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Cytological analysis of the effect of reactive oxygen species on sclerotia formation in
Sclerotinia minor

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Abstract

The Sclerotium is one of the most persistent organs in filamentous fungi. Control of sclerotial formation is promising in the prevention of sclerotial disease. In this study, cytological analyses of sclerotial development were conducted in *Sclerotinia minor*. Number and size of sclerotia were correlated with nutrient concentration of the media. Interruption of aeration by sealing with parafilm completely suppressed sclerotial formation. We also found that reactive oxygen species (ROS) generated two phases, i.e., hydrogen peroxide at sclerotial initial stage and O_2^- at outer layer of sclerotial development/mature stages, during sclerotial formation. Ultrastructural analyses revealed that ROS was prominently produced at the outer layer of sclerotia in sclerotial mature phase. Although most of the inhibitors for ROS generation enzymes were ineffective for

sclerotial formation, ascorbic acid, one of the scavengers of hydrogen peroxide, inhibited melanin biosynthesis during sclerotial maturation stage. The mycelia sealed with parafilm, when exogenously sprayed with hydrogen peroxide, could not produce sclerotium. These results indicated that ROS generation during sclerotial formation is mainly involved in the production of melanin layer.

Introduction

Sclerotium is defined as multiple hyphal aggregates that are persistent for long periods (Butler 1966). Since sclerotium is the primary source of infection for sclerotial producing fungi (Chet & Henis 1975), it is important to know the mechanism of sclerotial formation to control sclerotial disease. We used *Sclerotinia minor* as a model fungal pathogen, because this pathogen produces sclerotia uniformly on the agar medium, and in short time with a suitable sclerotial size (ca. 1 mm diameter) for electron microscopy analysis. *S. minor* is a phytopathogenic ascomycete fungus that infects a broad range of plant species (Melzer *et al.* 1997).

Georgiou *et al.* (2006) classified three sclerotial developing stages: sclerotial initials (SI), sclerotial developing (SD), and sclerotial mature (SM). SI comprises sparse hyphal aggregates with white colour, SD comprises dense hyphal aggregates with grey colour, and SM comprises matured hyphal aggregates with melanised black colour. SM is further differentiated into two parts: melanised surface layer (rind), and internal mycelial aggregates (medulla) (Willetts & Bullock 1992). Rind is responsible for stress tolerance and medulla acts as the nutrient reservoir containing glycogen and lipid body (Willetts & Bullock 1992).

Initiation signal of sclerotial formation is mediated by various factors such as light, temperature, air permeability, pH, nutrient contents, physical stress of hyphae, and chemical

compounds from other microorganisms (Chet & Henis 1975; Henis 1965; Mukiibi 1969; Willetts 1978). These factors may cause oxidative stress in mycelia. Indeed, accumulation of reactive oxygen species (ROS) and lipid hydroperoxides was correlated with sclerotial development (Georgiou *et al.* 2006). Moreover, antioxidant compounds (e.g., ascorbic acid and β -carotene), hydroxyl radical scavengers (e.g., salicylate and ethanol), and thiol redox state modulators (e.g., glutathione and *N*-acetyl cysteine) suppressed sclerotial formation (Georgiou *et al.* 2006). Therefore, oxidative stress is believed to be one of the initiation factors for sclerotial formation. However, these conclusions were mostly based on the measurement of oxygen stress-related compounds or the inhibitor study. Biochemical measurements of oxygen stress-related compounds were correlated with sclerotial formation. However, temporal/spatial distribution of ROS has not been studied in detail by cytological analysis. Moreover, it was difficult to conclude whether the correlation between sclerotial formation and accumulation of these compounds was a cause or effect. Inhibitor studies also had some problems; it was difficult to characterise the suppression of sclerotial formation, and old inhibitor studies exhibited low quality pictures and recent inhibitor studies exhibited only quantitative data without any picture. Sclerotial developmental stage was recently defined to be divided into three stages, i.e., SI, SD, and SM (Georgiou *et al.* 2006); the old inhibitor studies did not consider the sequential course of sclerotial development. It is imperative to know which sclerotial stage is blocked during the treatment with inhibitor. Some inhibitors might be targeted to SI, whereas others target between SI and SD. We have tried to examine the effects of inhibitors on sclerotial formation by taking into account all the sclerotial developmental stages.

In this study, we evaluated the localisation of ROS generation during sclerotial development by histochemical and ultrastructural analyses. We also evaluated the effects of inhibitors and scavengers on sclerotial formation. Furthermore, we evaluated the effects of

nutrient contents and air permeability on sclerotial formation in *S. minor*.

Materials and Methods

Fungal isolate

S. minor Jagger strain Sm-5 was isolated from an infected tomato plant in Tochigi prefecture, Japan. Culture medium used was either PDA medium containing 24 g potato dextrose broth (BD Biosciences, Franklin Lakes, NJ, USA) in 1.5% (w/v) agar, or sclerotial formation medium (Georgiou *et al.* 2001) containing 15 mM ammonium nitrate, 2 mM sodium chloride, 0.5 mM magnesium sulfate, 10 μ M thiamine, 0.1 M glucose, 0.1% (w/v) yeast extract, and 1.5% (w/v) agar in 10 mM potassium phosphate (pH 7.0).

Sclerotial formation test

PDA medium (normal volume was 10 ml) was solidified in a petri dish 5 cm in diameter, and cellulose membranes (PT #300, Rengo, Osaka, Japan), which were cut in the same size as the petri dish, were laid on the surfaces of the agar plate. Mycelial plugs (ca. 4 mm square) of *S. minor* were inoculated at the centre of the petri dish. When the mycelia covered the whole petri dish, cellulose membranes with mycelia were transferred to other agar media, which had different concentration of nutrients or inhibitors. To evaluate the effect of aeration, the petri dishes were sealed with parafilm. The number of sclerotia in each treatment was evaluated at different time points during the experiment. The experiments were demonstrated at least thrice. Significant differences were compared with control at the same time points by t-test ($P < 0.05$).

Chemicals

ROS scavengers and inhibitors of ROS generation in oxygen stress response pathways were used as follows: sodium ascorbate, diphenylene iodonium (DPI), 3-amino-1,2,4-triazole (3-ATZ), 4-amino-1,2,4-triazole (4-ATZ), sodium azide, salicylhydroxamic acid (SHAM), diethyldithiocarbamate (DDC). Hydrogen peroxide was used as an oxygen stressor. All chemicals were dissolved in distilled water (DW) or dimethyl sulfoxide (DMSO) at adequate concentrations and sterilised by syringe filtration. The sterilised chemicals were added to sclerotial formation medium after autoclaving.

Generation times (GT) were evaluated to assess the toxic effects of the chemicals on mycelial growth (Georgiou *et al.* 2001). GT was estimated using the following formula: $GT = (T2 - T1) / 3.3(\log DW2 - \log DW1)$, where T1 and T2 were two different points of incubation time, and DW1 and DW2 were the dry weight of mycelia at each time, respectively. Mycelia on the cellulose membranes at each time point were carefully removed and dried at 70°C for 12 hours.

Histochemical study

To detect ROS generation, nitro blue tetrazolium (NBT, for O_2^- detection) and diaminobenzidine (DAB, for hydrogen peroxide detection) staining were performed.

For macroscopic observation, mycelia grown on the agar media at different sclerotial development stages (i.e., mycelia, SI, SD, and SM) were treated with 0.05% (w/v) NBT stain in 50 mM sodium phosphate buffer (pH 7.4; PBS), vacuum-infiltrated, incubated for 1 hour at 25°C, washed thrice with DW, and observed.

For microscopic observation of each sclerotium, sclerotia at different developmental stages (SI, SD, and SM) were collected and rolled on the wet paper to remove air bubbles from the sclerotial surface. Then, the sclerotia were dipped in 0.05% (w/v) NBT stain or 0.01% (w/v) DAB stain in PBS, vacuum-infiltrated, incubated for 1 hour at 25°C, and washed thrice with

DW. The stained sclerotia were fixed in 100% ethanol and embedded with 4% (w/v) agar. The embedded sclerotia were trimmed and sliced into sections of 30 μ m thickness, using a Microslicer (Dosaka EM, Kyoto, Japan). The sections were observed under an optical microscope. To quantify ROS generation level, densities of NBT/DAB positive signals were scored as following; 0: no signal, 1: weak/sporadic, 2: intermediate, 3: strong signal from 10 to 30 sections in each treatment. Tukey's test ($P<0.05$) was examined the difference between treatments.

To confirm that each stain permeated into the core of sclerotia, red aqueous ink (Pilot, Tokyo, Japan) was used. Briefly, sclerotia at different developmental stages were collected and treated with or without rolling on the wet paper. The sclerotia were dipped in 1/10-diluted red ink, vacuum-infiltrated, and incubated for 1 hour at 25°C. Further steps were the same as those for NBT/DAB staining.

