



High spatio-temporal resolution imaging reveals brown margins associated with lesion restriction in finger millet blast caused by *Magnaporthe oryzae*

Kathryn A. Prado · Chang Hyun Khang 

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Abstract Finger millet blast, caused by the fungal pathogen *Magnaporthe oryzae*, is an important disease affecting finger millet, *Eleusine coracana*, a cereal grain grown across Africa and South Asia. Blast lesions on susceptible finger millet leaves are often associated with a brown margin surrounding a gray lesion center. However, the progression of finger millet blast disease over time and the potential role of the brown margin in lesion development remain unknown. We developed an innovative high-temporal resolution imaging system to document lesion development from 2 to 7 days post-inoculation. Our imaging system revealed that after an asymptomatic period of three days, gray lesions began to develop on the infected finger millet leaf. We discovered that the brown margin formed as partial segments of brown pigmentation separated from the gray lesion center, which then continued to develop to encircle the gray center, preventing further expansion. Cytological analyses revealed that invasive hyphal colonization was retained within the boundary of the brown margin-associated cells, and reactive oxygen species accumulation preceded brown margin formation.

Together, these findings indicate that the brown margin is associated with lesion restriction and suggest that even a susceptible host retains the capacity to limit lesion expansion.

Keywords Blast disease · Leaf clearing · Plant defense responses · Brown pigmentation · *Pyricularia oryzae* · ROS · Time-lapse imaging · WGA-Alexa Fluor 488

Introduction

Finger millet blast is a devastating disease affecting finger millet (*Eleusine coracana*), a highly nutritious cereal grain grown by sustenance farmers in many countries across Africa and South Asia, including Ethiopia, Uganda, Kenya, and India (Chela et al., 2021; Masaki et al., 2023; Mbinda & Masaki, 2020). The disease is caused by the blast fungus *Magnaporthe oryzae* (syn. *Pyricularia oryzae*), which comprises distinct host-adapted lineages, one of which is specialized to infect finger millet (Heath et al., 1990; Murakami et al., 2000; Tosa et al., 2006; Gladieux et al., 2018; Asuke et al., 2020; Odeph et al., 2020). The finger millet-adapted lineage of *M. oryzae* is a significant obstacle to global finger millet production due to its substantial impact on yield, with losses ranging from 50–90% (Chela et al., 2021; Mbinda and Masaki, 2020; Odeph et al., 2020). Beyond cultural practices and costly chemical controls, breeding

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K. A. Prado · C. H. Khang (✉)
Department of Plant Biology, University of Georgia,
Athens, GA 30602, USA
e-mail: ckhang@uga.edu

for genetic resistance remains one of the key strategies for managing the disease. However, even resistant finger millet cultivars often develop lesions, and the mechanisms underlying lesion formation remain understudied compared to other blast diseases. For example, rice blast, caused by rice-adapted *M. oryzae*, has been extensively characterized as the disease progresses through hemibiotrophic fungal colonization, where a period of initial asymptomatic biotrophic colonization in the first two to three days post-inoculation (dpi) is transitioned to necrotrophy with visible lesion formation (Berruyer et al., 2006; Jones et al., 2016, 2021; Kankanala et al., 2007; Khang & Valent, 2010; Khang et al., 2010; Mosquera et al., 2009). Similar disease progression has also been documented in goosegrass and weeping lovegrass, with visible symptoms appearing between 48 and 72 h (Heath et al., 1990). Whether finger millet blast follows a similar disease progression remains unknown. Most studies on finger millet blast have focused on lesion appearance at later stages, typically seven days after infection (Masaki et al., 2023; Qi et al., 2025). Blast symptoms in susceptible finger millet range from isolated lesions with gray necrotic centers surrounded by dark brown margins to coalesced necrotic areas leading to shriveled leaves. Blast lesions on other host species, such as rice, show a consistent phenotype with brown margins being most evident in isolated lesions, while areas without prominent browning show coalesced lesions (Heath et al., 1990; Valent et al., 1991). A better understanding of lesion development over time and the roles of brown margins could provide insights into preventing blast disease on finger millet as well as other host species.

This study characterized blast lesion development in susceptible finger millet using an innovative imaging platform to capture hourly photographs of infected leaves from 2 to 7 days post-inoculation (dpi). This time-lapse imaging showed that disease symptoms on finger millet did not appear on the leaf surface until late 2 dpi and then continued to develop over time. The brown margin formed as partial segments of brown pigmentation and developed around the edge of the gray lesions. Our high-temporal photography, together with cytological analyses, showed that the brown margin developed around the gray lesion and was associated with retained invasive hyphae. Early reactive oxygen species (ROS) accumulated where the brown margin later formed. Overall, this study

provides evidence that the brown margin is associated with restricted fungal colonization and suggests that even a susceptible host retains the capacity to limit lesion expansion.

