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Title: Potential of three forms of ascorbate as fungicides against the blast pathogen, *Pyricularia oryzae*

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ABSTRACT

Reactive oxygen species (ROS) are important as signal molecules in the pathogenicity of the blast fungus (*Pyricularia oryzae*). Therefore, the ROS signaling pathway could provide potential targets for developing fungicides. Ascorbates can degrade ROS produced in fungal cells, so three forms of ascorbate, L-ascorbic acid (AsA), dehydroascorbic acid (DHA) and sodium ascorbate (AsA-Na), were assessed for antifungal effects. All three at 20 mM inhibited mycelial growth in vitro, and AsA and DHA disrupted linear growth of hyphae. Because polarized growth of hyphae is regulated by *Tea* genes, the expression of three *PoTea* genes in mycelium exposed to AsA and DHA were analyzed. These analyses showed that (1) all *PoTea* genes in nontreated hyphae had a circadian-like pattern of expression and (2) circadian-like regulation of each *PoTea* gene was considerably disrupted after exposure to AsA and DHA. Expression of the circadian clock gene *PoFrq* was similarly affected, suggesting that the circadian rhythm in *P. oryzae* was disrupted by 20 mM AsA and DHA, resulting in inhibition of hyphal growth. As expected, the three ascorbates significantly suppressed infection of barley. DHA was also shown to act as a plant defense activator against the blast pathogen.

KEYWORDS

L-Ascorbic acid, circadian clock, L-dehydroascorbic acid, *Hordeum vulgare*, *Oryza sativa*, polarized hyphal growth, *Pyricularia oryzae*, reactive oxygen species

1 INTRODUCTION

The ascomycete fungus *Pyricularia oryzae* (Zhang et al., 2016) causes blast disease in rice, the most serious disease of cultivated rice (*Oryza sativa*) worldwide, and serious problems in wheat (*Triticum aestivum*) in southern Brazil, Paraguay, Bolivia, Bangladesh and Zambia (Singh et al., 2021). Barley, oats, ryegrass and millet can also be severely damaged (Martin-Urdiroz et al., 2016; Inukai et al., 2006). Thus, blast disease poses a serious threat to global production of cereals.

When conidia of *P. oryzae* are dispersed from blast lesions to the leaf surface of a host plant (Martin-Urdiroz et al., 2016), the conidium adheres to the leaf, germinates in a drop of water, forming a germ tube, which can differentiate into a dome-shaped infection structure called the appressorium. The mature, melanized appressorium can generate enormous turgor pressure (up to 8.0 MPa), which enables the narrow penetration hypha that forms at the base of the appressorium to penetrate the leaf cuticle and epidermal cell wall, then invaginate into the plasma membrane of the epidermal cell (Eseola et al., 2021). Invasive hyphae are produced in the infected cells and grow from cell to cell, and within days, leaves develop blast lesions.

The direction of hyphal growth is controlled by cell-end marker proteins at the tip of the hyphae (Riquelme et al. 2018). In *Aspergillus nidulans*, the cell-end marker TeaA (Tea1) is transported specifically to the hyphal tip and is anchored to the membrane by another cell-end marker, TeaR (Mod5) (Takeshita 2016). The kinesin motor protein KipA (Tea2) is also necessary for anchoring TeaA to the hyphal tip (Takeshita et al., 2008). TeaC (Tea4) physically interacts with TeaA in the hyphal tip (Higashitsuji et al., 2009). In *P. oryzae*, homologs of *Tea1* and *Tea4*, *PoTea1* and *PoTea4* (synonym for *MoTea4*) have been identified and shown to have important roles in polarized cell growth (Qu et al., 2022; Patkar et al., 2010; Rogers et al., 2024). A deletion mutant of *PoTea1* had slower hyphal growth, increased hyphal branching, delayed appressorium formation and less sporulation than the wild type (Qu et al., 2022). A *PoTea4* mutant had aerial hyphae with a zigzag morphology and greatly reduced

conidiation (Patkar et al., 2010). Thus, inhibition or loss of the *PoTea* genes causes abnormal growth and development and reduced virulence.

Conidia of *P. oryzae* generate reactive oxygen species (ROS) during germination, as do appressoria as they differentiate (Egan et al. 2007). This ROS production requires NADPH oxidases Nox1 and Nox2 as shown by a study of *nox1* and *nox2* mutants, which do not form normal appressoria. Thus, ROS production in the conidia and appressoria is essential for pathogenicity of *P. oryzae*. When Egan et al. (2007) applied antioxidants such as L-ascorbic acid (AsA), which degrades superoxide anions produced by the conidia, abnormal appressoria formed. Because AsA is also a major endogenous antioxidant in plants and present at millimolar levels in plant cells (Zechmann, 2011), endogenous AsA might also influence the infectivity of the blast fungus; however, there is yet little information on any antifungal effects of AsA.

AsA is synthesized in plants primarily by the mannose/galactose pathway and by several alternative pathways (Gallie, 2013; Quiñones et al., 2024). To protect cells, AsA, with or without the aid of an enzyme, reduces ROS that are generated during aerobic metabolism or by abiotic stress. Ascorbate peroxidase (APX) uses AsA as an electron donor to reduce hydrogen peroxide (H_2O_2). Plants also have evolved pathways to recycle oxidized forms of AsA, monodehydroascorbic acid (MDHA) and dehydroascorbic acid (DHA); MDHA and DHA are reduced back to AsA by MDHA reductase (MDHAR) and DHA reductase (DHAR), respectively (Gallie, 2013; Quiñones et al., 2024). DHA can also scavenge H_2O_2 and superoxide anions (Dewhirst and Fry, 2018). Some AsA is released to the apoplast, oxidized by ROS and converted into MDHA and DHA. Because DHA reductase is absent in the apoplast, DHA is oxidized by ROS, especially H_2O_2 (Dewhirst and Fry, 2018). Thus, AsA and DHA might both act as antioxidants in the apoplast where plants directly interact with hyphae of pathogenic fungi.

In the present study, we first examined the effects of three forms of ascorbate, AsA, DHA and AsA-Na on mycelial growth of blast fungus in vitro. All clearly inhibited mycelial growth, and AsA and DHA,

especially, disturbed the linear growth of hyphae. Because polarized hyphal growth is regulated by *Tea* genes, we investigated changes in the expression level of each *PoTea* gene in hyphae after exposure to AsA and to DHA. We next applied the three forms of AsA to barley and rice to determine whether they can suppress fungal infection. The results showed that infection was significantly suppressed in plants by all three ascorbates; however, they had different modes of action against the blast fungus.

2 MATERIALS AND METHODS

2.1 Fungal and plant materials

Rice blast isolates Ken54-20 and Ai79-142 used in this study were obtained from the collection maintained at the Paddy Crop Disease Laboratory at the National Agriculture Research Center, Japan. Susceptible barley cultivar Harrington was obtained from the Barley Germplasm Center at Okayama University, Japan, and susceptible rice cultivar Nipponbare from the Research Center of Genetic Resources, and the National Agriculture and Food Research Organization, Japan. Barley seeds were placed on moistened filter paper in a petri dish for 2 days at 22 °C to hasten germination, sown in peat pellets (Jiffy-7; SAKATA, Yokohama, Japan), and grown in an MLR-350 growth chamber (SANYO, Tokyo) at 22 °C with a 12 h photoperiod (150 $\mu\text{mol}/\text{m}^2/\text{s}$). Rice seeds were soaked in water for 2–3 days at 30 °C to hasten germination, sown in sterilized nursery soil in a plastic tray and grown in the greenhouse at Hokkaido University (Sapporo, Japan).

2.2 Fungal culture

The rice blast isolates used in this study were stored as dried mycelium on a small piece of filter paper in a freezer at –30 °C. The culture was revived by placing the filter paper into 50 mL of broth containing 2

g/L yeast extract and 10 g/L glucose, then cultured for 3–4 days in ambient light at 25 °C. A small quantity of the mycelia was then removed with a sterilized glass pipet, spread onto oatmeal agar containing 20 g/L sucrose, and incubated in the dark for about 10 days at 25 °C. For an in vitro test of fungal sensitivity to various chemicals, a small plug of mycelium was removed from the plate with a sterilized pasteur pipette and placed on plates of complete medium (CM) containing one of the test chemicals, 20 mM each AsA, DHA and L-ascorbic acid sodium salt (AsA-Na) (Wako Pure Chemical Industries, Osaka, Japan). For the control for CM containing AsA or DHA (pH 4.6), CM was prepared and adjusted to pH 4.6 using 1 N sodium chloride solution.

For extracting RNA from broth cultures for quantitative RT-PCR, the mycelium was revived as described above in the dark and harvested by vacuum filtration through filter paper. For extracting RNA from agar cultures for semiquantitative RT-PCR, the fungus was grown on a nylon membrane on each plate, then the mycelium was scraped off the membrane with a sterilized spatula (Schumann et al. 2013).

For synchronizing conidiation for plant infection assays, mycelium was cultured on oatmeal agar plates in the same conditions described above, brushed with a wet paintbrush, then incubated under fluorescent light for 3–4 days at 25 °C. Conidia were collected with a wet paintbrush and immediately used for inoculation.

To test the effects of the ascorbates on spore germination and appressorium formation, we added AsA, AsA-Na or DHA (final concentration: 20 mM) to a conidial suspension (5×10^4 spores/mL), which was then dropped onto a plastic coverslip; the coverslip was placed on a holder in a petri dish with a small amount of water and incubated for 17 h at 25 °C. At least 80–100 spores were checked for germination and whether germ tubes had formed appressoria using a BX51 optical microscope (OLYMPUS, Tokyo). All tests were done at least three times. Percentage germination was calculated as (Number of germinated spores / Total number of spores observed) $\times$ 100 and percentage appressorium formation as (Number of appressorium / Total number of observed spores) $\times$ 100.

2.3 Light microscopy of hyphae

A small amount of molten CM agar supplemented with 10 mM AsA, 10 mM DHA, or 20 mM AsA-Na was solidified on a glass slide, and a filter paper covered with mycelium was placed on the agar. The filter paper was covered with a cover slip, and the slides were placed on a holder in a petri dish filled with a small amount of water and incubated at 25 °C. At 24, 36, and 48 h after inoculation, the hyphae were observed on the agar using epi-illumination and a BX51 optical microscope (OLYMPUS, Tokyo). Images were captured using digital camera system DP71 (OLYMPUS).

2.4 Pretreatment of leaves with AsA, DHA and AsA-Na

AsA (20 mM), AsA-Na (20 mM) or either 10 or 20 mM DHA solutions, all with 0.2% (v/v) Tween 20, were sprayed on barley or rice leaves with a hand sprayer. After 6 and 24 h in a growth chamber or greenhouse with ambient temperature and light, the treated plants were then inoculated as described in Sect. 2.6.

2.5 ROS assay

For detecting superoxide anions in response to AsA or DHA treatment, conidia of isolate Ken 54-20 were collected from oatmeal agar plates and resuspended in sterile deionized water to a final concentration of 10^5 conidia/mL. A 50- μ L droplet was placed on a plastic coverslip, then 12.5 μ L of either 100 mM AsA, DHA, or sterile deionized water was added to the droplet. The coverslip was then incubated in a moist chamber at 25 °C for 2 h, then the droplet was removed from the coverslip and replaced with 100 μ L of 0.3 mM aqueous nitroblue tetrazolium (NBT). After 30 min, the samples were observed for formazan staining using a BX51 optical microscope (OLYMPUS, Tokyo) and bright-field optics.