Ultrastructure study

To detect hydrogen peroxide, fragments of sclerotia at different developmental stages (SI, SD, and SM) were collected and rolled on a wet paper to remove air bubbles from the sclerotial surface. Then, the sclerotia were vacuum-infiltrated in 50 mM 3-(*N*-morpholino) propanesulfonic acid (MOPS; pH 7.2) with or without 5 mM cerium chloride (CeCl_3) at 25°C for 1 hour. The fragments were prefixed in 2.5% glutaraldehyde, buffered with 0.1 M cacodylate buffer (pH 7.2), at 4°C overnight, washed thrice with the same buffer for 10 min at each time, and then fixed with 1% buffered osmium tetroxide at 4°C for 1 hour. The fragments were then dehydrated in a series of ethanol (50%, 70%, 90%, and 100%) and embedded in Spurr's resin mixture (Nissin EM, Tokyo, Japan). The Spurr's resin mixture was exchanged thrice with fresh resin mixture every 2 days at 4°C to permeate resin mixture into the core of sclerotia. The resin

mixtures with sclerotia were embedded with embedding mould and heated at 70°C for 24 hours to produce resin blocks. Sections with a thickness of 90–120 nm were cut from resin blocks, using an ultramicrotome (MT-1; Sorval, Norwalk, CT, USA) and a diamond knife (Diatome, Bienne, Switzerland). Unstained sections were observed under an H-7100 electron microscope (Hitachi, Hitachinaka, Japan). For image analysis, electron microscopic negatives were printed on the same printing papers as those described by Morita *et al.* (2013). Each of the micrographs was scanned at 600 dots per inch (dpi) and saved as a JPEG file. We analysed the area of cerium-reactive products on the electron micrographs, using ImageJ software (<http://rsb.info.nih.gov/ij/index.html>). Then, we measured the areas of cerium-reactive products at the sclerotial cell walls. The relative ratio of the product area per unit area (μm^2) of the sclerotial cell wall was determined as the ratio of the area of cerium peroxide deposit (μm^2)/the area of the sclerotial cell wall (μm^2). We measured at least 100 cells from three different blocks.

Results

Effect of nutrient concentration on sclerotial formation

To evaluate the effect of nutrient concentration on sclerotial formation, first, we inoculated mycelial plugs directly onto different strengths of PDA media (i.e., 1/4-, 1-, 2-, and 4-strength) and incubated for 12 days. Higher strength of PDA media tended to delay sclerotial formation (Fig. 1A). Number of sclerotia was increased with the increase in the concentration of potato dextrose broth (Fig. 1A and Supplementary Fig. S1A). Moreover, the size of sclerotia became larger as the concentration became higher (Supplementary Fig. S1A). To exclude the effect of different mycelial growth rate in each strength of medium, 2-day-old mycelia incubated on cellulose membranes laid on 1-strength of PDA media were transferred to different strengths of

PDA media (i.e., 1/4-, 1-, 2-, 4-strength). In the 1/4-strength of PDA media, sclerotial formation was rapid and smaller than 1-strength of PDA media, however, the number of sclerotia was not significantly different, respectively (Fig. 1B and Supplementary Fig. S1B). In contrast, mature sclerotia were not produced in the 4-strength of PDA media after 6 days of inoculation, although numerous developing sclerotia were observed (Fig. 1B and Supplementary Fig. S1B). The higher amount of media in the same petri dish tended to suppress sclerotial formation (Fig. 1C and Supplementary Fig. S1C).

We also evaluated the effect of aeration on sclerotial formation. When the petri dishes with growing mycelia were tightly sealed with parafilm, sclerotial formation was suppressed (Fig. 1D and Supplementary Fig. S1D). When the petri dishes sealed with parafilm were deprived of the parafilm after 9 days of incubation, which was long enough for the mycelia to grow towards the edge of the petri dish and to stop growing mycelia, sclerotia were never produced (Supplementary Fig. S1D).

Histochemical analyses of ROS generation during sclerotial development

To detect ROS generation during sclerotial development, ROS indicators, NBT (for O_2^- detection) and DAB (for hydrogen peroxide detection), were directly applied to the mycelial growing in the media. We divided sclerotial development into four stages: solely mycelia (MY), sclerotial initial (SI), sclerotial developing (SD), and sclerotial mature (SM) (Fig. 2A and B). NBT- and DAB-positive signals were seen at hyphal tips in the MY stage and in mycelial aggregates in the SI and SD stages (Fig. 2C and D). It was difficult to observe positive signals in the sclerotia at the SM stage because the sclerotia were highly melanised (Fig. 2C and D). DAB-positive signals were observed at the cytosolic region of hyphal cell (Fig. 2E). Since air bubbles were frequently observed around sclerotial developing structures (Supplementary Fig.

S2 arrowheads), NBT stain might not have permeated into the core of the sclerotia, as aqueous red ink also did not permeate (Supplementary Fig. S2). By rolling sclerotia on the wet paper, air bubbles were removed and red ink successfully permeated into the core of the sclerotia in the SI and SD stages, although it did not permeate in the SM stage (Supplementary Fig. S2). By this technique, NBT and DAB staining were performed on the sclerotial developing structures in the SI, SD, and SM stages. Although NBT-positive signals were mostly observed at the surface of the SD and SM stages, it was sporadic in the SI stage (Fig. 3A). In contrast, DAB-positive signals were observed in entire mycelial aggregates in the SI stage (Fig. 3A). DAB-positive signals in the SD stage were variable, i.e., some sclerotial developing structures were intensely stained at the surface, whereas others were sporadic (Fig. 3A). DAB-positive signals in the SM stage were intense and uniform at the surface region (Fig. 3A).

We evaluated the effects of various inhibitors on ROS generation. We focused on using the DAB stain in the SI stage, and the NBT stain in the SD stage, because these stains were stable in the respective stages and expressed strong signal. In case of the DAB stain for SI stage, sodium diethyldithiocarbamate (DETC), a super oxide dismutase inhibitor, suppressed DAB staining (Fig. 3B). In case of the NBT stain for SD stage, diphenyleneiodonium, an NADPH oxidase inhibitor, suppressed NBT staining (Fig. 3B). Other ROS generation inhibitors did not suppress either DAB or NBT staining (Fig. 3B).

Ultrastructural analyses of ROS generation during sclerotial development

Ultrathin sections were obtained from each sclerotial developmental stage. Sclerotia were divided into two layers. In SD and SM, we observed surface dense hyphal layer (outer) and loose hyphal layer (inner) (Fig. 4A). Since the length of the outer layers was approximately 25 μm , this means that the distance of the outer layer in SI from the surface to the internal was 25

μm. In the ultrathin sections from the specimen with CeCl₃ treatments, cerium peroxide pigmentations that were products of reacting with cerium and hydrogen peroxide, were observed on the sclerotial cell wall (Fig. 4B). No cerium signal was observed in the specimen without CeCl₃ treatment (Supplementary Fig. S3). We quantified the area of cerium peroxide pigmentations on the sclerotial cell wall by ImageJ. We found that cerium signals were significantly (t-test; $p < 0.05$) increased at the outer layers in SM (Fig. 4C).

Is ROS an initiation signal for sclerotial formation?

We evaluated the effects of inhibitors for ROS generation on sclerotial formation. Ascorbic acid (AsA) is a scavenger of superoxide radical (Som *et al.* 1983). During the treatment with AsA, formation of mature sclerotia (SM) was suppressed (Fig. 5A and B). Although GT was increased with the increase in the concentration (Supplementary Fig. S5), mycelia still spread to the edge of the petri dish. During the treatment with high concentration of AsA, the mycelia were rugged, suggesting that sclerotial formation might have suppressed in the middle of the sclerotial development stage. Therefore, we evaluated the dynamics of sclerotial development by treating it with AsA. During the treatment with 7.5 mg/mL AsA, sclerotial development was delayed compared with that of the control (Fig. 5C). During the treatment with 30 mg/mL AsA, sclerotial development was inhibited at the SI stage (Fig. 5C).

Other ROS generation inhibitors like DPI (NADPH oxidase inhibitor), allopurinol (xanthine oxidase inhibitor), and DETC (superoxide dismutase inhibitor) did not affect sclerotial formation (Fig. 5D and E). Although sclerotial formation was reduced during the treatment with 25 μM DPI, mycelial growth was also inhibited, since generation time was increased (Supplementary Figs. S4 and S5).