Materials and methods

Plant materials and fungal strains

Four to five seeds of the finger millet (*Eleusine coracana*) cultivars AAUFM-44 or TZA1637, were planted in 4-inch square plastic pots filled with Sun-gro Professional GrowMix soil (Qi et al., 2025). Pots were placed in plastic trays inside a Conviron PGC20 growth chamber to grow under long-day cycle conditions (14/10 h, day/night). The temperature in the growth chamber was set to 28 °C during the daytime hours and 24 °C during the night. Pots were watered from the bottom via flooding of the tray. Each pot was sprayed with ~5 mL of liquid iron chelate at the time of planting. One liter of fertilizer in water was added to the tray at the time of planting. An additional treatment of 500 mL of the same fertilizer in water was applied seven days later. Three finger millet-adapted *M. oryzae* strains were used: T34 from Tanzania, U2 from Uganda, and CKF4046, previously described as E2-GFP (Masaki et al., 2023; Qi et al., 2025). CKF4046 (E2-GFP) was generated by transforming the Ethiopian isolate E2 with a GFP construct (pBV126) and was confirmed to retain the same level of virulence as the original E2 isolate. The fungal strains were cultured on V8 agar medium under constant light at 24 °C in a Percival tissue culture incubator to induce conidiation.

Plant infection assays

Thirteen-day-old seedlings of finger millet cultivar AAUFM-44 were placed into a box container system consisting of a black Sterilite plastic tote (73.3 cm×48.3 cm×48.9 cm) lined with a clear plastic landscaping bag. For hourly time-lapse imaging, representative seedlings were selected and transplanted into cone-shaped pots prior to inoculation. The youngest and second youngest leaves of each plant were marked lightly with a black Sharpie for easier identification after seven additional days of growth. *M. oryzae* conidia were harvested in a 0.25%

gelatin solution and sprayed onto the leaf surfaces using an artist's airbrush, inoculating each pot with 2.5 mL of a 5×10^4 conidia/mL suspension. After inoculation, the box was closed tightly to maintain high humidity for 24 h. After this period, the inoculated plants were returned to the growth chamber. For inoculation with additional *M. oryzae* isolates on AAUFM-44 and TZA1637, one pot of AAUFM-44 and one pot of TZA1637 were placed together in a clear plastic bag for each treatment. To maintain high humidity, 10 mL of tap water was added to the bottom of each bag. One bag was mock inoculated with 5 mL of 0.25% gelatin solution, whereas the other bags were each inoculated with 5 mL of a conidial suspension of CKF4046, T34, or U2 at a concentration of 1×10^4 conidia/mL.

Manual daily time-lapse imaging of inoculated plants

The first photograph was taken at 1 day post inoculation (dpi) to document the plant growth stage and appearance as follows: One 13-day-old AAUFM-44 seedling was selected based on its growth orientation in relation to the side of the pot, and all other seedlings in the pot were removed, leaving a single plant for imaging. At 1 dpi, the pot was placed flush against the white board, and the target leaves (the youngest and the second youngest) were gently taped to the board using thin pieces of clear Scotch tape. Wide field-of-view images of the entire plant and close-up images of the target leaves were captured using a digital camera (Canon EOS Rebel T6 DSLR with EF-S 18–55 mm lens) mounted on a tripod. Once images were collected, the tape was carefully removed, and the plant was returned to the growth chamber. This process of taping the target and newly developed leaves to the white board, photographing the entire plant and target leaves, removing the tape, and returning the plant to the growth chamber was repeated daily through 7dpi. The positions of the tripod feet, white board, and pot were marked with strips of labeling tape on our lab bench to ensure consistent placement throughout the time course.

Automated hourly time-lapse imaging of inoculated leaves

At two days post inoculation, the transplanted seedlings in cone-shaped pots were placed in a holder

made from a recycled pipette tip box, which served as water reservoirs. A channel cut into a plastic grid allowed the target leaves to be held snugly without causing mechanical damage. The tip box holder was angled to align with the slanted board holding the leaves, minimizing stem bending. Non-target leaves were tucked out of the way using pieces of wire inserted into the board. The Canon EOS Rebel T6 DSLR camera with an EF-S 18–55 mm lens was mounted on a tripod and powered continuously by an AC adapter. The camera was set to a focal length of 45 mm, an aperture of f/5.6, an exposure time of 1/4000 s, and ISO 800. Hourly images were taken using an intervalometer controller until 7 dpi, generating a total of 122 images. A digital timer was placed in frame on the board to show the visual progression of time for reference. Images taken during the dark cycle were excluded, except for a single black frame used to indicate day transitions. The final set of 72 images were compiled into a time-lapse movie using ImageJ software.