2.6 Infection assays

Pretreated leaves of barley seedlings at the 2–3-leaf stage were sprayed with a suspension of 5×10^4 conidia/mL water + 0.5% (w/v) gelatin. Rice was similarly inoculated at the 6–7-leaf stage, but the suspension contained 0.02% (v/v) Tween 20. All plants were incubated in a dew chamber at 25 °C for 20 h, then transferred to the greenhouse or a growth chamber with a 12-h light/12-h dark at 25 °C; 7 days after inoculation, any lesions on the youngest expanding leaf of each seedling were scored as brown spots; small, round lesions; or large, spindle-shaped lesions; the number of each lesion type on one leaf was recorded.

2.7 Quantification of AsA and DHA

Leaves were harvested and immediately ground in 5 volumes of 6% v/v metaphosphoric acid with a mortar and pestle. The homogenate was centrifuged at 14,000 rpm for 20 min, and the supernatant was stored at –80 °C until analysis. The supernatant was diluted 10-fold with 5% metaphosphoric acid to measure AsA and 5-fold for DHA as described for the 2,4-diphenylhydrazine method (Bradley et al., 1973)

2.8 Identification of *PoTea2* gene in *P. oryzae*

Tea2 encoding kinesin-like protein had not yet been reported in *P. oryzae*, and so we searched for orthologs of the *A. nidulans* *KipA* (*Tea2*) gene in the genome database of *P. oryzae* in EnsemblFungi (https://fungi.ensembl.org/Magnaporthe_oryzae/Info/Index) and found MGG_04366, which contained a kinesin motor domain and shared sequence identity of 57% and similarity of 70% with that of *KipA*. Therefore, we identified MGG_04366 as a possible ortholog of *Tea2* in *P. oryzae* and named it *PoTea2* in this study.

2.9 Gene expression analysis by quantitative and semi-quantitative RT-PCR

Total RNA was extracted from mycelia and leaves using TRIzol reagent (Invitrogen, Tokyo) and the manufacturer's instructions, then treated with RNase-free DNase-I (Roche Diagnostics, Mannheim, Germany) to degrade contaminating DNA. Expression levels of the fungal and plant genes studied here were determined using the primers summarized in Supplementary Table S1 and multiplex RT-qPCR with the GenomeLAB GeXP Start Kit (Beckman Coulter, Fullerton, CA, USA) as described previously (Kim et al., 2008). Expression levels of the genes were calculated relative to the geometric mean of the internal standard genes described in Table S1 (Che Omar et al. 2016).

For semiquantitative RT-PCR, cDNA was synthesized using the PrimeScript II 1st strand cDNA Synthesis Kit (Takara, Tokyo) and the manufacturer's protocol, and the PCR was performed using ExPremier DNA polymerase (Takara, Tokyo) with the primers and the PCR program for each gene summarized in Table S2. *P. oryzae* tubulin gene (MGG_00604) was used as an internal standard. The PCR products were separated using 2% agarose gel electrophoresis, and the density of each band was quantified using Photoshop 2022 (Adobe, San Jose, CA, USA). The pixel intensity of each band was measured, and the background pixel intensity was deducted from the raw measurement for each band.

2.10 Statistical analyses

Significant differences ($P < 0.05$) in the means between two or more groups were evaluated using Student's *t* test or Tukey's multiple comparison test

3 RESULTS

3.1 AsA, DHA and AsA-Na inhibit hyphal growth of blast pathogen

Mycelial growth of the fungus after 7 days on CM amended with 20 mM AsA was inhibited by 73% and by 89% on CM with 20 mM DHA compared with pH 7 CM (Figure 1a and b). To verify the possibility

that the inhibitory effects of AsA and DHA might be due to the low pH (pH 4.6 in CM), the fungus was also grown on CM containing 20 mM AsA sodium salt (AsA-Na) at pH 7.0 and control CM at the same pH as the CM containing AsA or DHA. On pH 7 CM containing AsA-Na, the growth of mycelia was moderately inhibited, and pH 4.6 CM also inhibited mycelial growth at a level similar to that of AsA-Na (Figure 1). These results suggested that the effects of AsA and/or DHA on mycelial growth was due to both its antioxidant capacity and acidic nature, but the antioxidant action alone can inhibit growth. We also used nitroblue tetrazolium staining to examine whether superoxide anions generated in fungal cells were sufficiently degraded by the treatments with AsA and DHA. Superoxide anions were mostly absent in the germ tubes and conidia cells treated with AsA and DHA but present in the untreated control (Figure S1).

When hyphae grown on CM with either AsA or DHA were observed with a light microscope, the hyphae frequently grew in a zig-zag pattern (Figure 2). Such zig-zag growth was not observed for hyphae on pH 4.6 CM or on pH 7 CM with AsA-Na (Figure 2). Thus, AsA and DHA inhibited overall mycelial growth in acidic and neutral conditions and caused the zig-zag growth only at low pH.

3.2 Zig-zag hyphal growth caused by AsA and DHA is related to disruption of cyclic, fluctuating expression of *PoTea* genes

Polarized hyphal growth is strictly regulated by the *Tea* cell-end marker genes, so a mutation in each *Tea* gene in *A. nidulans* and *P. oryzae* often causes a zig-zag type of hyphal growth (Brand and Gow, 2009; Patkar et al., 2010). Because the growth pattern of hyphae exposed to AsA and DHA in *P. oryzae* closely resembled the phenotypes of the *Tea* mutants of *A. nidulans* and *P. oryzae*, we investigated the expression of *PoTea1*, *PoTea2* and *PoTea4* (synonym for *MoTea4*) in mycelia cultured in CM broth containing 20 mM AsA or DHA in the dark, then analyzed expression of each *PoTea* gene in mycelia at 6, 12, 24 and 48 h after addition of AsA and DHA. Expression levels of all *PoTea* genes in the controls for the AsA and

DHA treatments changed remarkably over time in the dark (Figure 3). For instance, after exposure to AsA, the expression of *PoTea1* in the control at 24 h post treatment (HPT) was about 2.5-fold higher than at 12 HPT, whereas the expression level at 48 HPT was lower than at 12 HPT (Figure 3). *PoTea2* and *PoTea4* also had an expression pattern similar to that of *PoTea1*. On the other hand, the expression level of *PoTea1* at 24 HPT in the control for the DHA treatment was about 0.5-fold lower than at 12 HPT, and expression at 48 HPT was higher than at 12 HPT (Figure 3); the expression patterns for *PoTea2* and *PoTea4* were similar. Although the expression patterns for the *PoTea* genes were different between those of the controls for the AsA and DHA treatments, these differences appeared to be due to the shift in circadian rhythm caused by the differences in the duration of light exposure of the fungal starter cultures. More importantly, such fluctuations in the expression of the three *PoTea* genes were considerably weakened by the AsA and DHA treatments (Figure 3). Thus, the zig-zag hyphal growth caused by AsA and DHA seems closely associated with the disorder of the fluctuating expression in the *PoTea* genes.

3.3 Expression of circadian oscillator *PoFrq* is also affected by AsA and DHA

The circadian rhythm in the blast fungus might be disturbed by exposure to AsA and DHA. To clarify if the circadian rhythm could be fluctuated under such culture condition, we analyzed the expression pattern of the circadian oscillator gene *PoFrq* (*FRQ* of Deng et al. [2015]) using the same set of samples described above. The results showed that expression of *PoFrq* was significantly affected by the exposure of AsA and DHA (Figure 3), indicating that both AsA and DHA could interfere with the circadian regulation of gene expression in the blast fungus. We also evaluated changes in the expression of *PoFrq* in light/dark (LD) conditions at 4-h intervals. The results showed apparent diurnal oscillation in *PoFrq* expression in the controls for the AsA and DHA treatments; however, *PoFrq* expression was out of rhythm by 24 h after treatment with AsA or DHA (Figures 4 and 5). Thus, the circadian clock system in the blast fungus appeared to be disrupted by exposure to AsA or DHA. In the control for the AsA

treatment, the expression of all *PoTea* genes also showed a slight to moderate diurnal oscillation; however, AsA disrupted rhythms in different way than it did for *PoFrq* (Figure 4). In the control for the DHA treatment, each *PoTea* gene was expressed with a more complicated oscillation pattern than that of *PoFrq* (Figure 5). The exposure to DHA also modified the rhythmic patterns in *PoFrq* and *PoTea* genes expression.

3.4 Three forms of ascorbate have different modes of action for suppressing the blast fungus

To investigate the inhibitory effects of AsA, DHA and AsA-Na on infection by the blast fungus, we added AsA, DHA or AsA-Na at a final concentration of 20 mM to the spore suspension just before inoculation of barley plants. AsA and AsA-Na significantly inhibited infection by blast isolate Ken 54-20, but DHA was not inhibitory (Figure 6). Although the reason for the lack of antifungal effects in this experiment is unclear, we suspect that DHA on leaves is degraded more readily than AsA.

The addition of AsA or AsA-Na to the spore suspension significantly suppressed infection of barley plants (Figure 6). Therefore, we next tested the effects of the three ascorbates on spore germination and appressorium formation. Only 20 mM AsA and DHA strongly inhibited spore germination and appressorium formation (Figure S2). Because fungal spores germinated in the control solution adjusted to the same pH as that of 20 mM AsA and DHA (Figure S2a), the difference between AsA and AsA-Na in the inhibition rate of spore germination was apparently not due to the acidic nature of AsA. Although most spores germinated in the solution containing 20 mM AsA-Na, the germ tubes never formed an appressorium (Figure S2b). Because 20 mM NaCl was not inhibitory to the fungus, the antifungal effect of AsA-Na appeared to be due to its antioxidant capacity.

When AsA, AsA-Na or DHA was added to CM, AsA severely inhibited accumulation of pigments in mycelium, but mycelium in CM containing AsA-Na changed from black to brown (Figure S3a). DHA did not have any observable effects on mycelial pigmentation. Quantitative RT-PCR analyses showed that the

expression of the genes *Rsy1* and *Alb1*, which are related to melanin biosynthesis, was suppressed by AsA but not DHA (Figure S3b). On the other hand, AsA-Na enhanced melanin biosynthesis because *Alb1* was upregulated, whereas *CNFI*, the negative regulator of melanin biosynthetic genes, was downregulated (Figure S3b). Thus, AsA and AsA-Na had opposite effects on melanin biosynthesis.

When barley plants were pretreated with the ascorbates 6 or 24 h before inoculation, AsA and DHA inhibited development of symptoms (Figure 6). By 24 h after the treatment with 20 mM AsA or DHA, the AsA content in barley leaves had significantly increased about 2-fold compared to that in their respective controls, but DHA content had barely increased (Figure 7). Therefore, the inhibition of fungal infection by the DHA pretreatment was unlikely due to DHA accumulation in plant cells. Because DHA in the apoplast could be transported into plant cells (Kollist et al., 2001) and reduced to AsA by DHAR, two hypotheses might explain why pretreatment with DHA inhibited infection. Either the higher level of AsA in plant cells inhibited the fungus, or the recycling pathway for DHA is linked to a pathway(s) that is involved in inducing a defense reaction. To clarify whether a defense reaction was induced, we compared the expression levels of three pathogenesis-related (PR) genes in barley plants that were treated with 20 mM DHA with those in the control plants treated with water. The results showed that the expression of *PR1b*, *PR4* and *PR5* gradually increased from 6 h after the DHA treatment to a level about 10- to 36-fold higher than the respective controls by 24 HPT (Figure 8). Thus, DHA can function as a defense activator against blast pathogen in barley.