Supplementation of hydrogen peroxide on agar media did not increase the number of sclerotia

(Fig. 6A and B). Hydrogen peroxide (10 mM) could not assist in the growth of mycelia (Fig. 6A and Supplementary Fig. S5). We then sprayed hydrogen peroxide on the growing mycelia sealed with or without parafilm. During the treatment with 10 mM hydrogen peroxide, sclerotial formation was increased in the absence of parafilm, however, sclerotium was never produced during the treatment with 10 mM hydrogen peroxide in the presence of parafilm (Fig. 6C and 6D)

Discussion

It is important to control sclerotial formation for preventing sclerotial diseases. In this study, we evaluated the crucial factors for sclerotial formation in *S. minor*, especially under oxidative stress, nutrient, and aeration conditions.

ROS are known to act as signalling molecules in cellular proliferation and morphological changes (Hansberg & Aguirre 1990; Aguirre *et al.* 2005). Georgiou *et al.* (2006) reported that oxygen stress is an initiation factor required for sclerotial formation. We monitored localisation of ROS during sclerotial development. Intense positive signals were observed at the hyphal tips of the mycelia and developing sclerotia with NBT and DAB staining. Growing hyphal tips, the active area of metabolism, are known to generate ROS (Takemoto *et al.* 2006; Semighini & Harris 2008). The mycelia developing sclerotia also seemed to be the active site of metabolism. However, the accumulation patterns of NBT and DAB positives during development of the sclerotia were different. In NBT staining, although positive signals were uniformly accumulated at the outer layer of developing sclerotia in SD and SM, positive signals were sporadically detected at the outer layer of mycelial aggregation in SI stage. In DAB staining, although positive signals were detected at the entire region of mycelial aggregation in SI stage, they were

absent at the inner region in SD and SM stages. In ultrastructural analysis, hydrogen peroxide-reactive signals were specifically accumulated in the outer layers of SM stage.

The different patterns of ROS localisation during sclerotial development might be explained by the characteristics of ROS indicators and, possibly, by the source of ROS generation. The two hydrogen peroxide indicators, DAB and CeCl_3 , have different cell permeability (Bestwick *et al.* 1997; Ueda *et al.* 2003; Liss *et al.* 2015). DAB signals during the SI stage were mostly detected at the cytosolic region. Since cerium could not permeate into the cytosolic region, ultrastructural analysis could not detect positive signals in the SI stage. On the contrary, cerium-positive signals in the SM stage might be derived from the cell membrane or cell wall. Moreover, inhibitor studies revealed that DAB-positive signals in the SI stage were inhibited by the superoxide dismutase inhibitor, DETC; and NBT-positive signals in the SD stage was partially inhibited by the NADPH oxidase inhibitor, DPI. Based on these results, we conclude that different types and sources of ROS might be accumulated during sclerotial development.

The internal region of sclerotia in the SD and SM stages could not detect ROS-positive signals, indicating that the internal hyphal cells were dormant, because it was reported that accumulation of hydrogen peroxide was correlated with the activity of cell proliferation (Aguirre *et al.* 2005).

Treatment with ascorbic acid inhibited sclerotial formation. Ascorbic acid is known to be a scavenger of various kinds of ROS, including hydrogen peroxide, O_2^- , hydroxyl radical, and singlet oxygen (Aver'yanov & Lapikova 1988; Bendich *et al.* 1986; Kwon & Foote 1988; Yamasaki & Grace 1998). This result indicated that ROS is involved in sclerotial formation. Antioxidant compounds might be promising fungicides for regulating sclerotial formation. However, we noticed that mycelia treated with ascorbic acid produced numerous mycelial aggregates that were equivalent to SI stage. Ascorbic acid might suppress the maturation step,

but not the initiation step of sclerotial development. In another inhibitor study, treatment with DPI, a specific inhibitor of NADPH oxidase (Nox), did not suppress sclerotial formation (Bokoch & Knaus 2003; Vignais 2002), suggesting that Nox might be dispensable for sclerotial formation. This result was consistent with those reported by Papapostolou & Georgiou (2010), where they found that the source of superoxide radical was not Nox, but xanthine oxidase. However, our inhibitor study revealed that the xanthine oxidase inhibitor, allopurinol, could not suppress sclerotial formation.

Moreover, exogenous supplementation with hydrogen peroxide could not initiate sclerotial formation. This result indicated that specific ROS other than hydrogen peroxide could initiate sclerotial formation. Alternatively, specific localisation of hydrogen peroxide could initiate sclerotial formation, since exogenous treatment with hydrogen peroxide was uniform throughout the mycelia.

Based on these results, we conclude that ROS mainly contributed in the sclerotial maturation step, especially for melanisation. The outer layer of sclerotia was highly melanised in the SM stage. The melanin layer was biosynthesized from the oxidation cycles of 1,8-dihydroxynaphthalene (DHN) or dihydroxyphenylalanine (DOPA) (Bell & Wheeler 1986). Antioxidant compounds were reported inhibitory effect on melanin production (Lan *et al.* 2013).

Nutrient condition affected the sclerotial number and duration of sclerotial maturation. When *S. minor* mycelia were grown on nutrient-poor media, sclerotia were produced earlier than those on regular nutrient media, although the number of sclerotia was small. In contrast, on nutrient-enriched media, sclerotial formation was delayed; instead, number and size of sclerotia were increased. The amount of media in the petri dish also affected the number of sclerotial formation. It was a matter of fact that nutrient was essential for mycelial growth and was the resource for sclerotial formation. Subsequently, the mycelia were occupied with substrate and

the exhausted nutrient from substrate. Nutrient deficiency signal might have triggered sclerotial formation. Alternatively, high concentrations of glucose might act as hydroxyl radical scavenger (Sagone *et al.* 1983).

Aeration was also an essential factor in sclerotial formation. The growing mycelia sealed with parafilm showed suppressed sclerotial formation. When the sealed parafilm was removed after 9 days of incubation, sclerotia were never produced. This result indicated that coexistence of factors (nutrient deficiency and aeration) at the same time could trigger sclerotial formation. It was difficult to infer the mechanism of the effect of aeration on sclerotial formation. The petri dish sealed with parafilm seemed to maintain the moisture of mycelia or restrict oxygen gas to generate ROS.

In summary, mycelia of *S. minor* might help maintain the balance between mycelial growth and sclerotial formation by sensing nutrient and aeration conditions. These sensing processes might be exclusively orchestrated and might subsequently regulate ROS level. The ROS generation pathway might be diverse and redundant during sclerotial development. More detailed analysis of ROS generation pathways to control sclerotial formation is required to arrive at a robust conclusion.

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Figure legends

Figure 1 Effect of nutrient and aeration conditions on sclerotial formation in *S. minor*. **(A)** Number of sclerotia on different strength of PDA media (green: 1/4-, orange: 1-, pink: 2-, and blue: 4-strength of PDA media). Asterisks indicate significantly different sclerotia from 1-strength of PDA media (t-test; *: $p < 0.05$ and **: $p < 0.01$). Error bars: standard deviation. **(B)**

Number of sclerotia in different strength of PDA media (green: 1/4-, orange: 1-, and blue: 4-strength of PDA media) grown on cellulose membranes, which were transferred from 1-strength of PDA media after two days incubation (indicated by arrow). Asterisks indicate the number of sclerotia, which were significantly different from 1-strength of PDA media (t-test; **: $p < 0.01$). Error bars: standard deviation. (C) Number of sclerotia on different amounts of PDA media after 6 days of incubation. Different letters indicate the number of sclerotia, which were significantly different from the sclerotia grown in 5 mL of PDA media (Tukey's test, $p < 0.05$). Error bars: standard deviation. (D) Effect of aeration sealed with parafilm on sclerotial formation after 6 days of incubation. Asterisk indicates the number of sclerotia, which were significantly different from the sclerotia which were grown without parafilm (t-test; **: $p < 0.01$). Error bars: standard deviation.

Figure 2 Developmental stages of sclerotial formation and ROS formation in *S. minor*. (A) Full view of mycelia at different stages of development, e.g., mycelia (MY), sclerotial initials (SI), sclerotial developing (SD), and sclerotial mature (SM). (B) Magnified views of unstained mycelia. Bars = 1 mm. (C) Magnified views of mycelia stained with NBT. Bars = 1 mm. (D) Magnified views of mycelia stained with DAB. Bars = 1 mm. (E) Hyphae stained with DAB. Bar = 3 μ m.

Figure 3 Localisation of ROS generation during development of sclerotia in *S. minor*. (A) Localisation of ROS in each sclerotial developmental stage (SI, SD, and SM) stained with NBT and DAB. Control: unstained. Left pictures: surface view; right pictures: sectional view (30 μ m). (B) Sectional view of sclerotia treated with ROS generation inhibitors. The sclerotia were stained with DAB (in SI) and NBT (in SD). Sorbent control: 0.5% DMSO. ROS generation

inhibitors: 25 μ M diphenyleneiodonium (DPI), 1 mM 3-aminotriazole (3ATZ), 1 mM 4-aminotriazole (4ATZ), 1 mM sodium azide (NaN_3), 1 mM salicylhydroxamic acid (SHAM), and 1 mM sodium diethyldithiocarbamate (DETC).