Time-lapse image analyses

All image analyses were performed manually by reviewing relevant frames or using ImageJ measurement tools, and final graphs were generated using Microsoft Excel. The timing of lesion developmental stages was determined by analyzing frame by frame ten individual lesions across two replicate time-lapse experiments. First, we recorded the hour that gray discoloration became visible on the leaf surface for each of the target lesions and denoted that as 'lesion appearance'. Then we followed the progression and recorded when brown discoloration first appeared in association with the lesion, designating that as 'brown margin appearance'. Lastly, we recorded when we last observed any further development or change in the brown margin within the time-lapse window and marked that as 'brown margin fully expanded'. These time points were graphed to visualize the progression of lesion development over time.

Lesion expansion was measured using time-lapse images and ImageJ measurement tools, tracking the expansion of the gray lesion area from the time when brown deposits first become visible until the gray lesion area reached the margin. To set the scale for the measurement tool, the size of the grid square in the grid channel holder was measured and used to

covert pixel to millimeters in ImageJ. Radial measurements were taken at seven different time points shown in Fig. 3 starting from the center of the gray discoloration and ending at the edge of the discoloration towards both left and right edges. Measurements for each direction were repeated three times and averaged. These averages were graphed to show the trend of lesion expansion over time. Lesion length area expansion over time was measured in ImageJ using the polygon tool to trace the gray lesion length at each time point. This was repeated three times for each time point and averaged to obtain the approximate lesion area. Brown margin length was measured using the line tool, with three measurements recorded and averaged for each time point. Brown margin completion and lesion isolation were determined by analyzing three separate time-lapse experiments. Brown margin completion was assessed at 6–7 dpi for each lesion based on the presence of a distinct dark brown delineation consistently surrounding the entire lesion. Lesions lacking distinguishable brown margins in any region were scored as incomplete. To determine whether lesions were singular or merged, any lesion whose gray area had coalesced with that of another lesion was classified as merged.

Cytological analysis of infected finger millet tissue

Hyphal colonization at the cellular level was determined by analyzing finger millet leaves harvested at 6–9 dpi. At this time point, brown margins had fully formed and lesions had ceased visibly expanding. The harvested leaves were cut into 4 cm segments and placed into glass tubes. Leaf clearing was conducted according to Murakami et al. (2000) as follows: leaf segments in the glass tube were boiled for 2 min in 5 mL of clearing solution (lactic acid/phenol/glycerol/distilled water/ethanol = 1:1:1:1:8 vol/vol/vol/vol/vol). After clearing, 1 cm portions of the leaf tissue were cut and placed into 2 mL centrifuge tubes. Samples were stained with Wheat Germ Agglutinin-Alexa Fluor 488 conjugate (AAT Bioquest Cat#25530) dye solution (10 µg/mL in 1×PBS buffer with 0.1% Tween 20) overnight at 4 °C, modified from a protocol used by Mochizuki et al. (2011). Stained samples were observed using a Zeiss Axio Observer 7 fluorescence microscope. WGA-Alexa Fluor 488 was excited using a 488 nm LED, and emission was collected between 500–550 nm. Single focal plane and Z-stack

multifocal plane images were taken at the edge of hyphal colonies and in association with brown margin cells. Colonization patterns were scored based on the location of invasive hyphae relative to the darkened brown margin-associated cells.