The effect of pretreatment with DHA against the blast pathogen was also examined in susceptible rice cultivar Nipponbare. By 24 h after the pretreatment with 10 mM DHA, total foliar AsA+DHA content was about 2-fold higher than in the control (Figure S4a). Only about a third of the DHA taken up by the cells seemed to be converted to AsA. The expression of the defense-related genes *PR2*, *PR5* and *WRKY45* was moderately upregulated by 24 h after the DHA treatment (Figure S4b), and the DHA-treated plants had significantly fewer lesions than on the control, indicating at least some resistance to the pathogen

(Figure S4c).

4 DISCUSSION

On CM agar containing antioxidants AsA or DHA, the hyphae of *P. oryzae* slowly grew with a zig-zag pattern. AsA-Na moderately inhibited hyphal growth, but hyphae retained their typical linear growth pattern. Only AsA and DHA strongly inhibited spore germination. These results suggest that the acidic nature of the AsA and DHA solutions was important for inhibiting spore germination and disrupting polarized hyphal growth. However, in the acidic solution without any ascorbate, the spores germinated, and the pattern of hyphal growth was not affected. These results suggest that both the acidity and antioxidant ability were necessary to inhibit spore germination and interfere with hyphal growth. Because the antioxidant activity of AsA is greater at lower pH (Gramlich et al., 2002), we consider that the antioxidant activity is essential for the antifungal action of ascorbates.

We also showed that *PoTea1*, *PoTea2* and *PoTea4* were expressed with a similar fluctuating pattern in continuous dark and in 12-h light/12-h dark, suggesting that their expression is regulated by the same pathway(s). Considering the possible roles of *PoTea1*, *PoTea2* and *PoTea4* in polarized growth of hyphae (Patkar et al., 2010; Qu et al., 2022), their expressions should be strictly coordinated to avoid misdirecting hyphal tip growth. Therefore, the changes in the expression of the *PoTea* genes could cause abnormal hyphal growth such as the zig-zag pattern.

Circadian rhythms are common features of almost all eukaryotes including fungi (Liu and Bell-Pedersen, 2006). The circadian clock, an endogenous oscillator with a 24-h period, is used by organisms to anticipate the daily light/dark cycle. In *P. oryzae*, the circadian rhythm is needed for asexual development and pathogenesis (Deng et al., 2015). In the present study, it is noteworthy that the expression of the circadian oscillator gene *PoFrq* was also disrupted by the antioxidants AsA and DHA, suggesting that the cellular redox state is linked to the circadian rhythm in *P. oryzae*. In *Neurospora*

crassa, increased ROS levels are known to enhance rhythmic expression of *FRQ*, whereas lower ROS levels inhibit that effect (Yoshida et al. 2011). Thus, the ROS signaling pathway might regulate the circadian clock in *P. oryzae*, and quenching of ROS by antioxidants such as ascorbates might change the expression of circadian-regulated genes, leading to a loss of pathogenicity or decreased virulence. Further analyses should give new insights into ROS-dependent circadian gene regulation in the blast fungus.

We here found that the three forms of ascorbate were effective in suppressing disease caused by the blast fungus; however, their modes of action differed. Although we showed that AsA and AsA-Na acted as antifungal agents against the fungus, pretreatments of barley plants with AsA or AsA-Na led to low levels of inhibition of lesion development although the AsA content was about 2-fold higher than in the control. In this case, AsA might have permeated the cuticle layer and immediately been transported to the cytoplasm of the epidermal cells and not entered the apoplast. Therefore, the penetration hypha might not encounter AsA after penetrating the cell wall. Because the hypha invaginates the plasma membrane without breaching it during infection (Eseola et al., 2021), if AsA accumulates in the host cytoplasm, it cannot act directly on the fungus.

AsA severely inhibited melanin biosynthesis in hyphae and considerably suppressed expression of the melanin biosynthesis-related genes *RsyI* and *AlbI*. Because melanin is important in appressorium formation and function of the blast fungus (Howard et al. 1991), the inhibitory effect of AsA on melanin biosynthesis might be involved in the suppression of appressorium formation by AsA. Recently, Huang et al. (2022) reported that melanin promotes sporulation of the blast fungus, suggesting that AsA might also inhibit spore production in this fungus. In contrast to AsA, AsA-Na did not inhibit melanin accumulation, but upregulated *AlbI* and downregulated *CNFI*, a negative regulator of the melanin biosynthetic genes. Because melanins enhance the survival of fungi under stress conditions such as high salinity (Pedras and Yu, 2009), Na⁺ rather than AsA might be involved in the activation of melanin synthesis on CM containing 20 mM AsA-Na.

Pretreatment of barley and rice plants with DHA also suppressed lesion development. Because DHAR activity in the apoplast of barley leaves represents only a small fraction of its total activity in the leaf (Vanacker et al., 1998a, 1998b), exogenous DHA should be transported into the host cells and converted to AsA by intracellular DHAR. In fact, only the level of endogenous AsA had increased 24 h after the DHA treatment in barley. In rice, about a third of DHA was converted to AsA. Although DHA was immediately converted to AsA, the DHA pretreatments of barley and rice plants effectively suppressed lesion formation by blast pathogen, and in response to DHA, the expression of the defense-related genes was also upregulated. These results suggest that the reduction of DHA might be more crucial to inducing a defense reaction than DHA itself. Because DHA reduction catalyzed by DHAR is coupled with the oxidation of glutathione, the so-called ascorbate–glutathione cycle (Foyer and Noctor, 2011), the rapid increase of the oxidized form of glutathione (GSSG) caused by exogenous DHA could be associated with induction of blast resistance. The reduced form of glutathione (GSH) generally predominates at a ratio of 20:1 GSH:GSSG in Arabidopsis (Mhamdi et al., 2010). In Arabidopsis, an imbalance in this glutathione redox homeostasis leads to a heightened defense response including accelerated activation of defense-related genes (Ge et al., 2007; Ge and Xia, 2008). In the Nudix-domain-containing hydrolase7 (*nudt7*) mutant of Arabidopsis, more GSH molecules are likely being used to reduce oxidized molecules, causing the consistently lower ratio of GSH to GSSG. This redox imbalance caused by the *nudt7* mutation enhances two distinct signaling pathways for defense responses: one independent of and the other dependent on NPR1 and SA. Thus, exogenous DHA could enhance the oxidation of glutathione, perturbing the cellular redox status, leading to induction of resistance in barley and rice. Interestingly, Chavan et al. (2022) reported that DHA caused accumulation of ROS and activation of the SA signaling pathway leading to induced systemic resistance against the root-knot nematode *Meloidogyne graminicola* in rice. Considering that DHA, which was exogenously applied, was immediately transported into the plant cells and converted to AsA as shown in our study, the exogenous DHA could presumably regulate

the cellular redox status linked to the activation of the SA signaling pathway. Further analysis will give more insight into the function of DHA as a plant defense activator.

In conclusion, all three forms of ascorbate had antifungal effects against the blast pathogen, but quite different modes of action not only on the blast fungus, but also on the host plants. These three ascorbates are good antifungal candidates against a wide range of pathogenic fungi, and the ROS signaling pathway and/or the circadian clock in pathogenic fungi could also be targets of these antifungal compounds.

ACKNOWLEDGEMENTS

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

CONFLICT OF INTEREST

The authors declare that they have no competing interests.

REFERENCES

- Bradley, D. W., Emery, G., & Maynard, J. E. (1973) Vitamin C in plasma: a comparative study of the vitamin stabilized with trichloroacetic acid or metaphosphoric acid and the effects of storage at -70 degrees, -20 degrees, 4 degrees, and 25 degrees on the stabilized vitamin. *Clinica Chimica Acta*, 44, 47–52.
- Brand, A., & Gow, N. A. (2009) Mechanisms of hypha orientation of fungi. *Current Opinion in Microbiology*, 12, 350–357.
- Chavan, S. N., De Kesel, J., Desmedt, W., Degroote, E., Singh, R. R., Nguyen, G. T. et al. (2022)

Dehydroascorbate induces plant resistance in rice against root-knot nematode *Meloidogyne graminicola*. *Molecular Plant Pathology*, 23, 1303–1319.

Che Omar, S., Bentley, M. A., Morieri, G., Preston, G. M., & Gurr, S. J. (2016) Validation of reference genes for robust qRT-PCR gene expression analysis in the rice blast fungus *Magnaporthe oryzae*. *PLoS ONE*, 11, e0160637.

Deng, Y. Z., Qu, Z., & Naqvi, N. I. (2015) Twilight, a novel circadian-regulated gene, integrates phototropism with nutrient and redox homeostasis during fungal development. *PLoS Pathogens*, 11, e1004972.

Dewhirst, R. A., & Fry, S. C. (2018) The oxidation of dehydroascorbic acid and 2,3-diketogulonate by distinct reactive oxygen species. *The Biochemical Journal*, 475, 3451–3470.

Egan, M. J., Wang, Z. Y., Jones, M. A., Smirnoff, N., & Talbot, N. J. (2007) Generation of reactive oxygen species by fungal NADPH oxidases is required for rice blast disease. *Proceedings of the National Academy of Sciences of the United States of America*, 104, 11772–11777.

Eseola, A. B., Ryder, L. S., Osés-Ruiz, M., Findlay, K., Yan, X., Cruz-Mireles, N. et al. (2021) Investigating the cell and developmental biology of plant infection by the rice blast fungus *Magnaporthe oryzae*. *Fungal Genetics and Biology*, 154, 103562.

Foyer, C. H., & Noctor, G. (2011) Ascorbate and glutathione: the heart of the redox hub. *Plant Physiology*, 155, 2–18.

Gallie, D. R. (2013) The role of L-ascorbic acid recycling in responding to environmental stress and in promoting plant growth. *Journal of Experimental Botany*, 64, 433–443.

Ge, X., Li, G. J., Wang, S. B., Zhu, H., Zhu, T., Wang, X. et al. (2007) AtNUDT7, a negative regulator of basal immunity in Arabidopsis, modulates two distinct defense response pathways and is involved in maintaining redox homeostasis. *Plant Physiology*, 145, 204–215.

Ge, X., & Xia, Y. (2008) The role of AtNUDT7, a Nudix hydrolase, in the plant defense response. *Plant Signaling and Behavior*, 3, 119–120.

Gramlich, G., Zhang, J., & Nau, W. M. (2002) Increased antioxidant reactivity of vitamin C at low pH in model membranes. *Journal of the American Chemical Society*, 124, 11252–11253.

Higashitsuji, Y., Herrero, S., Takeshita, N., & Fischer, R. (2009) The cell end marker protein TeaC is involved in growth directionality and septation in *Aspergillus nidulans*. *Eukaryotic Cell*, 8, 957–967.

Howard, R. J., Ferrari, M. A., Roach, D. H., & Money, N. P. (1991). Penetration of hard substrates by a fungus employing enormous turgor pressures. *Proceedings of the National Academy of Sciences, of the United States of America*, 88, 11281–11284.