Figure 4 Ultrastructural study of ROS localisation on each sclerotial developmental stage with cerium chloride treatment. **(A)** Ultrastructural views of sclerotia in SI, SD, and SM phases (without cerium chloride treatment). 25 μ m of surface areas were defined as “outer” and representative inside areas were defined as “inner”. Bars = 25 μ m. **(B)** Ultrastructural view of sclerotia in SI, SD, and SM phases (with 5 mM cerium chloride treatment). Cerium positive signals were indicated by arrowheads. Bars = 25 μ m. **(C)** Quantification of ROS generation at outer (blue) or inner (red) areas in SI, SD, and SM. Error bars: standard deviation. Asterisk indicates the amount of ROS at outer area, which was significantly different, as compared with the inner one (t-test; *: $p < 0.05$).

Figure 5 Effects of ROS generation inhibitors on sclerotial formation in *S. minor*. **(A)** Entire view of mycelia treated with ascorbic acid (AsA). Enlargement views (bottom). **(B)** Number of matured sclerotia treated with AsA (blue: 0, pink: 7.5, orange: 15, and green: 30 mg/ml). Error bars: standard deviation. **(C)** Proportion of sclerotial development stages (SI, SD, and SM), which were treated with ascorbic acid. Blue, pink, orange, and green bars correspond to control, 7.5, 15, and 30 mg/ml AsA, respectively. Error bars: standard deviation. **(D)** Number of sclerotia treated with DPI (10 and 25 μ M). DMSO was used as sorbent control. Error bars: standard deviation. Asterisk indicates the number of sclerotia that were significantly different from the control (t-test; *: $p < 0.05$). **(E)** Number of sclerotia, which were treated with allopurinol (100 μ M) and DETC (1 mM). Error bars: standard deviation.

Figure 6 Effect of exogenous supplement, containing hydrogen peroxide on sclerotial formation. (A) Entirety views of mycelia grown on hydrogen peroxide containing media 9 days after incubation. (B) Number of sclerotia treated with hydrogen peroxide (pink: 0 and blue: 1 mM). (C) Full view of mycelia sprayed with hydrogen peroxide (1 and 10 mM) and sealed with or without parafilm. (D) Number of sclerotia sprayed with hydrogen peroxide (0, 1, and 10 mM, respectively) sealed with or without parafilm. Asterisk indicates significantly different sclerotia number from that of the control (t-test; *: $p < 0.05$). Error bars: standard deviation.

Supplemental figure S1 Effects of nutrient and aeration on sclerotial formation in *S. minor*. (A) Entire view of mycelia on different strength of PDA media. NE: not examined. Bottom photos: enlarged images of mycelia 18 days after incubation. (B) Entire view of mycelia in different strengths of PDA media, grown on cellulose membranes, which were transferred from 1-strength of PDA media after two days of incubation. (C) Entire view of mycelia in different amounts of PDA media after five days of inoculation. (D) Entire view of mycelia sealed with or without parafilm. The word “re-open” means parafilm was removed after 9 days of incubation.

Supplemental figure S2 Effect of air bubble removal from sclerotia through permeation of aqueous solution into the core of the sclerotia. Sclerotia in SI, SD, and SM were treated with (Removed) or without (Control) rolling on wet paper and dipped with red ink. Top pictures: surface view of sclerotia. Arrowheads indicate air bubbles. Bottom pictures: sectional view (30 μm) of sclerotia.

Supplemental figure S3 Ultrastructural pictures of sclerotia in SI, SD, and SM without cerium

chloride treatment. Left: outer layers; right: inner layers.

Supplemental figure S4 Effect of chemicals on the sclerotial number. Entire view of mycelia treated with DPI (10, 25, and 50 μ M, respectively), Allopurinol (100 μ M), and DETC (1 mM).

Supplemental figure S5 Effects of chemicals on mycelial growth. Generation times (Georgiou *et al.* 2001) were evaluated during the treatment with AsA, DPI, hydrogen peroxide, allopurinol, and DETC. N.D.: generation times that could not be calculated because the mycelia did not grow at those concentrations of the chemicals.

