cucullus*. On the other hand, it has been reported that influx of Ca^{2+} into target cells triggers in vitro excystment in metacercaria of *Paragonimus ohirai* (Ikeda, 2001; Ikeda, 2004), indicating that Ca^{2+} might be a candidate for the excystment inducing factor in *P. ohirai*. However, it is not applicable to *C. cucullus*. Since Ca^{2+} was added when encystment was induced (see Materials and methods), Ca^{2+} can be regarded as a factor for inducing encystment rather than excystment. It has also been reported that removal of Ca^{2+} which binds to the cyst wall is required for induction of excystment in *C. cucullus* (Maeda et al., 2005). For these reasons, Al and Ca contained in the soil extract were excluded from the candidate for factors in the induction of excystment and inhibition of encystment in *C. cucullus*. ICP-OES also showed the existence of trace elements in the soil extract (P, 0.05 mM; B, 0.01 mM; Mn, 0.002 mM; Ba, 0.00053 mM; Sr, 0.00052 mM. Data are not shown in Fig. 2). The possibility that these trace elements act as soil factors, either solely or together with fulvic acid, cannot be ruled out.

Generally, soil organic matter consists of humic and non-humic substances (Stevenson, 1994). The humic substances are further divided into three groups based on their solubility in water; humin, humic acid, and fulvic acid, whereas non-humic substances include fats, sugars, and amino acids. As solubility of the humic substances, humin is insoluble in water, humic acid is soluble in base solution but insoluble in acid solution, and fulvic acid is soluble in both base and acid solutions (Duan et al., 2020; Hartenstein, 1981; Pham et al., 2021; Winkler and Ghosh, 2018; Xu et al., 2018). It is highly unlikely that humin

and humic acid play a role as a factor for excystment induction. Due to its insolubility, it is unlikely that humin and humic acid would be present in water seeped into the soil. For these reasons, among the humic substances, only fulvic acid is a candidate for inducing excystment in *C. cucullus*.

After the addition of soil extract to induce excystment, excysted vegetative cells of *C. cucullus* encysted spontaneously within a few days even in the presence of the soil extract. The spontaneous encystment occurs normally even when vegetative cells are excysted by the addition of culture medium (aqueous extract of dried wheat leaves). However, the rate of spontaneous encystment in the soil extract-induced excysted cells was lower than that in the culture medium-induced excysted cells (data not shown). Actually, as shown in Figs. 5 and 6, soil extract has an ability to inhibit encystment. The fact that excysted vegetative cells by adding soil extract are hard to be encysted again in the presence of soil extract suggests the possibility that soil extract might contribute to make the period for growing and proliferating of vegetative cells of *C. cucullus* longer in the harsh environment with limited water.

Detailed morphological alterations during excystment process in *C. cucullus* were well described in Funadani et al. (2013). No significant difference of the excystment process between ExIM-induced and soil extract-induced excysted cells was found when observed under an optical microscope. It is likely that a unique intracellular signal transduction system would be activated even when excystment is induced by different factors such as chlorophyll-derived certain molecules in cereal extract or certain organic molecules like fulvic acid in soil extract. Otherwise, molecules commonly existing in both extract might be a strong candidate for the factor for excystment induction in natural environment in *C. cucullus*. Further studies are needed to identify the excystment-inducible factor that also has encystment-inhibitory effect for *C. cucullus* by assessing reaction of resting cysts and encystment-induced vegetative cells, respectively, after adding components of cereal extract and soil extract.

CONCLUSION

In the present study, we demonstrated for the first time that soil extract has an ability to induce excystment of resting wet and dried cysts and to inhibit encystment of vegetative cells in the terrestrial ciliated protozoan *C. cucullus*. Fulvic acid, one of the humic substances contained in soil, was found to play an important role in the effect of soil extract on *C. cucullus* but the relationship between fulvic acid and soil inorganic elements must be investigated for further understanding a role of soil in adaptive strategy of the small underground inhabitant, *C. cucullus*, in the natural environment.

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CONFLICT OF INTEREST

All authors declare no conflict of interest in the study.

SUBMISSION DECLARATION AND VERIFICATION

The authors declare that this manuscript is original, has not been published before and is not currently being considered for publication elsewhere.

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