DAB staining analysis of infected finger millet tissue

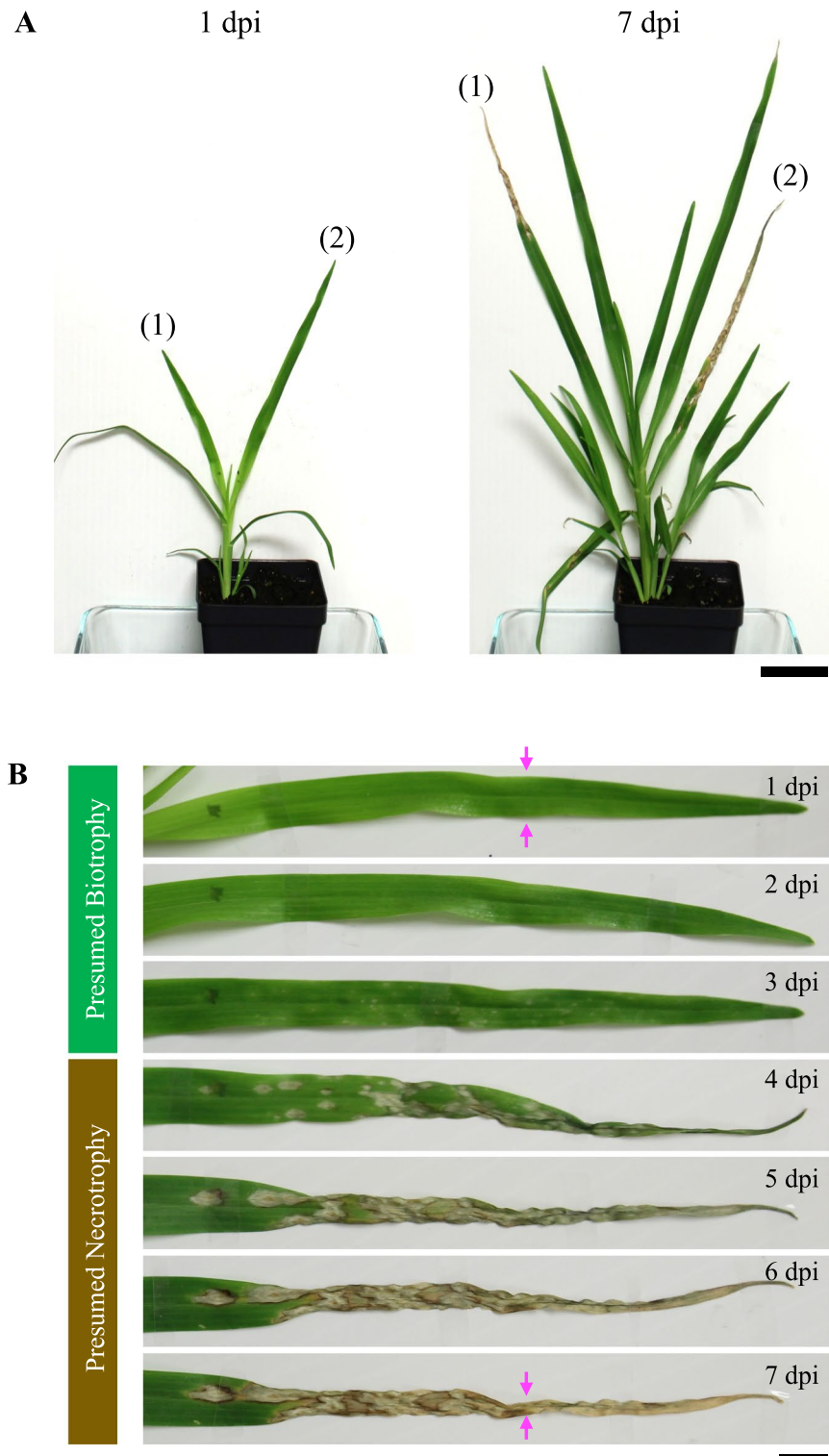
The presence of ROS in inoculated finger millet leaves was determined by 3,3'-diaminobenzidine (DAB; 91–95-2 Acros Organics) staining. Inoculated finger millet leaves were harvested at 3 and 5 dpi and imaged using a computer scanner before being transferred into glass test tubes covered with tin foil. The leaves were submerged in 20 mL of DAB solution (1 mg/mL, pH adjusted to 3.8 using 0.1 N HCl) and incubated overnight at room temperature (Kumar et al., 2014). The following day, leaves were removed from the DAB staining solution and cut into approximately 4 cm segments before being transferred to clean glass test tubes for leaf clearing as described above. Mock-inoculated leaves were processed in parallel as controls. After clearing, the leaf segments were arranged on a laminated background and scanned again. Scanned images were then uploaded into ImageJ for analysis. The images were converted to 8-bit grayscale, and line scans were drawn through target lesions to measure relative gray intensity. Values were graphed in Microsoft Excel. DAB-stained area and distance from the gray lesion edge were measured using the polygon and line tools, respectively, in ImageJ.