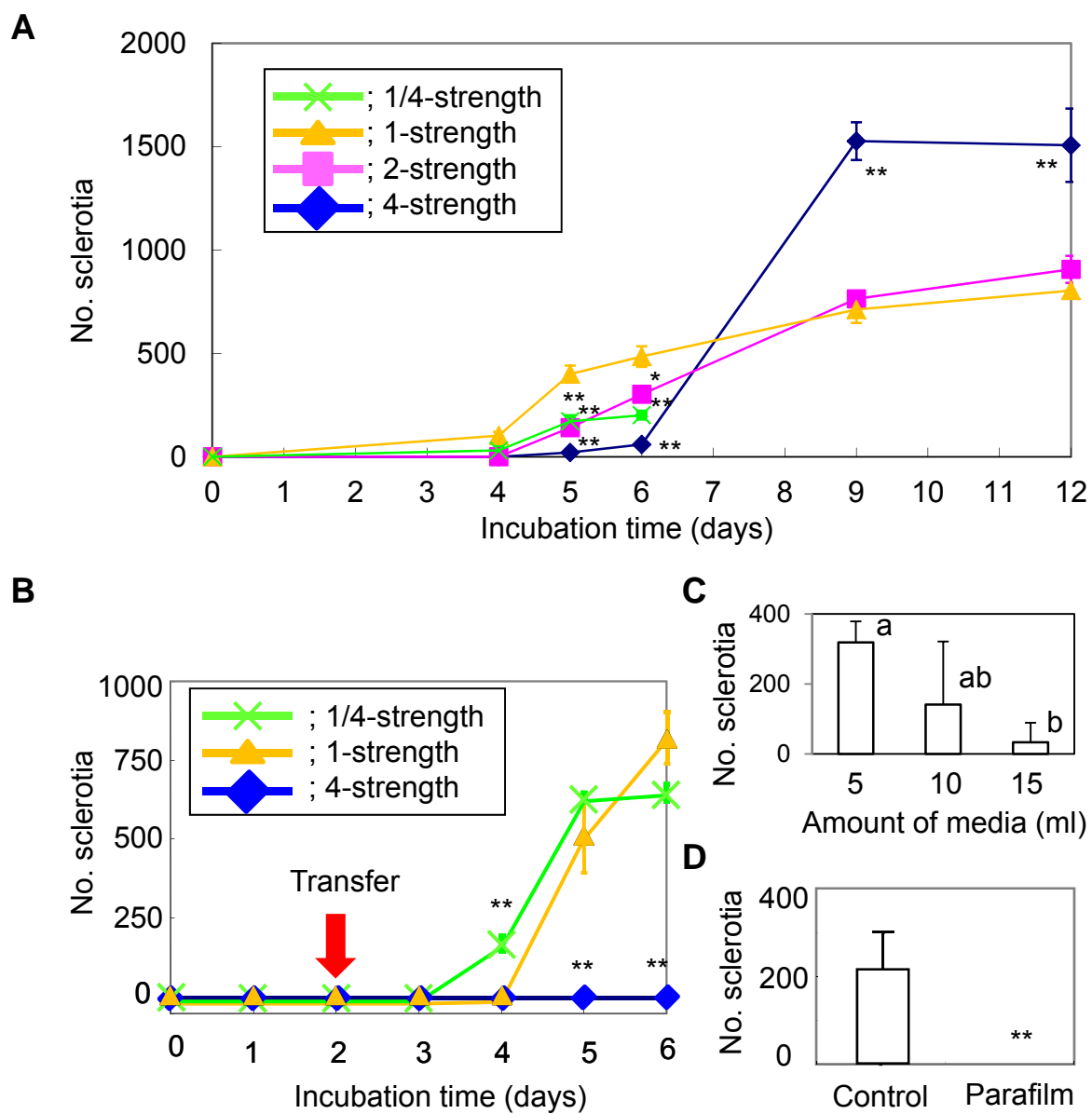


Fig. 1

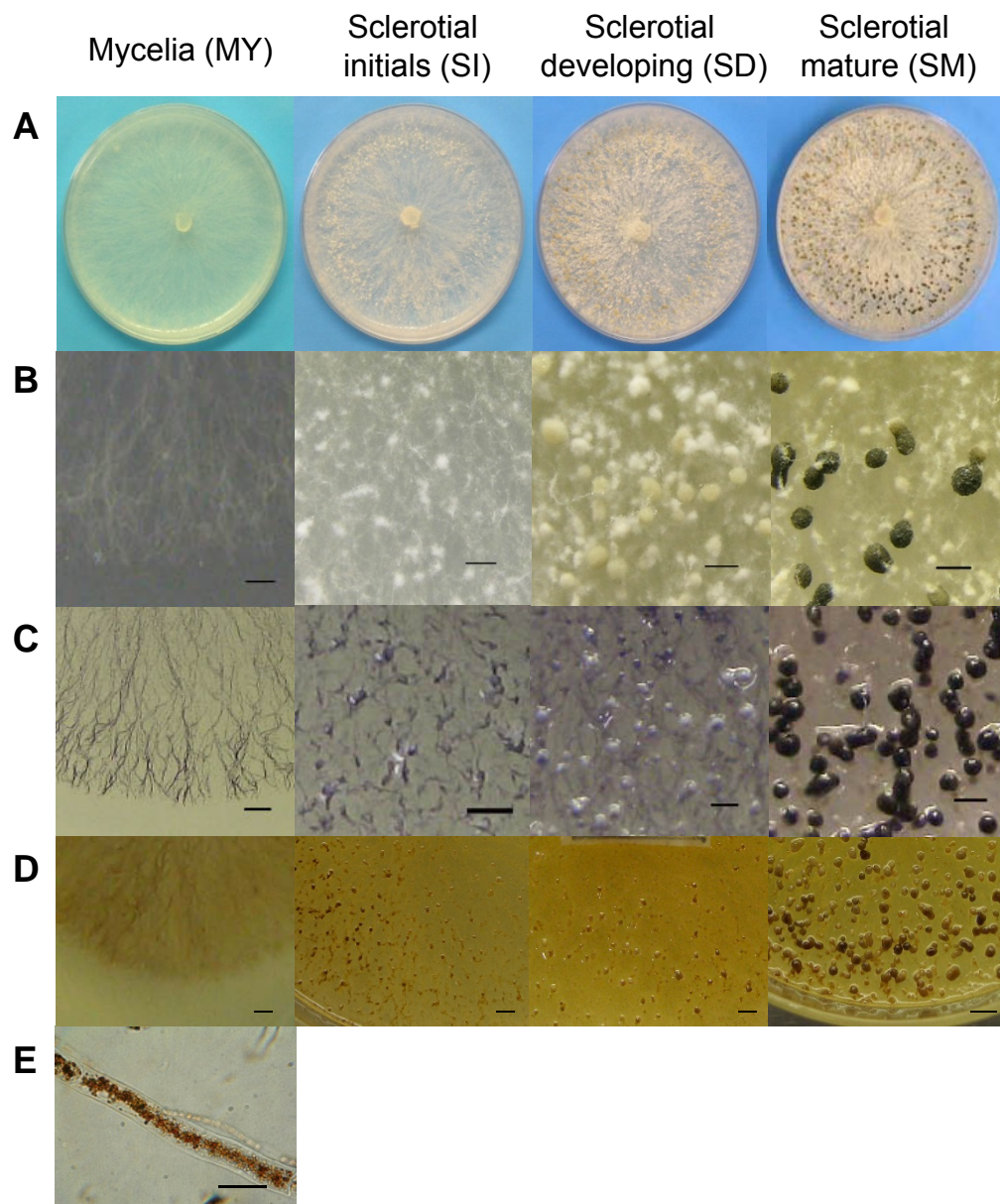


Fig. 2

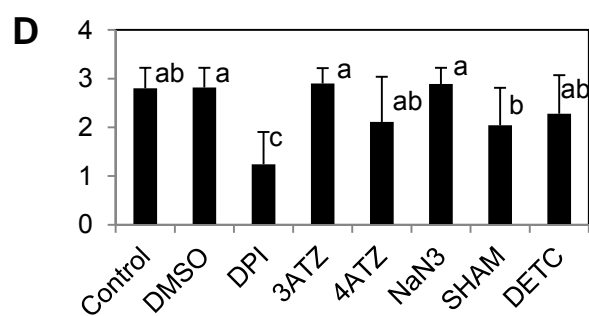
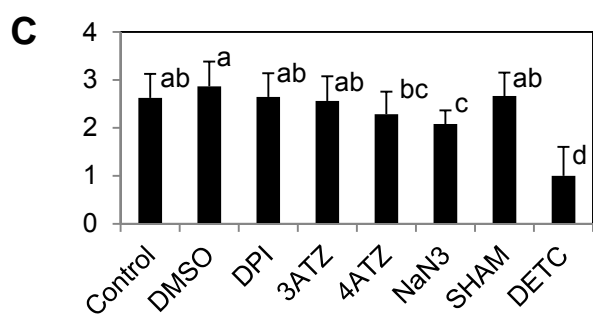
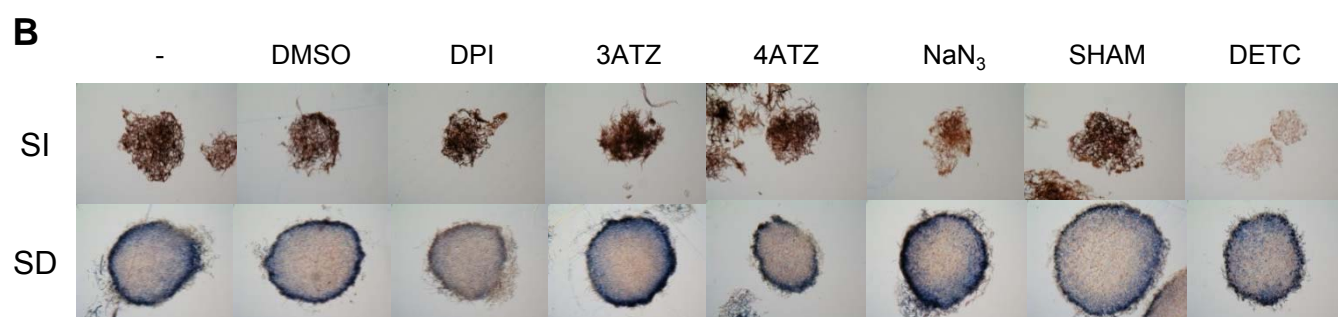
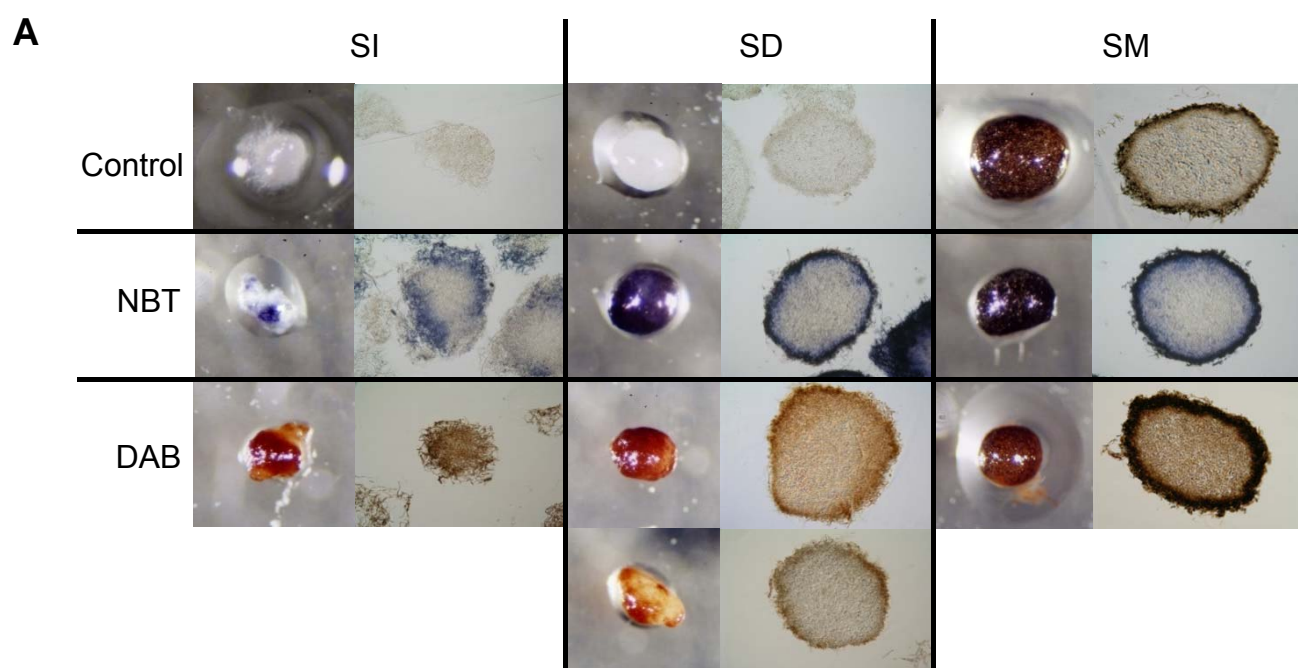