- Huang, P., Cao, H., Li, Y., Zhu, S., Wang, J., Wang, Q., Liu, X., Lin, F. C., & Lu, J. (2022) Melanin promotes spore production in the rice blast fungus *Magnaporthe oryzae*. *Frontiers in Microbiology*, 13:843838.
- Inukai, T., Vales, M. I., Hori, K., Sato, K., & Hayes, P. M. (2006) *RMo1* confers blast resistance in barley and is located within the complex of resistance genes containing *Mla*, a powdery mildew resistance gene. *Molecular Plant-Microbe Interactions*, 19, 1034–1041.
- Kim, B., Masuta, C., Matsuura, H., Takahashi, H., & Inukai, T. (2008) Veinal necrosis induced by turnip mosaic virus infection in *Arabidopsis* is a form of defense response accompanying HR-like cell death. *Molecular Plant-Microbe Interactions*, 21, 260–268.
- Kollist, H., Moldau, H., Oksanen, E., & Vapaavuori, E. (2001) Ascorbate transport from the apoplast to the symplast in intact leaves. *Physiologia Plantarum*, 113, 377–383.
- Liu, Y., & Bell-Pedersen, D. (2006) Circadian rhythms in *Neurospora crassa* and other filamentous fungi. *Eukaryotic Cell*, 5, 1184–1193.
- Martin-Urdiroz, M., Osés-Ruiz, M., Ryder, L. S., & Talbot, N. J. (2016) Investigating the biology of plant infection by the rice blast fungus *Magnaporthe oryzae*. *Fungal Genetics and Biology*, 90, 61–68.
- Mhamdi, A., Hager, J., Chaouch, S., Queval, G., Han, Y., Taconnat, L. et al. (2010) *Arabidopsis* GLUTATHIONE REDUCTASE1 plays a crucial role in leaf responses to intracellular hydrogen peroxide and in ensuring appropriate gene expression through both salicylic acid and jasmonic acid signaling pathways. *Plant Physiology*, 153, 1144–1160.
- Patkar, R. N., Suresh, A., & Naqvi, N. I. (2010) *MoTea4*-mediated polarized growth is essential for proper asexual development and pathogenesis in *Magnaporthe oryzae*. *Eukaryotic Cell*, 9, 1029–1038.
- Pedras, M. S., & Yu, Y. (2009) Salt stress induces production of melanin related metabolites in the phytopathogenic fungus *Leptosphaeria maculans*. *Natural Product Communications*, 4, 53–58.
- Qu, Y., Cao, H., Huang, P., Wang, J., Liu, X., Lu, J. et al. (2022) A kelch domain cell end protein, PoTea1, mediates cell polarization during appressorium morphogenesis in *Pyricularia oryzae*. *Microbiological Research*, 259, 126999.
- Quiñones, C. O., Gesto-Borroto, R., Wilson, R. V., Hernández-Madrigal, S. V., & Lorence, A. (2024) Alternative pathways leading to ascorbate biosynthesis in plants: lessons from the last 25 years. *Journal of Experimental Botany*, 75, 2644–2663.
- Riquelme, M., Aguirre, J., Bartnicki-García, S., Braus, G. H., Feldbrügge, M., Fleig, U. et al. (2018) Fungal morphogenesis, from the polarized growth of hyphae to complex reproduction and infection

structures. *Microbiology and Molecular Biology Reviews*, 82, e00068-17.

Rogers, A. M., Taylor, R., & Egan, M. J. (2024) The cell-end protein *Tea4* spatially regulates hyphal branch initiation and appressorium remodeling in the blast fungus *Magnaporthe oryzae*. *Molecular Biology of the Cell*, 35, br2.

Schumann, U., Smith, N. A., & Wang, M.-B. (2013) A fast and efficient method for preparation of high-quality RNA from fungal mycelia. *BMC Research Notes*, 6, 1-5.

Singh, P. K., Gahtyari, N. C., Roy, C., Roy, K. K., He, X., Tembo, B. et al. (2021) Wheat Blast: A disease spreading by intercontinental jumps and its management strategies. *Frontiers in Plant Science*, 12, 710707.

Takeshita N. (2016) Coordinated process of polarized growth in filamentous fungi. *Bioscience, Biotechnology, and Biochemistry*, 80, 1693–1699.

Takeshita, N., Higashitsuji, Y., Konzack, S., & Fischer, R. (2008) Apical sterol-rich membranes are essential for localizing cell end markers that determine growth directionality in the filamentous fungus *Aspergillus nidulans*. *Molecular Biology of the Cell*, 19, 339–351.

Vanacker, H., Carver, T. L., & Foyer, C. H. (1998a) Pathogen-induced changes in the antioxidant status of the apoplast in barley leaves. *Plant Physiology*, 117, 1103–1114.

Vanacker, H., Harbinson, J., Ruisch, J., Carver, T. L. W., & Foyer, C. H. (1998b) Antioxidant defenses of the apoplast. *Protoplasma*, 205, 129–140.

Yoshida, Y., Iigusa, H., Wang, N., & Hasunuma, K. (2011) Cross-talk between the cellular redox state and the circadian system in *Neurospora*. *PLoS ONE*, 6, e28227.

Zechmann, B. (2011) Subcellular distribution of ascorbate in plants. *Plant Signaling and Behavior*, 6, 360–363.

Zhang, N., Luo, J., Rossman, A. Y., Aoki, T., Chuma, I., Crous, P. W. et al. (2016) Generic names in *Magnaporthales*. *IMA Fungus*, 7, 155–159.

FIGURE LEGENDS

FIGURE 1

Colony area of *Pyricularia oryzae* isolate Ken 54-20 on complete medium (CM) amended with 20 mM L-ascorbic acid (AsA), 20 mM AsA-Na, or 20 mM dehydroascorbic acid (DHA) and on control CM at pH 7.0 or adjusted to pH 4.6 with HCl. (a) Colonies after 4 days. (b) Colony area after 3 to 7 days. Error bars: SD of the mean of biological triplicates.

FIGURE 2

Hyphae of *Pyricularia oryzae* isolate Ken 54-20 after 36 to 48 h on complete medium (CM) amended with 10 mM L-ascorbic acid (AsA), 10 mM AsA-Na, or 10 mM dehydroascorbic acid (DHA) and on control CM at pH 7.0 or adjusted to pH 4.6 with HCl. Insets: Higher magnification of hyphae to show typical growth pattern. Scale bars = 50 μ m.

FIGURE 3

Changes in relative expression of the three *PoTea* genes and circadian oscillator gene *PoFrq* in mycelia of *Pyricularia oryzae* isolate Ken 54-20 cultured in complete medium (CM) broth amended with 20 mM L-ascorbic acid (AsA) or 20 mM dehydroascorbic acid (DHA) for 48 h in continuous dark (DD). Transcript levels were determined using RT-qPCR. Error bars: SD of means for biological triplicates. Significant differences between means were determined using Student's *t*-test (* $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$).

FIGURE 4

Changes in relative expression of the circadian oscillator gene *PoFrq* and three *PoTea* genes in *Pyricularia oryzae* isolate Ken 54-20 cultured on complete medium (CM) amended with 20 mM L-

ascorbic acid (AsA) during 40 h with 12-h light/12-h dark (LD). Transcript levels in mycelia were determined with semiquantitative RT-PCR. Left: Agarose gel of amplicons from cDNA of each gene. Right: Relative expression calculated using data from left panel.

FIGURE 5

Changes in relative expression of the circadian oscillator gene *PoFrq* and three *PoTea* genes in *Pyricularia oryzae* isolate Ken 54-20 cultured on complete medium (CM) amended with 20 mM dehydroascorbic acid (DHA) during 40 h with 12-h light/12-h dark (LD). Transcript levels in mycelia were determined with semiquantitative RT-PCR. Left: Agarose gel of amplicons from cDNA of each gene. Right: Relative expression calculated using data from left panel.

FIGURE 6

Effects of 20 mM L-ascorbic acid (AsA), AsA-Na or dehydroascorbic acid (DHA) sprayed on plants 24 or 6 h before inoculation or added to the spore suspension on number of lesions on third leaf of susceptible barley (*Hordeum vulgare*) cv. Harrington 7 days after inoculation. Plants were inoculated with *Pyricularia oryzae* isolate Ken 54-20. For pretreatments, 20 mM AsA, AsA-Na or DHA with 0.2% Tween 20 was sprayed on barley plants before inoculation. When added to the spore suspension, AsA, AsA-Na and DHA were adjusted to a final concentration of 20 mM. C: Control, A: AsA, AN: AsA-Na, D: DHA. Error bars: SD of the means of six replicates.

FIGURE 7

Total ascorbic acid content (L-ascorbic acid [AsA] and dehydroascorbic acid [DHA]) in third leaves of *Hordeum vulgare* cv. Harrington 24 h after leaves were sprayed with 20 mM AsA or DHA (each with 0.2% Tween 20). Error bars: SE of means for biological triplicates. Significant differences among means

were determined using Student's *t*-test (** $P \leq 0.01$).

FIGURE 8

Relative expression of three pathogenesis-related genes in *Hordeum vulgare* cv. Harrington at 6, 12, 24 and 48 h after treatment with 20 mM dehydroascorbic acid (+0.2% v/v Tween 20; DHA). HPT: hours post treatment. Transcript levels in the third leaves were determined with RT-qPCR using *GAPDH*, *tub7*, *18SrRNA* as internal standards. Error bars: SD of means for five biological replicates. Significant differences among means were determined using Student's *t*-test (* $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$).

SUPPORTING INFORMATION

Table S1. List of primers used for multiplex RT-qPCR of genes from different species in this study

Table S2. List of primers and PCR programs used for semiquantitative RT-PCR in this study.

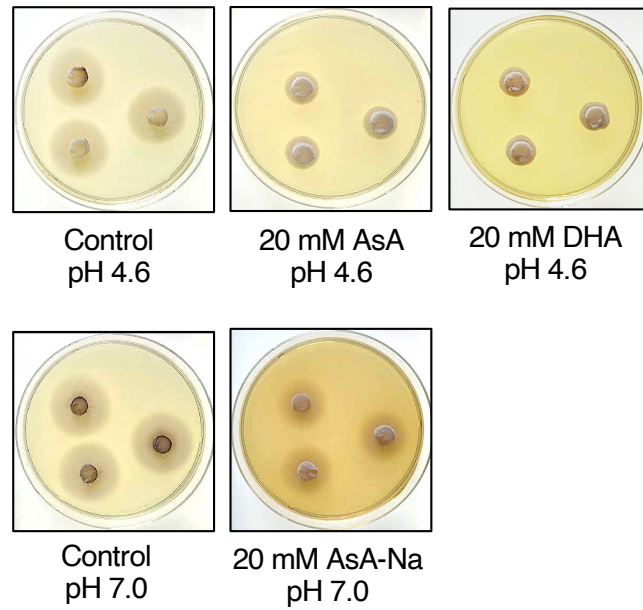
Figure S1. Degradation of reactive oxygen species generated during germination of conidia of *Pyricularia oryzae* that were treated with 20 mM ascorbic acid or 20 mM dehydroascorbic acid.

Figure S2. Percentage of conidia that germinated and the percentage of germ tubes that formed an appressorium after 17-h exposure of conidia to 20 mM L-ascorbic acid (AsA), AsA-Na, dehydroascorbic acid, NaCl or HCl adjusted to the same pH as 20 mM AsA.

Figure S3. Effect of L-ascorbic acid (AsA), AsA-Na or dehydroascorbic acid on melanin biosynthesis in *Pyricularia oryzae* isolate Ken 54-20.