Results

Finger millet blast displays delayed onset of lesions on infected leaf surface

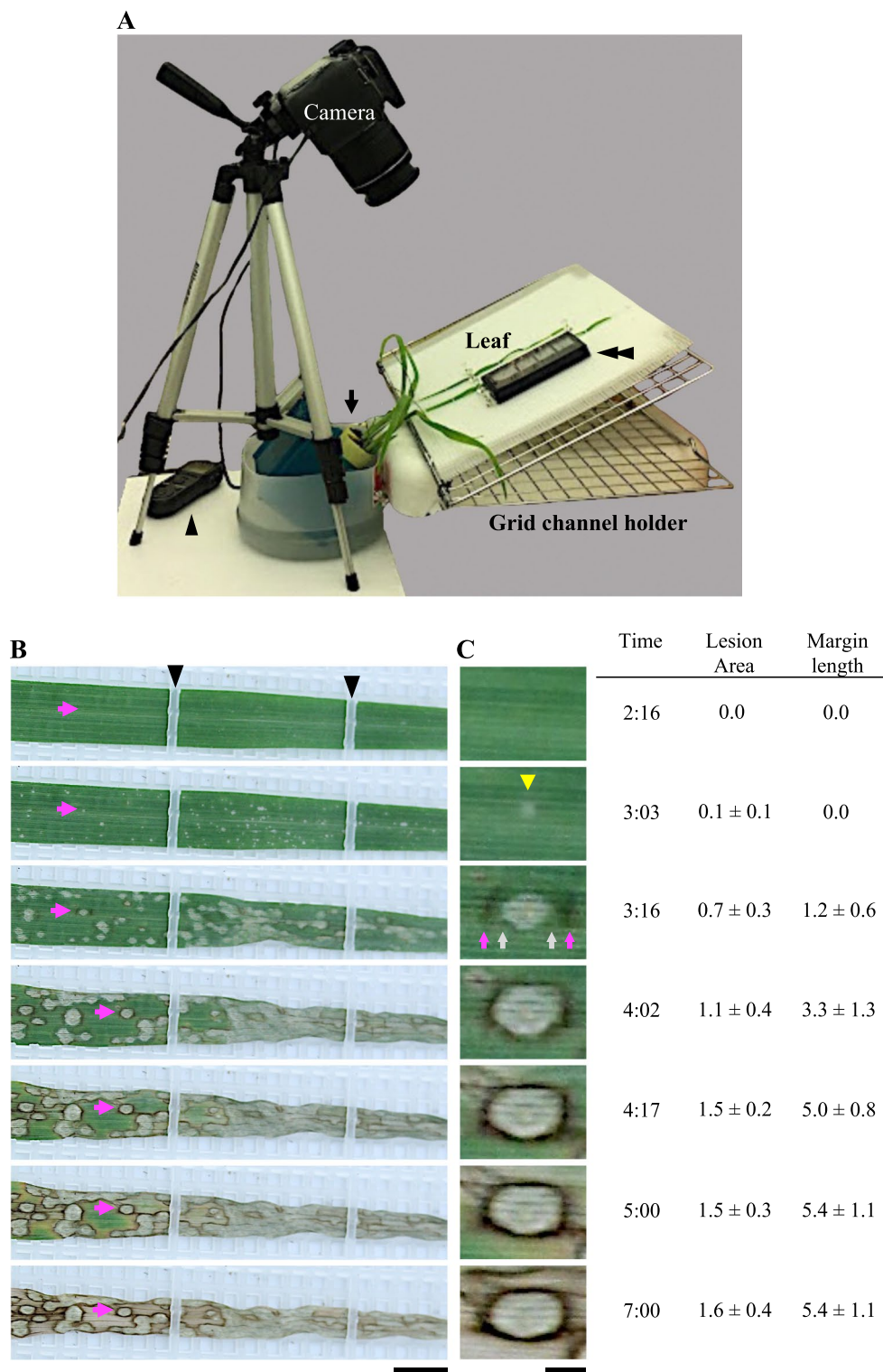
To document the progression of blast disease, we photographed the highly susceptible finger millet cultivar AAUFM-44 daily for seven days following inoculation with the finger millet-adapted *M. oryzae* strain CKF4046 (Qi et al., 2025). At the time of whole-plant spray inoculation, the youngest and second youngest leaves were partially expanded, which further grew approximately 15 cm and 5 cm of new tissue, respectively, during the seven days post-inoculation (dpi) (Fig. 1A). Both leaves showed similar

Fig. 1 Manual daily time-lapse imaging of finger millet AAUFM-44 inoculated with *M. oryzae* CKF4046 captured using a digital camera. Finger millet leaves were gently taped to a board during photography to make most leaves visible (see [Materials and Methods](#) for details). **(A)** Images of the same plant at 1 day post inoculation (dpi; left) and 7 dpi (right). Severe infection, characterized by dense lesions and shriveled tissue with a straw-colored appearance, is evident in the youngest and second youngest leaves present at the time of inoculation, labeled as (1) and (2), respectively. In contrast, other tissues largely remained green with no noticeable infection. Bar = 5 cm. **(B)** Close-up images of the youngest leaf in **(A)** depicting disease development over seven days from 1 to 7 dpi. Magenta arrows in the 1 dpi and 7 dpi images highlight the shriveling of the severely infected leaf tissue. Bar = 1 cm



patterns of infection when observed at 7 dpi (Fig. 1A: 7 dpi). Straw-colored leaf shriveling with merged

lesions was evident exclusively in the leaf tissue that was present at the time of inoculation, whereas the



newly expanded tissue lacked lesions. In addition, new leaves that emerged after inoculation also did

not develop lesions. These observations suggest that, under our infection assay conditions, lesion formation