Fig. 3

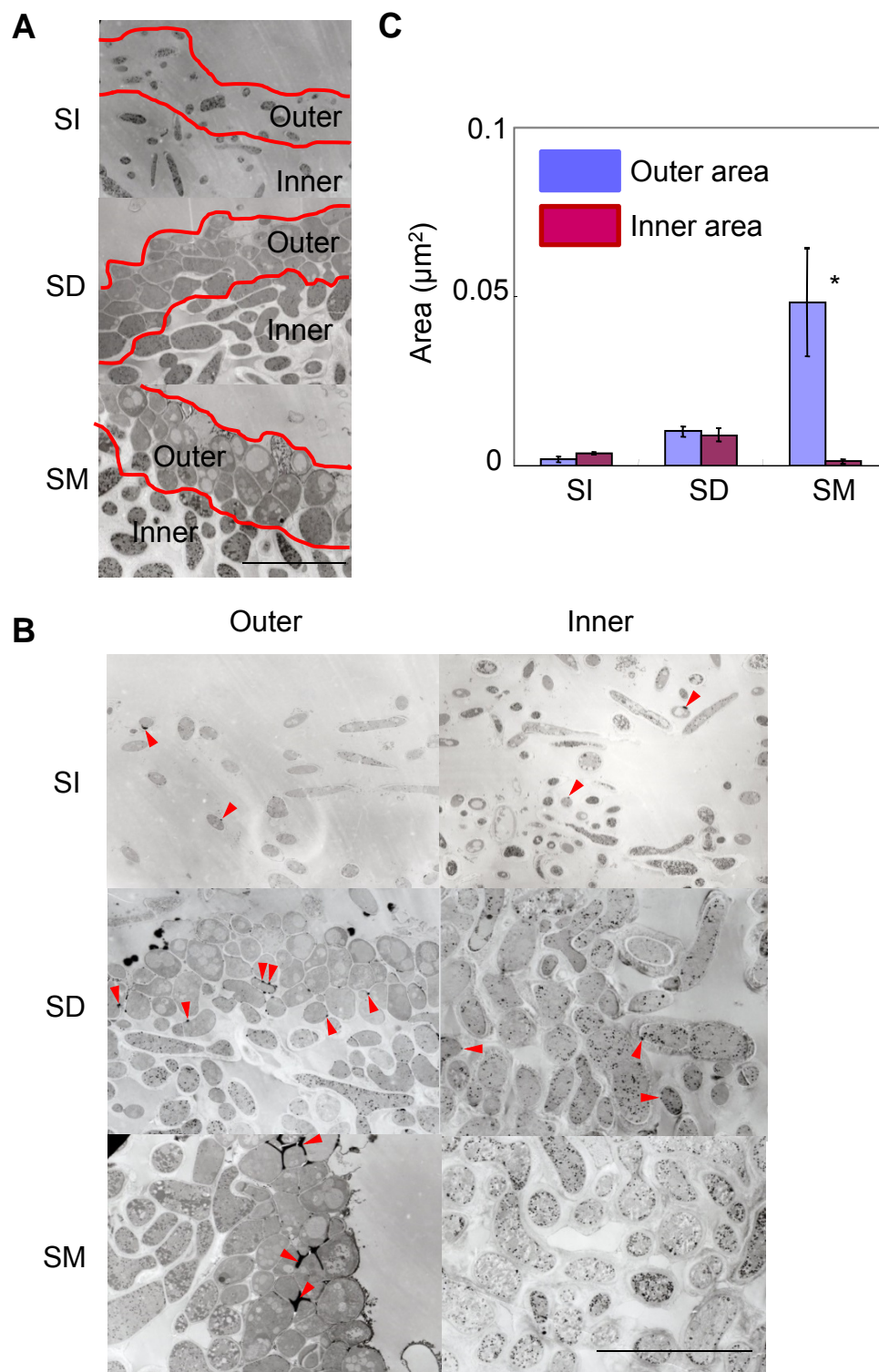


Fig. 4

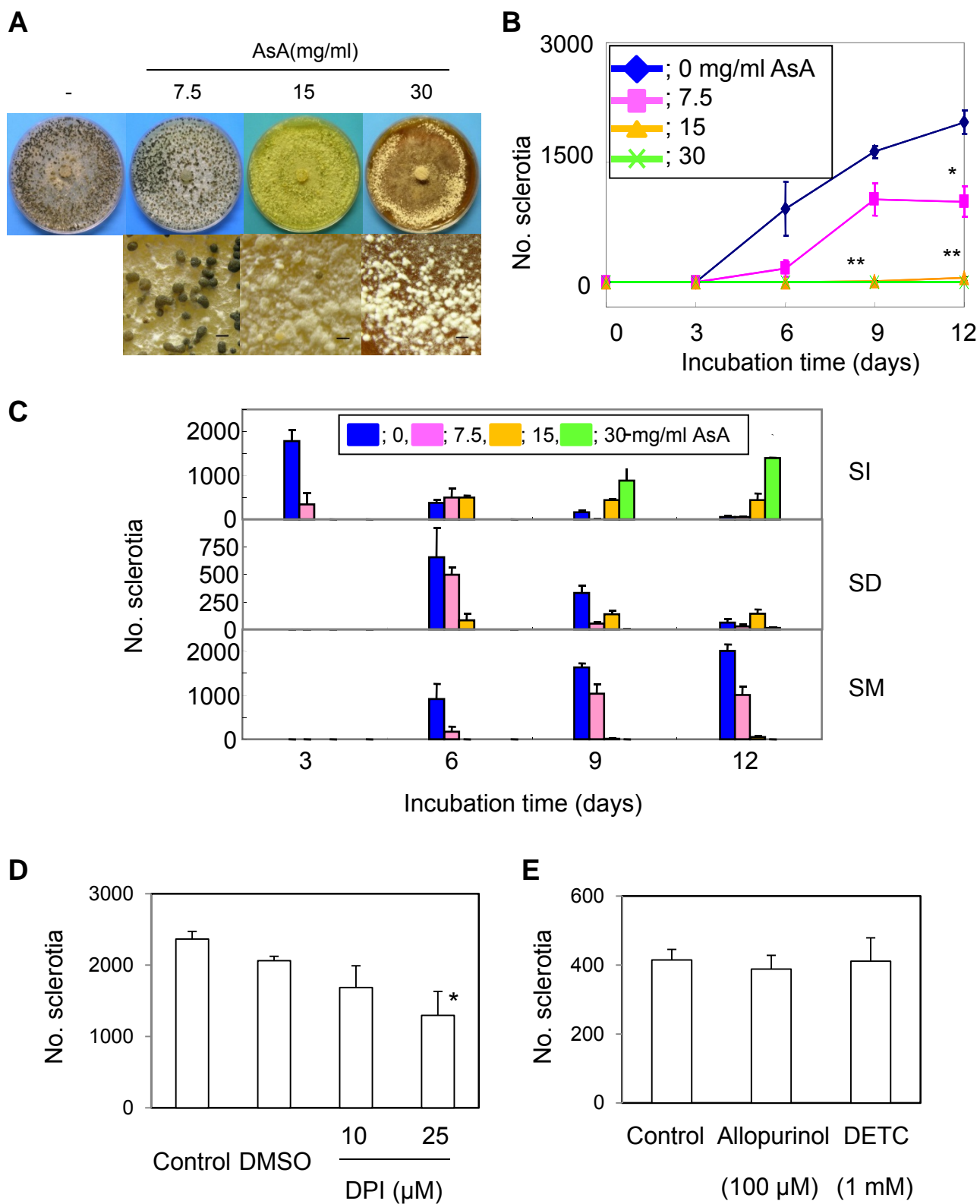


Fig. 5

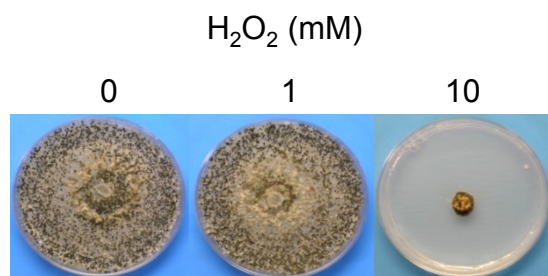
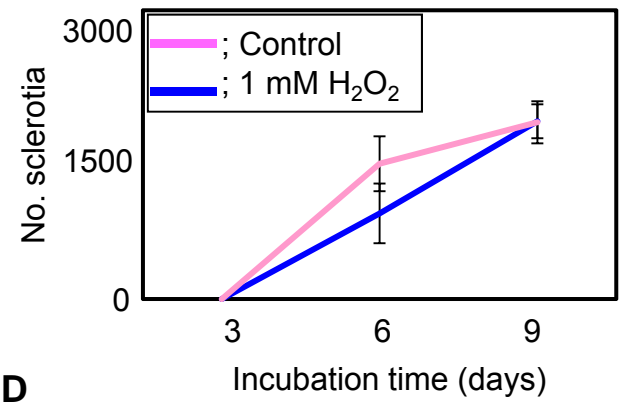
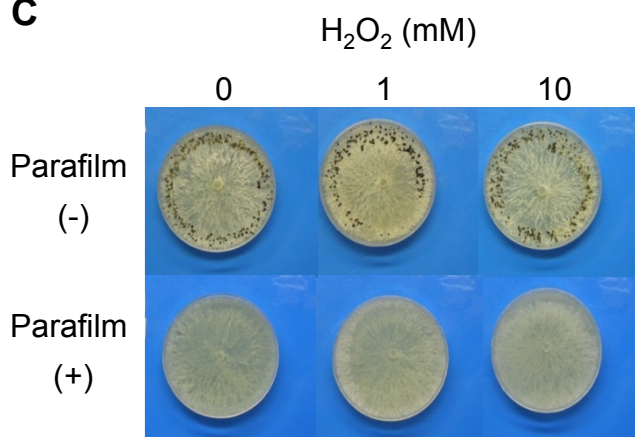
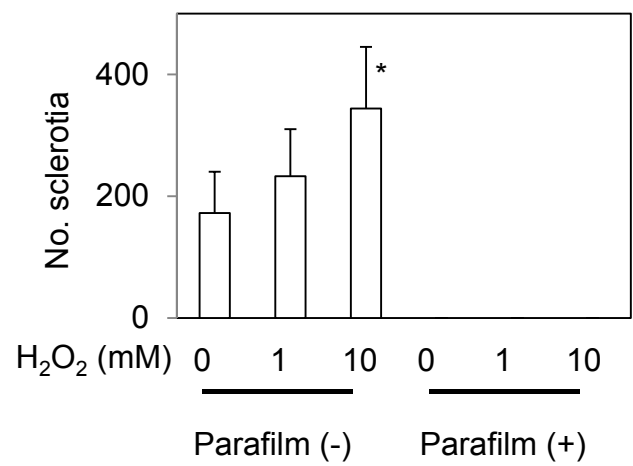