Figure S4. Effect of pretreatment of *Oryza sativa* cv. Nipponbare with exogenous dehydroascorbic acid after 24 h on total ascorbic acid content, expression of defense-related genes in sixth leaves that were not inoculated with the blast fungus and on blast lesion formation on sixth leaves after inoculation.

a



b

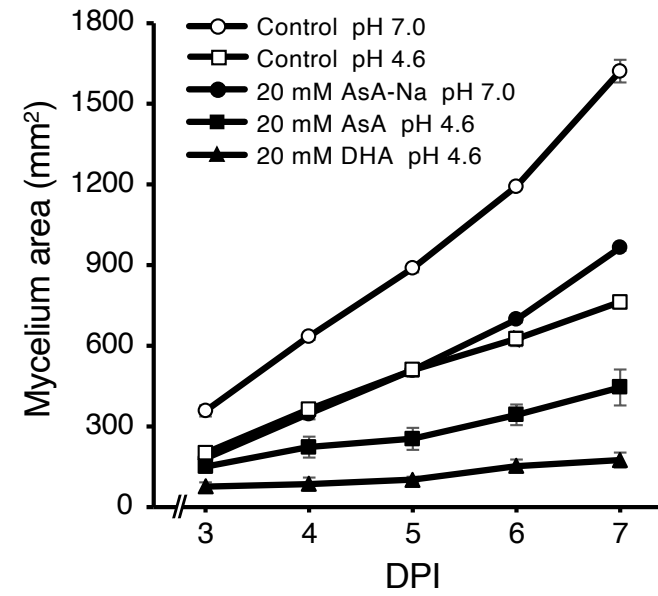


Fig. 1

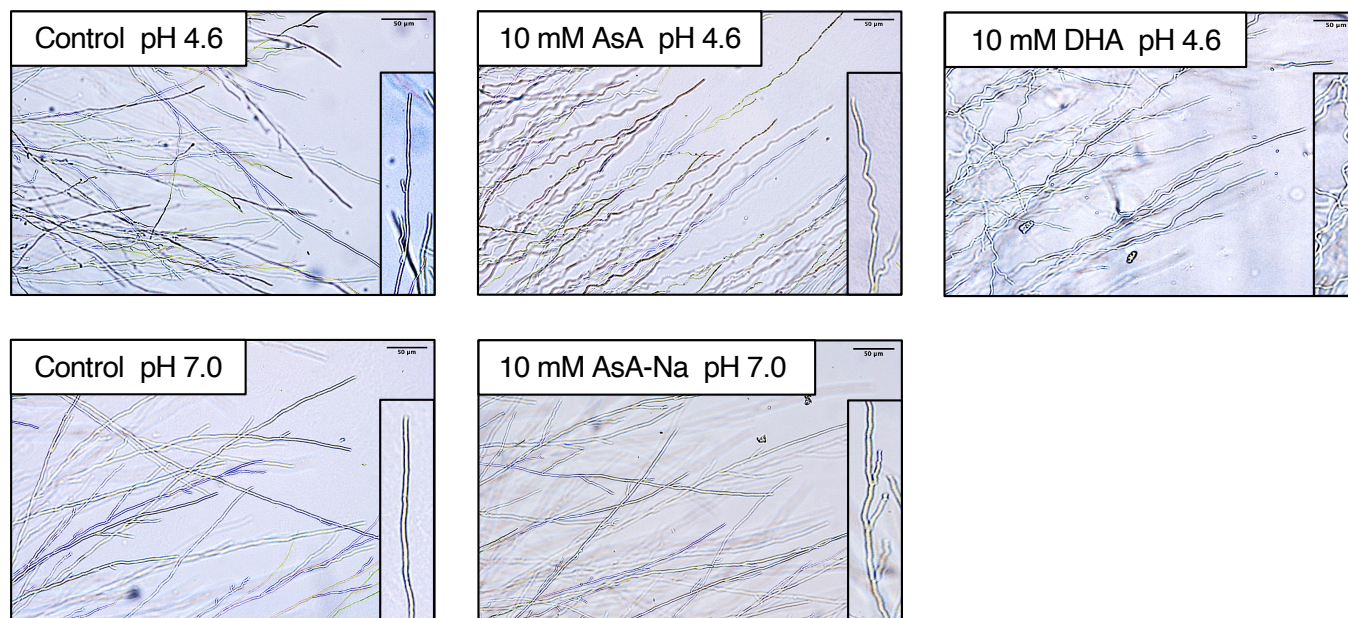


Fig. 2

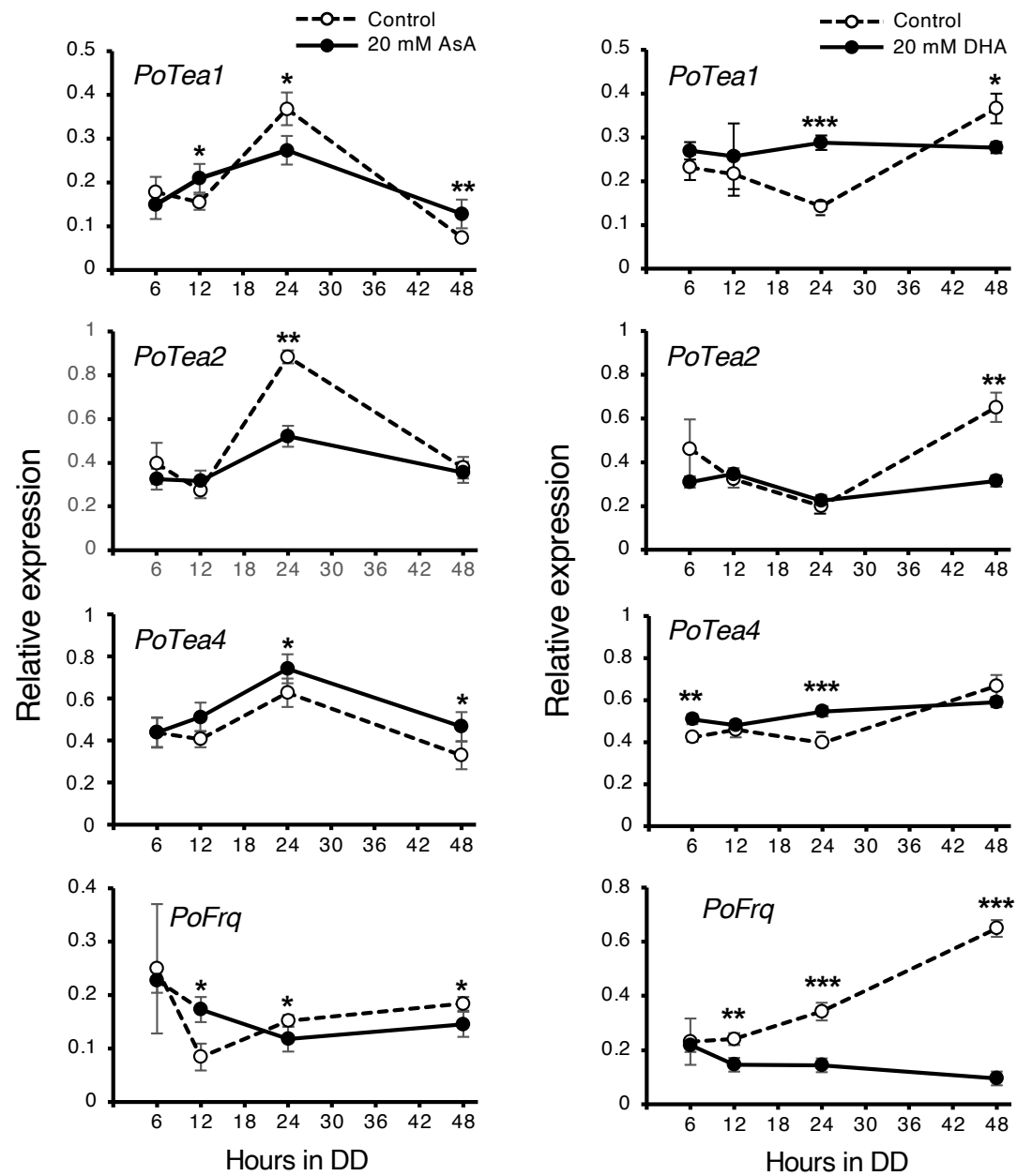


Fig. 3

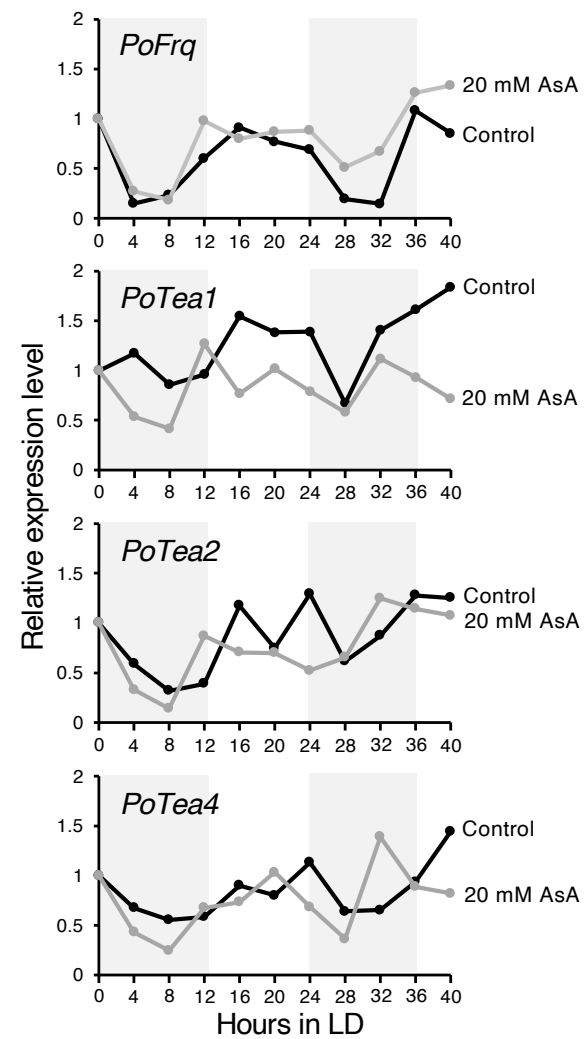
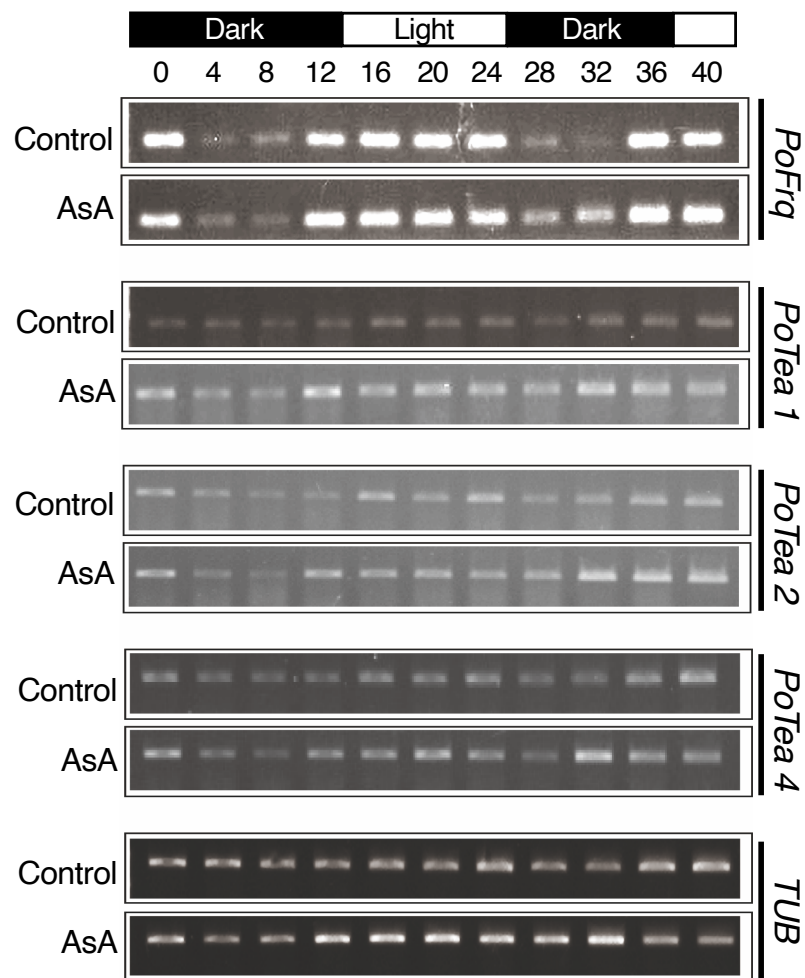