Fig. 2 Automated hourly imaging of finger millet AAUFM-44 leaves inoculated with *M. oryzae* CKF4046. **(A)** Photograph showing the setup of our automated imaging system for inoculated leaves. The second youngest leaf (labeled as "Leaf") at the time of inoculation, grown in a yellow cone-shaped pot (arrow), was placed on a grid channel holder. The holder provided an approximate 30-degree angle, aligning with the natural angle of the leaf growing from the tilted pot. The camera, mounted on a tripod, was positioned to face the leaf from the side, with the lens approximately 40 cm from the leaf. A single arrowhead indicates an intervalometer attached to the camera to automatically capture images at a set interval. Double arrowheads indicate a digital timer placed next to the target leaf to track time progression. The imaging system was set up in a growth chamber. The background of the photograph was adjusted using Photoshop to enhance clarity. **(B)** Selected photographs from the automated imaging system shown in **(A)**, captured hourly from 2 to 7 dpi. These images were chosen to highlight noticeable changes in lesion development. Time points are labeled as day:hour after inoculation. For the complete time-course visualization, refer to Movies 1 and 2. Arrowheads indicate the grid channels used to keep the inoculated leaf in focus while allowing repositioning as the leaf continued to grow. Note that the leaf shifted to the right between 3:16 and 4:02, as indicated by the changing positions of the magenta arrows. Bar = 10 mm. **(C)** Development of an isolated lesion (marked by magenta arrows in **B**), shown at representative time points for quantification. At 3:03, the lesion appeared as a circular gray area of discoloration (yellow arrowhead). By 3:16, brown pigmentation (magenta arrows) formed on opposite sides, away from the edge of the expanding gray lesion (white arrows). Over time, the gray lesion continued to expand but did not extend beyond the brown pigmentation, which developed into a distinct margin delineating the boundary of the gray lesion. Areas beyond the brown margin showed signs of chlorosis, noticeably from 5:00 onward. The gray lesion area and brown margin length at each time point were quantified for this lesion and two additional lesions of similar size and developmental stage and are shown as mean $\pm$ SD. Bar = 1.25 mm

was confined to the tissue exposed to the inoculum, consistent with previous infection results at 7 dpi (Masaki et al., 2023; Qi et al., 2025).

Our daily photographs taken from 1 to 7 dpi further showed that lesions did not form immediately after inoculation (Fig. 1B). Visible symptoms first appeared at 3 dpi, initially as small pinpricks of gray discoloration. By 4 dpi, this discoloration became more prominent and expanded into larger oval-shaped lesions, with some lesions coalescing. Continued discoloration and lesion coalescence resulted in straw-colored, shriveled leaves. The notable change from asymptomatic to lesion formation resembled hemibiotrophic fungal colonization as reported in other blast diseases, such as rice blast. Taken together, our data suggest that finger

millet blast begins with an initial presumed biotrophic fungal colonization, followed by a transition to presumed necrotrophy at around 3 dpi.

Custom-designed imaging system tracks hourly progression of finger millet blast lesions

Building on our manual photography of lesion progression, taken at approximately 24-h intervals (Fig. 1), we wanted to develop an automated imaging system using a digital camera that captures lesion progression at customizable intervals, such as hourly over several days. Early prototypes inadvertently damaged the leaves. For example, the youngest leaves, which were held fixed in place for camera focus, continued to grow over time, resulting in bending and damage. Our final imaging system was developed by incorporating several features that addressed the leaf damage issue and enabled the automated photography of inoculated leaves (Fig. 2A). First, we chose to photograph the second-youngest leaf at the time of inoculation. This leaf typically grew less than the youngest leaf after inoculation, thus minimizing the chance of growth-related damage while still exhibiting consistent lesion development as the youngest leaf (Fig. 1A). Second, we implemented a grid channel holder to keep the target leaf in-camera focus. The holder was designed to allow the leaf to expand and be easily adjusted for its angle to match the plant's natural position. The plant was also grown in a rotatable cone-shaped pot for easy orientation of the target leaf without twisting when placed on the grid holder. Finally, our imaging system was equipped with a digital camera connected to an intervalometer, enabling photographs to be taken at customizable intervals. Using our imaging system, we automatically photographed AAUFM-44 leaves inoculated with *M. oryzae* hourly from 2 to 7 dpi (Movies 1 and 2). These hourly observations showed lesion appearance around 3 dpi, progressive coalescence of lesions, and severe chlorosis of the leaf tissue by 7 dpi (Fig. 2B), consistent with our manual daily time-lapse photography (Fig. 1B). This consistent disease progression confirms that our automated imaging system enables reliable, high-temporal-resolution tracking of blast lesion development.

Hourly time-lapse imaging reveals the progressive formation of brown margins around gray lesions

From our daily time-lapse images, we noticed that the edges of the gray discolored lesions were associated with dark brown pigmentation, which we referred to as "brown margins" (Fig. 1B). To determine how these brown margins develop in spatial relation to the gray lesions, we analyzed our hourly time-lapse images of over 300 individual lesions. We found that the brown margins did not appear fully formed around the gray lesions but instead developed in partial segments of brown pigmentation (Fig. 2C). These brown segments initially appeared spatially separated from the developing gray lesions at varying distances, ranging from 0.5 to 0.8 mm. These image analyses also showed that the initial brown segments appeared to form seemingly randomly on any side of the gray lesion and subsequently expanded outward from these points, gradually accumulating along the edge of the lesion to form a continuous margin (Fig. 2C and 3A). Our quantification of gray lesion area and brown margin expansion showed that the expanding gray lesion became contained within the developing brown margin, which continued to extend until it fully enclosed the lesion (Fig. 2C). To determine a timeline for brown margin development, we analyzed a sequence of hourly time-lapse photographs of 10 independent lesions. We found that brown pigmentation became visible on average 20 h after the appearance of the gray lesions and continued to expand over an average of 50 h before the brown margin reached completion (Fig. 4, as an example).