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Fig. 6

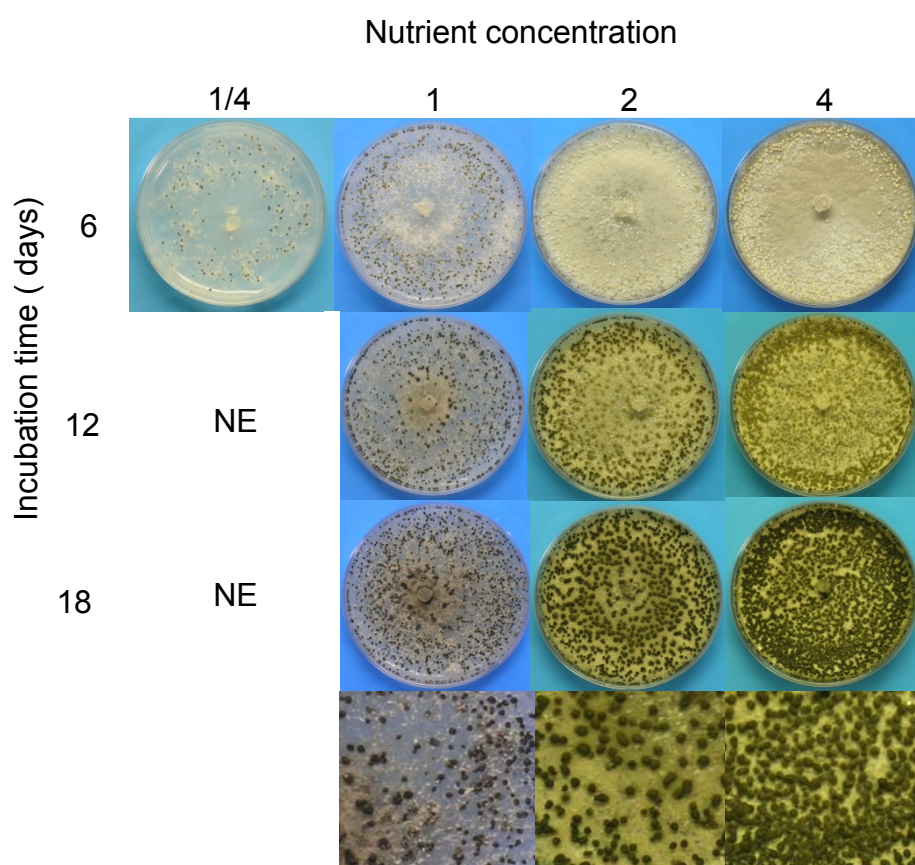
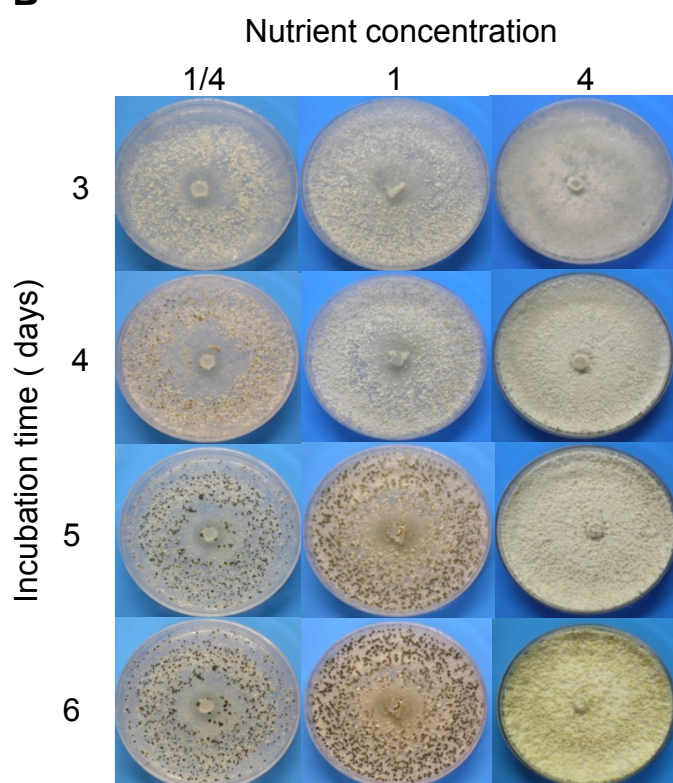
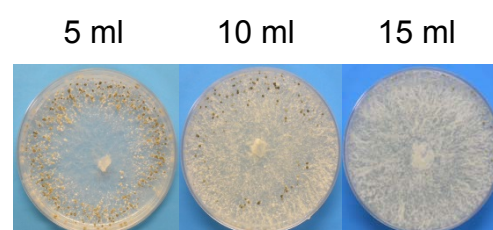
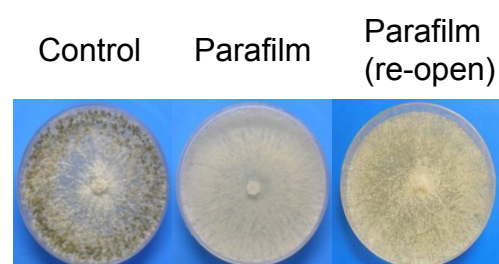
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Fig. S1