Fig. 4

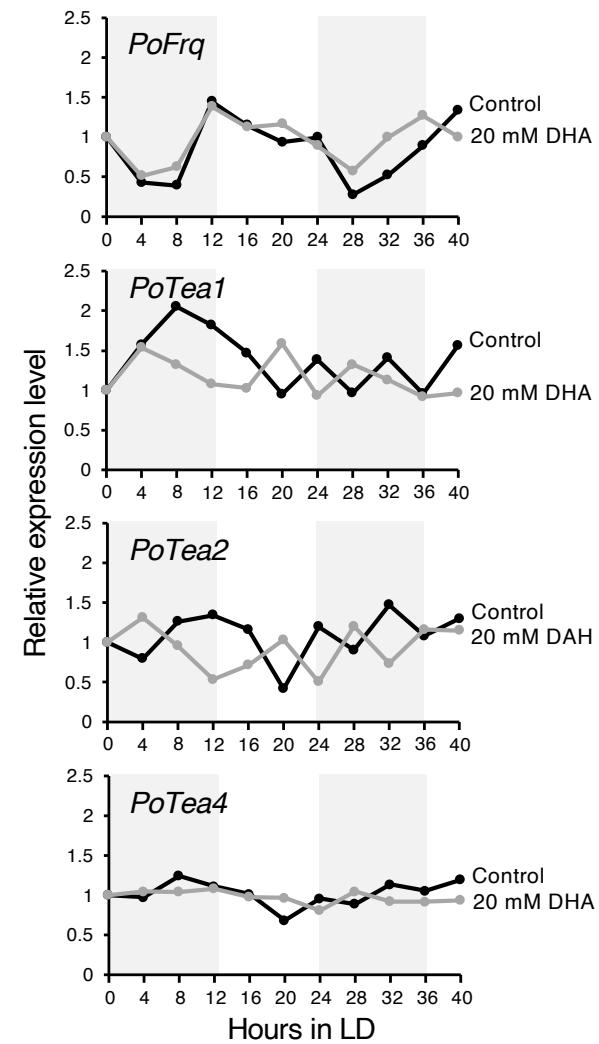
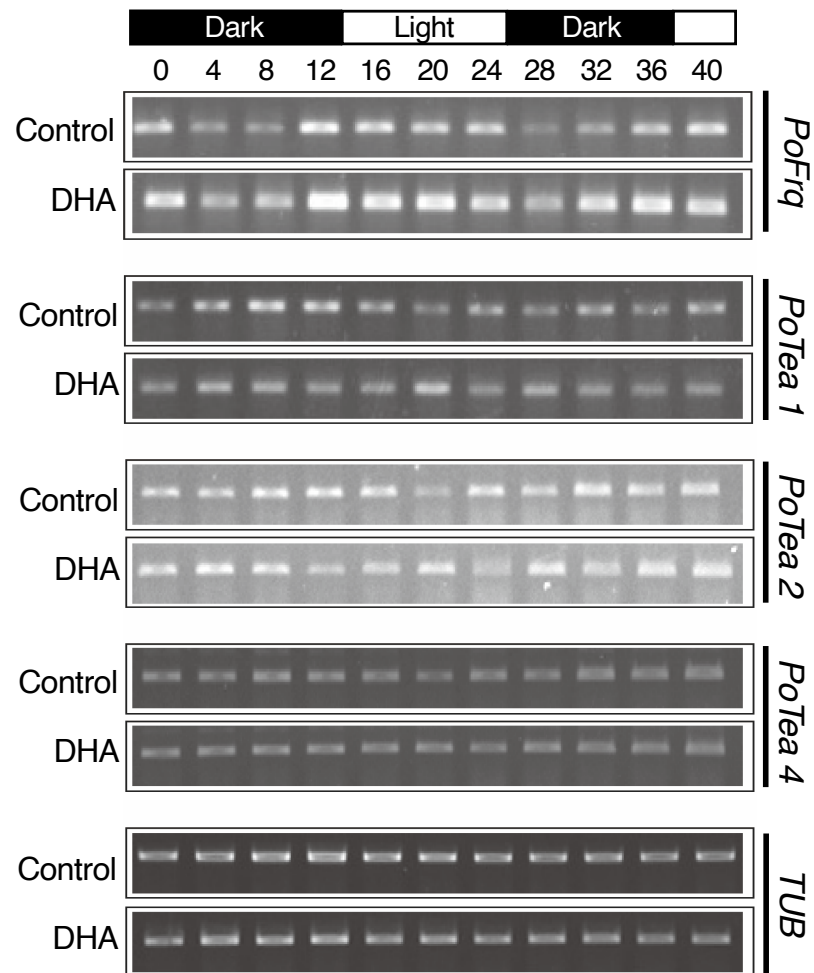