Brown margin impedes lesion expansion

During the tracking of lesion development, we also observed that the brown margin delineated the boundary of the gray lesion, appearing to prevent the lesion from expanding beyond it. To quantify this pattern, we measured the distance from the center of the gray discoloration to its edge at multiple time points (Fig. 3; $n=6$ lesions). Our analysis revealed that once the expanding gray lesion reached the point where brown pigmentation had formed, it did not further expand in that direction. However, the gray lesion continued to grow in areas where brown pigmentation had not yet formed. As the brown margin progressively encircled more of the lesion, expansion of

the gray lesion in those directions ceased (Fig. 3C). These observations suggest that the brown margin plays a role in limiting the expansion of gray lesions, thereby influencing the eventual size and shape of the lesion.

The incomplete formation of the brown margin allows for the merging of lesions

At 7 dpi, susceptible finger millet leaves often exhibited large areas of heavily infected and shrunken tissue (Figs. 1B and 2B) (Masaki et al., 2023; Qi et al., 2025). In our hourly imaging, this symptom appeared to result from the merging of gray lesions, along with the collapse of leaf tissue between the lesions. In the majority of lesions we observed ($n=77$), the brown margin did not completely encircle the gray lesion, leaving gaps through which the lesion could "escape" (Fig. 3A). We found that lesions with open sections in their margins, particularly when in close proximity, were able to merge into larger lesions (Fig. 4). Nearly 70% of the lesions we tracked ($n=52$) merged with neighboring lesions. Interestingly, new brown margins formed along the edges of some of these merged lesions, which appeared to restrict further expansion of the larger lesion (Fig. 4). We also noticed changes in plant tissue outside of the lesions enclosed by the brown margin over time. Areas without visible lesions beyond the brown margin began showing signs of chlorosis around 4 dpi (Figs. 2C, 3A, and 4). Over time, these chlorotic areas transitioned to a necrotic tan color, and the leaf tissue began visibly pulling inward (Figs. 1B and 2B).

Brown margins are associated with isolated lesions on both resistant and susceptible finger millet cultivars

We first observed brown margin formation on the susceptible finger millet cultivar AAUFM-44 with one *M. oryzae* strain, CKF4046. We next asked whether this pattern would also be observed with other fungal strains of similar or higher virulence to CKF4046. To answer this, we inoculated AAUFM-44 with two additional *M. oryzae* strains, T34 and U2, alongside CKF4046 for comparison (Fig. 5) (Masaki et al., 2023; Qi et al., 2025). On AAUFM-44, inoculation with CKF4046 and T34, which showed similar virulence, resulted in brown