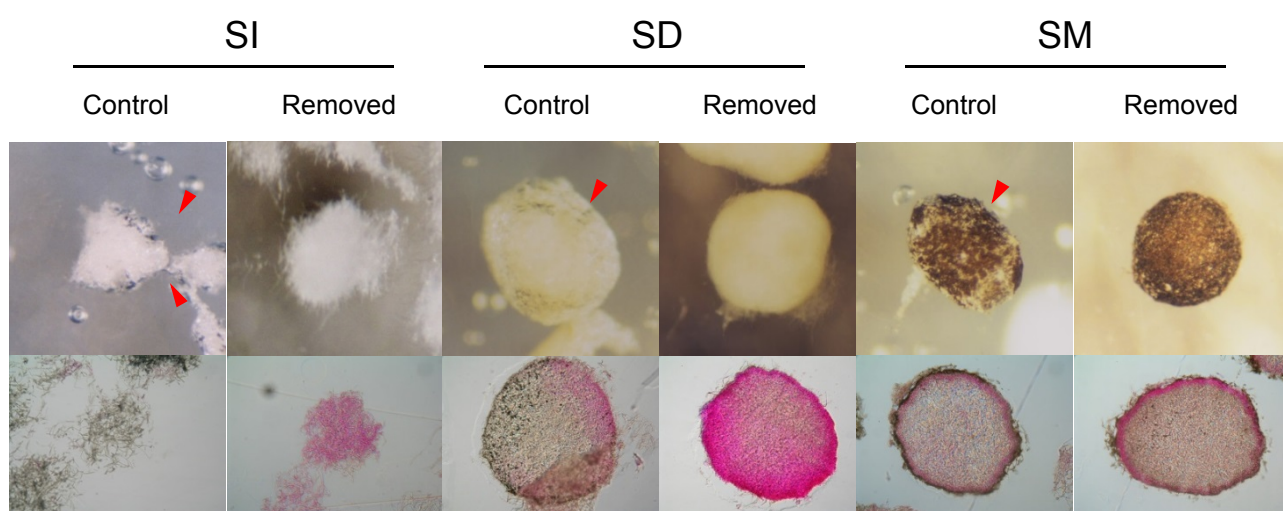


Fig. S2

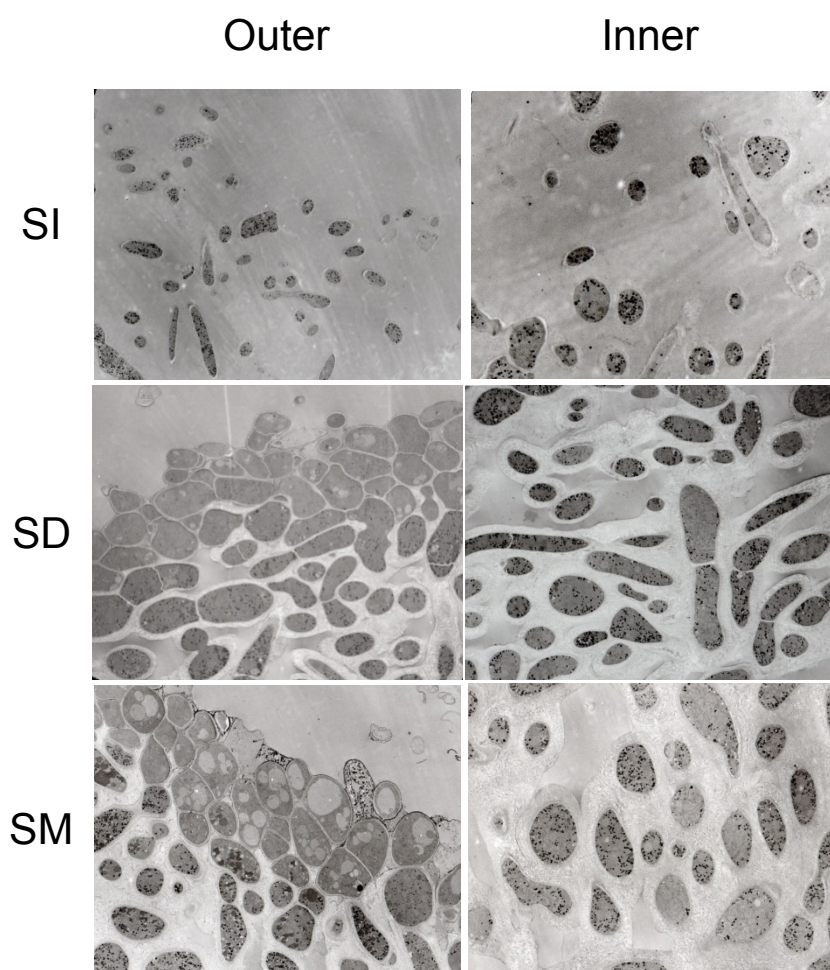


Fig. S3

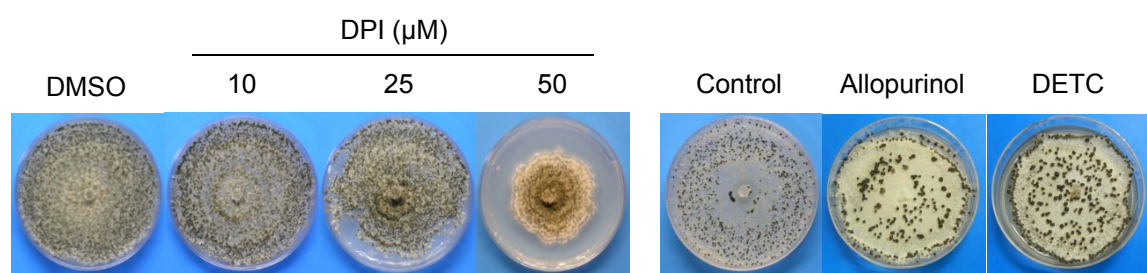


Fig. S4

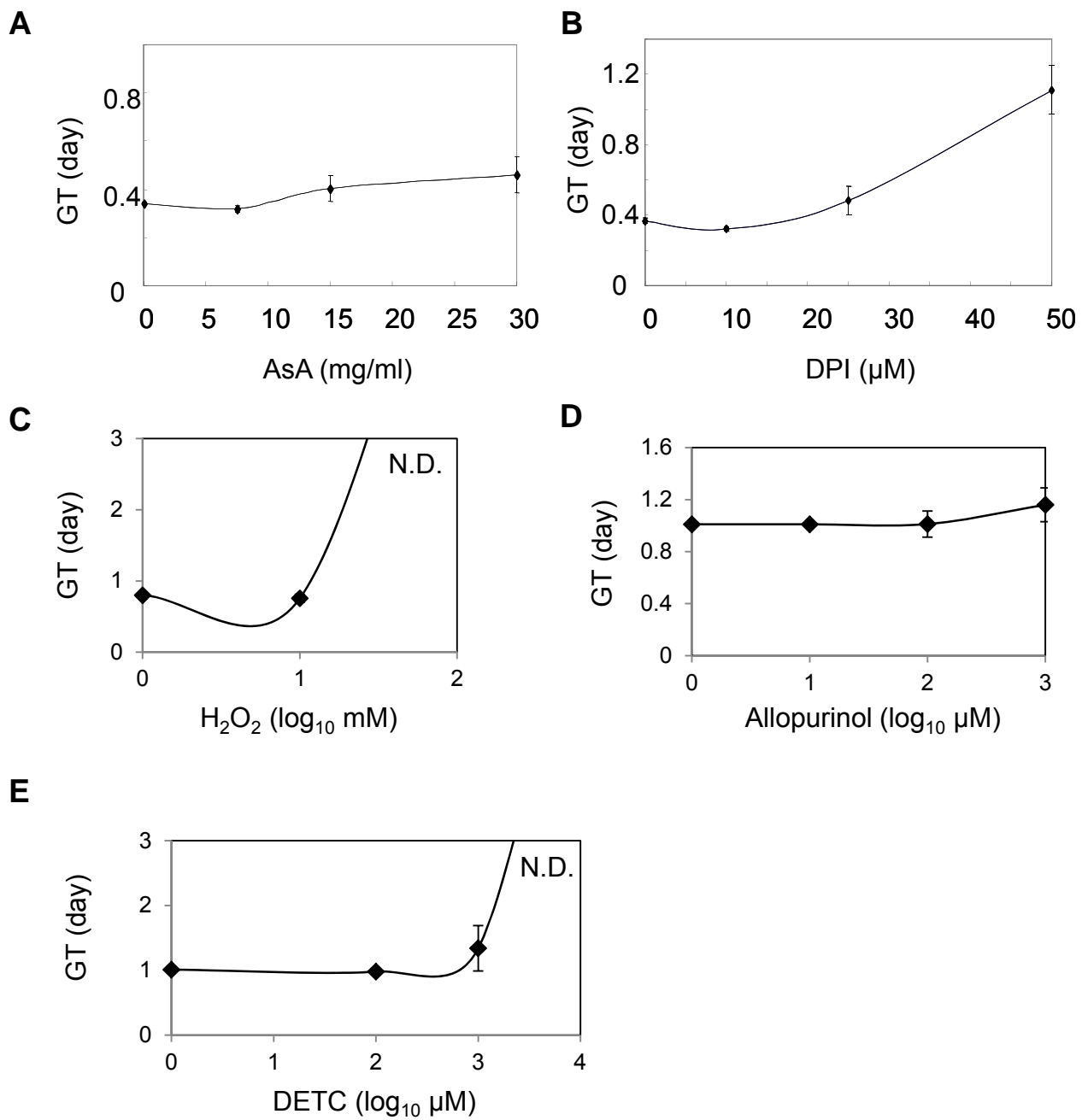


Fig. S5