Fig. 5

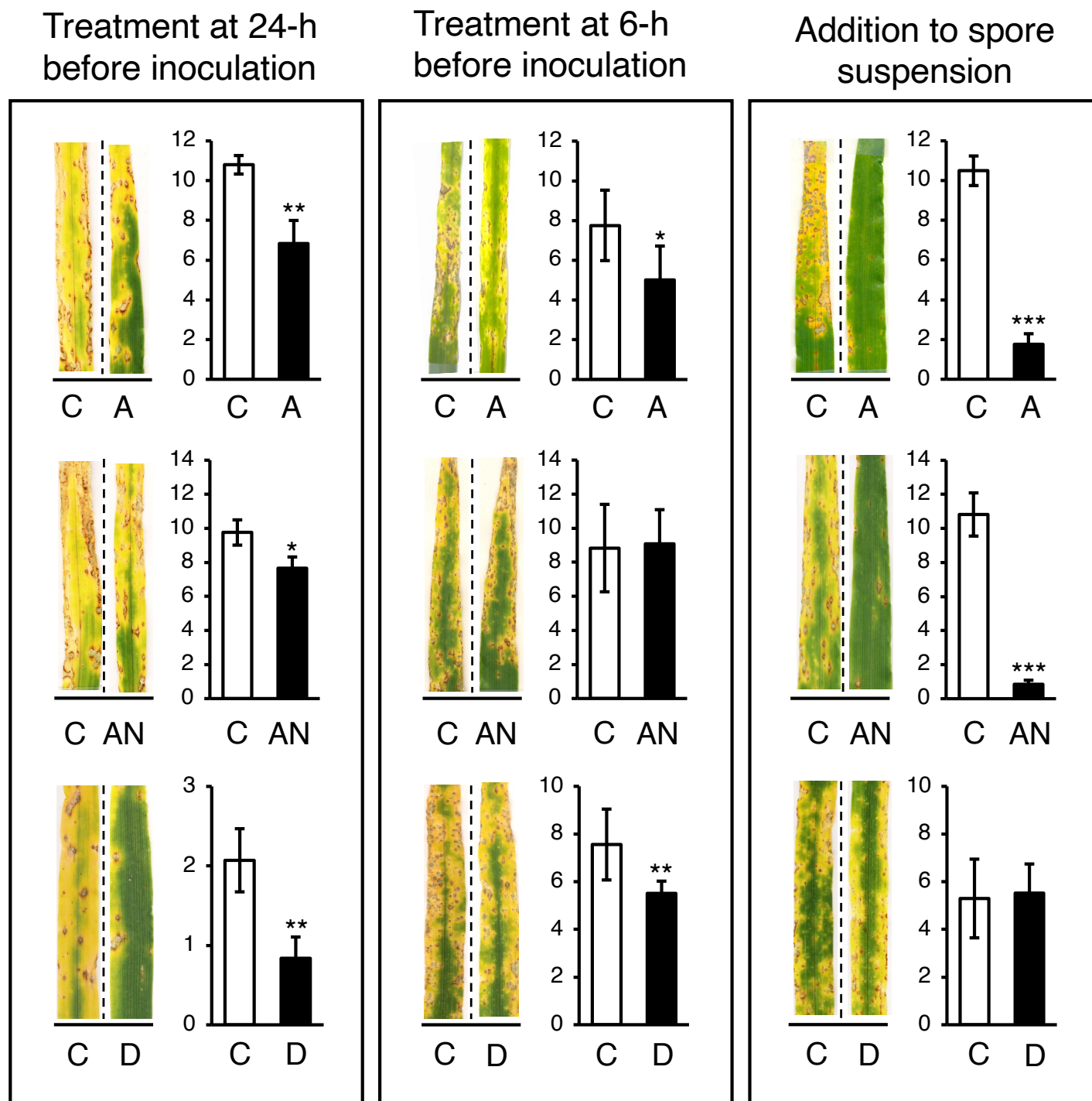


Fig. 6

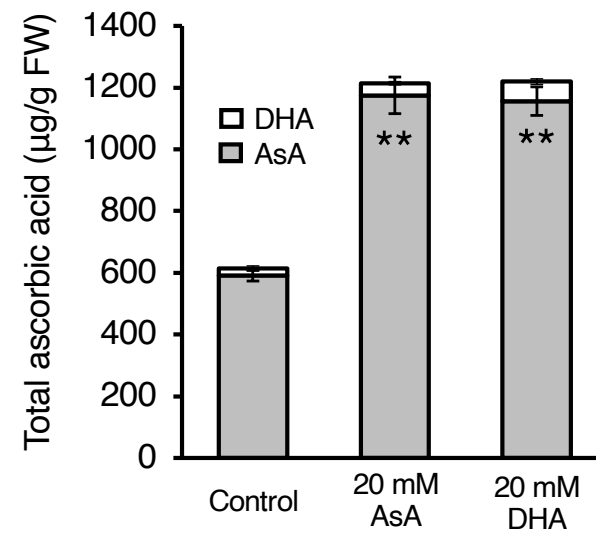


Fig. 7

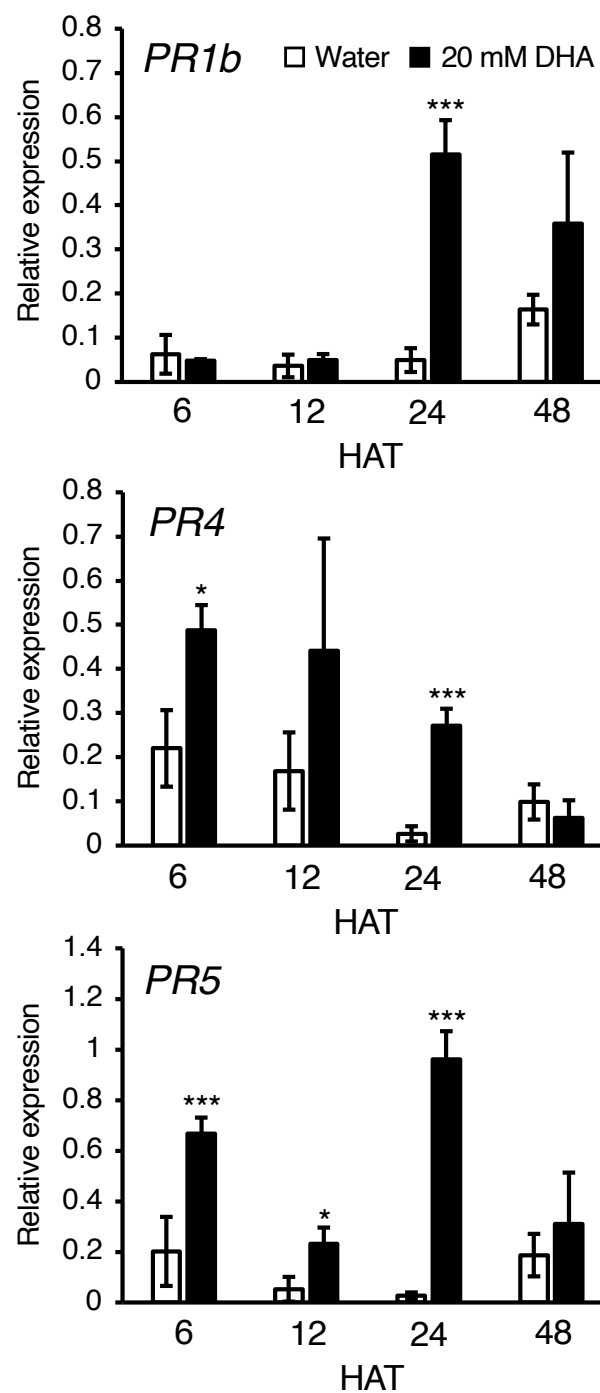


Fig. 8

Table S1. List of primers used for multiplex RT-qPCR of genes from different species in this study

Species	Gene function	Gene	Forward primer (5' to 3')	Reverse primer (5' to 3')
<i>Pyricularia oryzae</i>	Polarized growth	PoTea1 (MGG_02875)	AGGTGACACTATAGAATACTACTAGCCAAGGTGCTGGC	GTACGACTCACTATAGGGAAGAGCTATCCACCGACCCTT
		PoTea2 (MGG_04366)	AGGTGACACTATAGAATAATCAGTGTAGGATCACCGCC	GTACGACTCACTATAGGGAGATGTTCTCTTGCTTTCGCC
		PoTea4 (MGG_06439)	AGGTGACACTATAGAATACTAGCCAGGAGGACCATTCA	GTACGACTCACTATAGGGATGTTGATGTCGTGACCGAGT
	Circadian clock	PoFrq (MGG_17345)	AGGTGACACTATAGAATACTCGACAAGTCGCTTCAAAA	GTACGACTCACTATAGGGAACGACGGCTTCCATGTTATT
	Melanin biosynthesis	CNF1 (MGG_02962)	AGGTGACACTATAGAATAACCACCACAACCTCAAGACC	GTACGACTCACTATAGGGACGGAGCATAACCACCATCTT
		Rsy1 (MGG_05059)	AGGTGACACTATAGAATAATGGGTTTCGCAAGTTCAAAA	GTACGACTCACTATAGGGAGAAGGAGCGGTAGTCAATGC
		Alb1 (MGG_07219)	AGGTGACACTATAGAATACCCGAGATGATTGACACCTT	GTACGACTCACTATAGGGAATGATGAGGCACGACTTTCC
	Internal control	S8 (MGG_03251)	AGGTGACACTATAGAATAGAGGCGTCGACTTCGTTTAC	GTACGACTCACTATAGGGATGTTGATTTTGTGACCGA
		S27a (MGG_02872)	AGGTGACACTATAGAATACTTCACGCCAAGATCTGGTT	GTACGACTCACTATAGGGAGGCACTGTTTCATGGAGGATT
		Ef1 (MGG_03641)	AGGTGACACTATAGAATATACAAGGGCTGGACCAAGAC	GTACGACTCACTATAGGGATTGTAGACATCTGGAGGGG
<i>Hordeum vulgare</i>	Defense response	PR1b	AGGTGACACTATAGAATATACTAACCCTGATGCACGTC	GTACGACTCACTATAGGGAGGTGCATGAGATTAGGAACG
		PR4	AGGTGACACTATAGAATACGCTTTCACCAAGATCGACA	GTACGACTCACTATAGGGAACAATCTAGTCGCGGCAGTC
		PR5	AGGTGACACTATAGAATAGACATCTCGTTATCGACGG	GTACGACTCACTATAGGGAGGGCAGAAGGTGATCTGGTA
	Internal control	GAPDH	AGGTGACACTATAGAATATGTTGAGGGTTTGATGACCA	GTACGACTCACTATAGGGAGATGTTGAAGCTAGCAGCCC
		tub7	AGGTGACACTATAGAATATGCACCAGCTTGTTGAGAAC	GTACGACTCACTATAGGGACGAGATGAGGTGGTTCAGGT
		18SrRNA	AGGTGACACTATAGAATAGTGACGGGTGACGGAGAATT	GTACGACTCACTATAGGGAGACACTAATGCGCCCGGTAT
<i>Oryza sativa</i>	Defense response	PR2	AGGTGACACTATAGAATATCCATGCGCATCTACTTCC	GTACGACTCACTATAGGGAGAACGCCTGGATGTTGTTCT
		PR5	AGGTGACACTATAGAATACAGCCAGGACTTCTACGACC	GTACGACTCACTATAGGGAGCATTATGGGCAGAAGACGA
		WRKY45	AGGTGACACTATAGAATAAGAAATAATTAGCGGCAGCG	GTACGACTCACTATAGGGAGACCCCAGCTCATAATCAA
	Internal control	actin1	AGGTGACACTATAGAATATTGCTGACAGGATGAGCAAG	GTACGACTCACTATAGGGACACATCTGCTGGAATGTGCT
		eEF-1a	AGGTGACACTATAGAATATCTCTGGGTTTGAGGGTGAC	GTACGACTCACTATAGGGAGTACACGTCCTGAAGGGGAA
		eIF-4a	AGGTGACACTATAGAATATGACAGCAAGATGCAGGAAC	GTACGACTCACTATAGGGACAATCCCCCTTTGCTGAATA
		α -tubulin	AGGTGACACTATAGAATAGTCAGCTCTTCCACCCTGAG	GTACGACTCACTATAGGGACACCAACAGCATTGAACACC

Table S2. List of primers and PCR programs used for semiquantitative RT-PCR in this study

Species	Function	Gene	Forward primer (5' to 3')	Reverse primer (5' to 3')	PCR program		PCR product size (bp)
					No. of cycles	Annealing temperature (°C)	
<i>Pyricularia oryzae</i>	Polarized growth	PoTea1 (MGG_02875)	ACAGTAACCCGAATCGCAGGTCATC	TCCATCTGAGGCGTACTTGACAAGC	24	57	400
		PoTea2 (MGG_04366)	GTCACCTGGAGTTATTCCACTAGCG	CTAGTCAACTTGCTGTCTCGGTACG	24	57	639
		PoTea4 (MGG_06439)	ATCACGACGCACCCTCAGCTAAGAG	CCGAGTAGTCATGGAAGGTTGGTGC	24	59	732
	Circadian clock	PoFrq (MGG_17345)	ATGCAACGTATGCCCAAAGCTATCG	CGACGGCTTCCATGTTATTCATATC	25	55	265
	Internal control	Tubulin (MGG_00604)	AAACAACTGGGCCAAGGGTCACTACA	CCGATGAAAGTCGACGACATCTTGAG	21	57	813

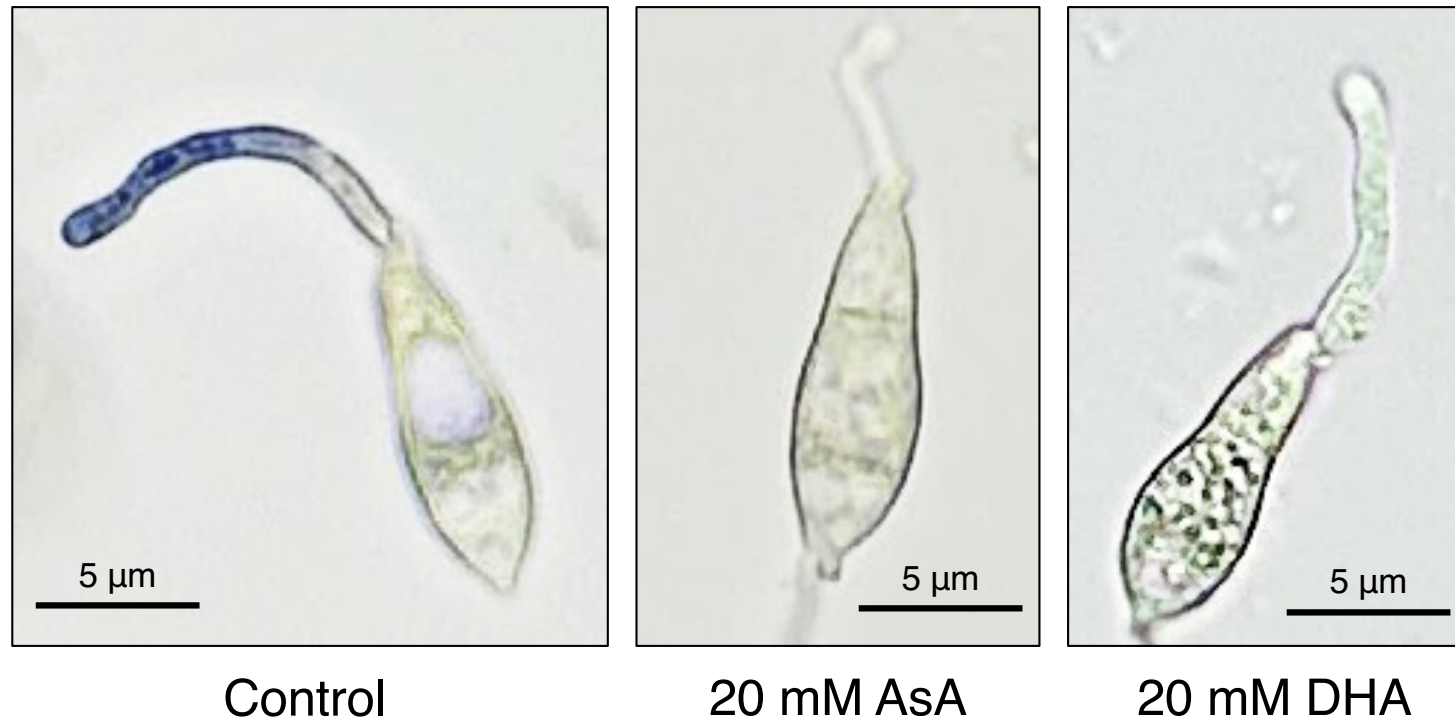


FIGURE S1 Degradation of reactive oxygen species generated during germination of conidia of *Pyricularia oryzae* during treatment with 20 mM L-ascorbic acid (AsA) or 20 mM dehydroascorbic acid (DHA). Cells that stained blue after staining with nitroblue tetrazolium (NBT) were positive for superoxide anions. Conidia were placed on glass coverslips and incubated in a moist chamber at 25°C for 2 h, then stained with 0.3 mM aqueous NBT for 30 min, then observed with bright-field optics and a light microscope.

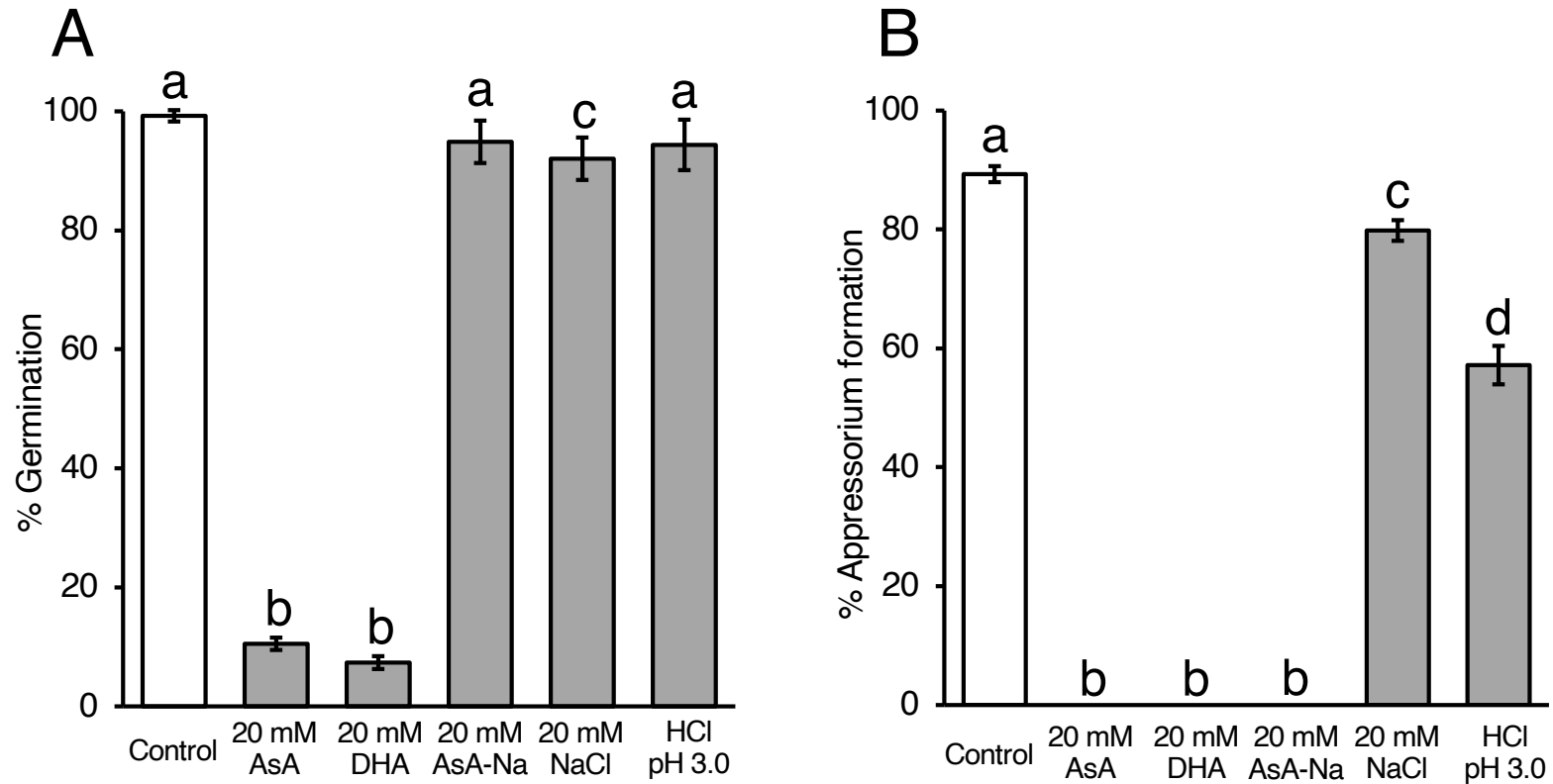
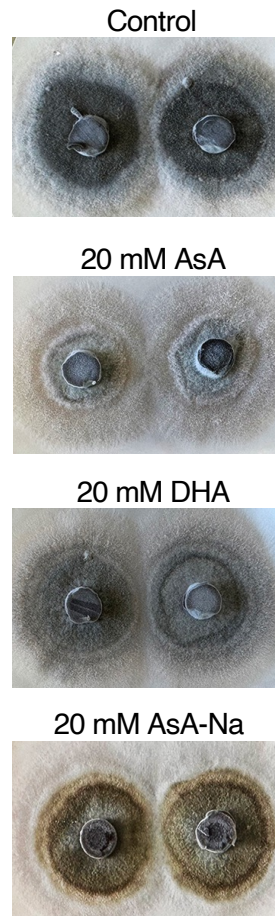


FIGURE S2 Percentage of conidia that germinated (A) and the percentage of germ tubes that formed an appressorium (B) after 17-h exposure of conidia to 20 mM L-ascorbic acid (AsA), AsA-Na, dehydroascorbic acid (DHA), NaCl or HCl adjusted to the same pH as 20 mM AsA. Error bars: SD of means for three biological replicates. Significant differences among means were determined using Tukey's multiple comparison test and are indicated by different letters above the means.

A



B

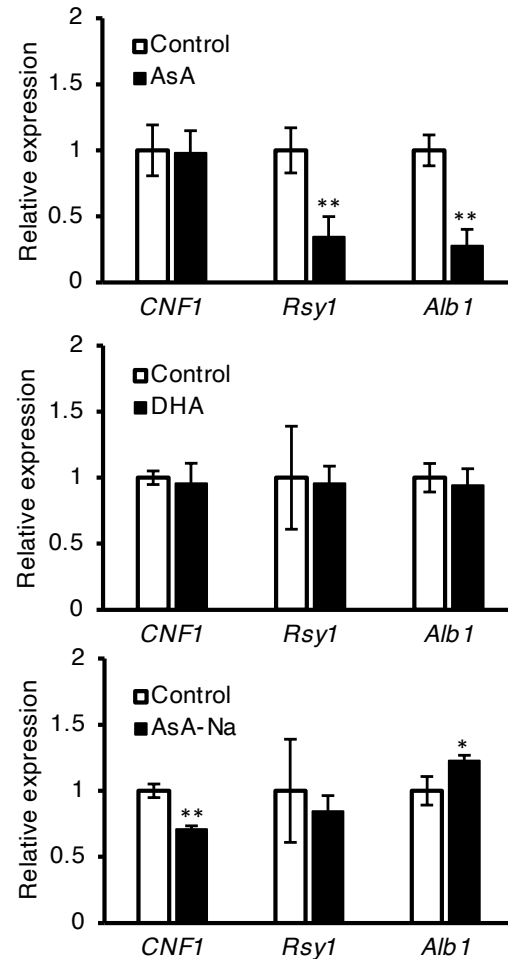


FIGURE S3 Effect of L-ascorbic acid (AsA), AsA-Na or dehydroascorbic acid (DHA) on melanin biosynthesis in *Pyricularia oryzae* isolate Ken 54-20. (A) Colonies on complete medium (CM) amended with 20 mM AsA, AsA-Na or DHA. (B) Expression of melanin biosynthetic genes at 4 days after transfer of mycelia that had been grown on a nylon membrane on CM to CM amended with 20 mM AsA, AsA-Na or DHA. Relative expression was determined using qRT-PCR with *S8* (MGG_03251), *S27a* (MGG_02872), *Ef1* (MGG_03641) as internal standards. Error bars: SD of means for three biological replicates. Significant differences among means were determined using Student's *t*-test (* $P \leq 0.05$, ** $P \leq 0.01$).

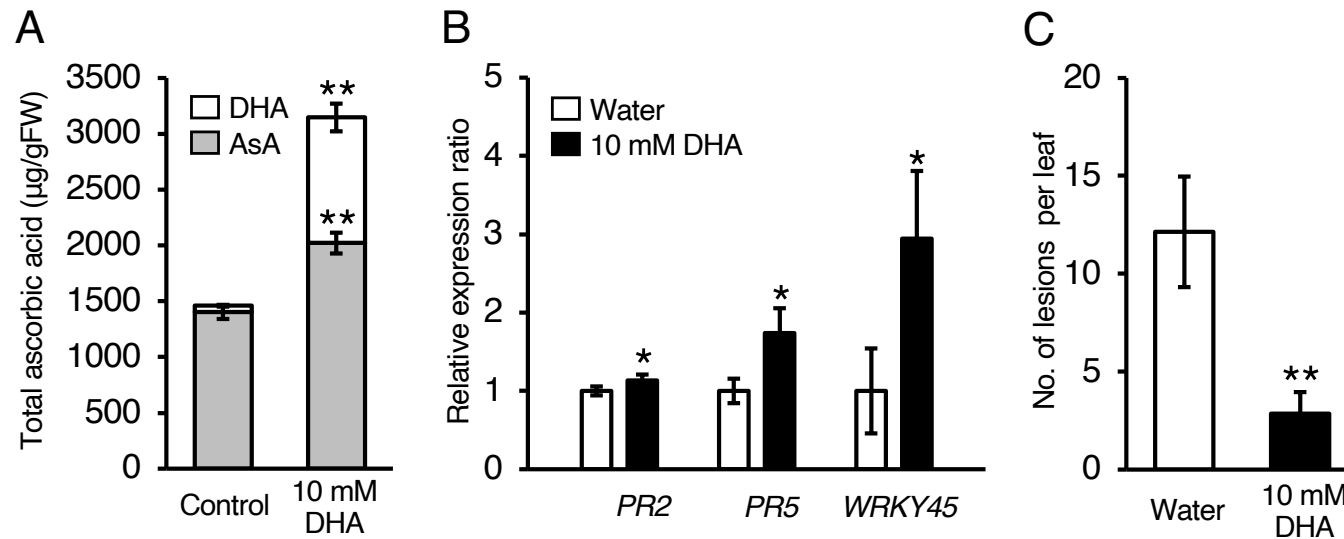


FIGURE S4 Effect of pretreatment of *Oryza sativa* cv. Nipponbare with exogenous dehydroascorbic acid (+0.2% v/v Tween 20; DHA) after 24 h on total ascorbic acid content (L-ascorbic acid [AsA] and DHA), expression of defense-related genes in sixth leaves that were not inoculated with the blast fungus, and on blast lesion formation on sixth leaves after inoculation. (A) Total AsA content after 24 h. (B) Relative expression of defense-related genes determined with qRT-PCR using *actin1*, *eEF-1a*, *eIF-4a*, *α-tubulin* as internal standards. Error bars: SE of means for three biological replicates. Significant differences among means were determined using Student's *t*-test (* $P \leq 0.05$, ** $P \leq 0.01$). (C) Number of lesions 7 days after inoculation with *Pyricularia oryzae* isolate Ai79-142.