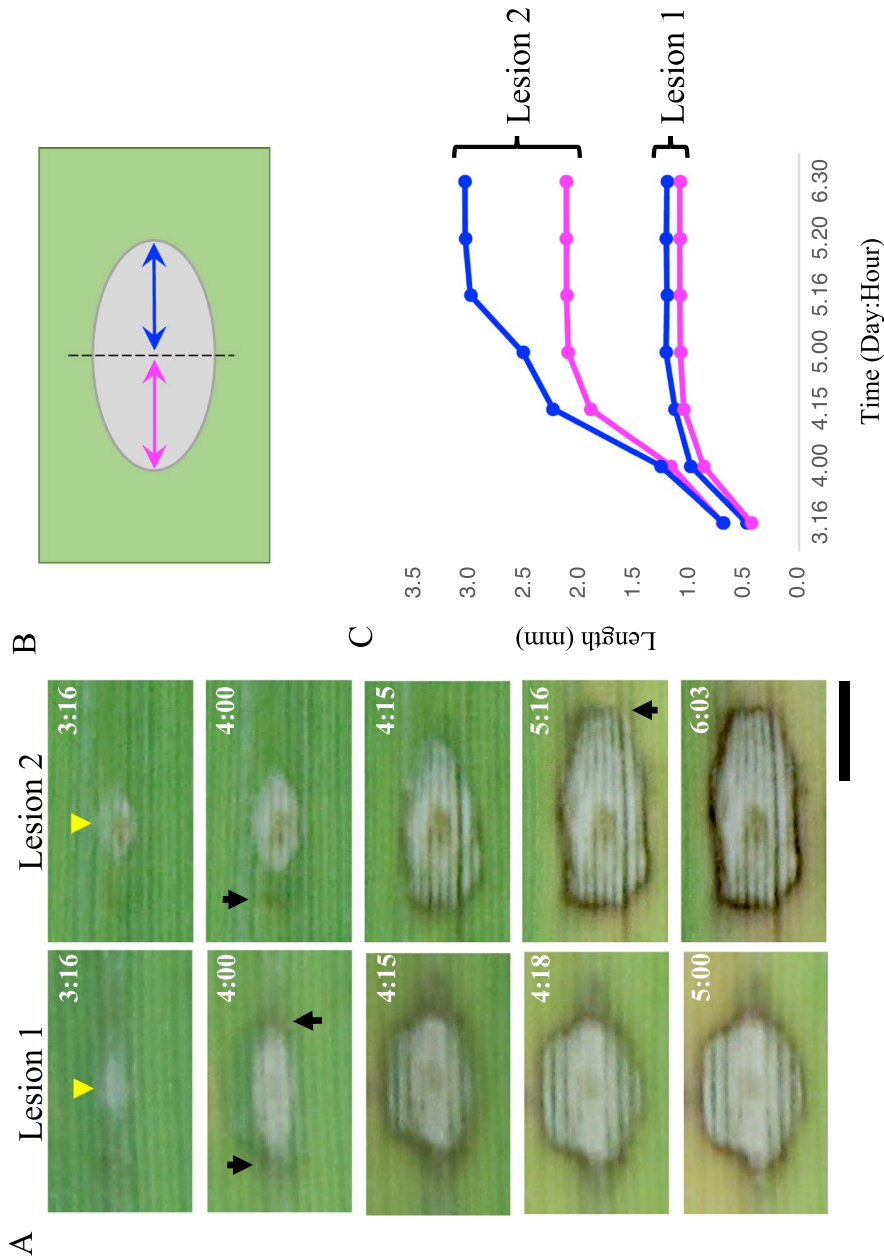
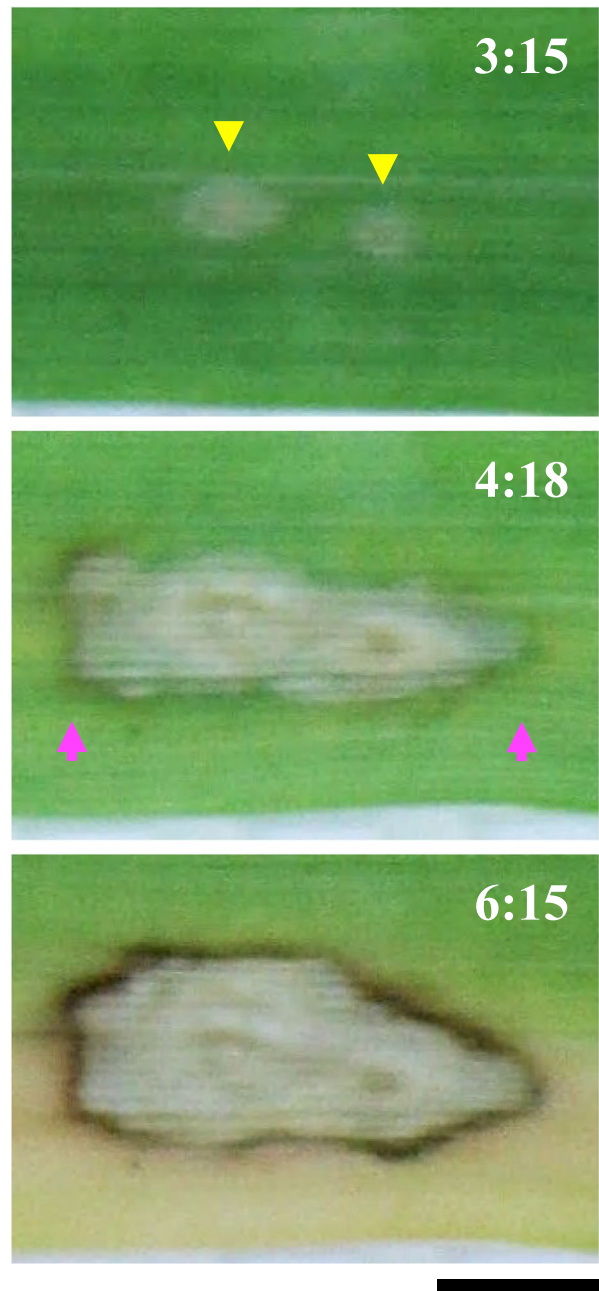


Fig. 3 Gray lesion expansion ceases upon encountering the brown margin. (A) Hourly time-lapse images taken of two isolated lesions on finger millet AAUFM-44 inoculated with *M. oryzae* CKF4046, showing the expansion of the gray lesion and the formation of the brown margin. At 3:16 (day:hour), the gray lesion (yellow arrowheads) formed. By 4:00, brown pigmentation (black arrows) first appeared on both sides of Lesion 1 and the left side of Lesion 2. At 5:16, a brown margin (black arrow) developed at the right end of Lesion 2. The gray lesion continued to expand until it reached the areas where the brown margin had formed. By 5:00, gray lesion expansion for Lesion 1 had ceased, with chlorosis visible beyond the brown margin. Lesion 2 ceased expansion by 6:03. Bar = 2.5 mm. (B) Diagram illustrating how gray lesion length measurements were taken. The green area denotes healthy leaf tissue, and the gray oval represents the gray lesion. Distances were measured from the lesion center (dashed vertical line) to the left edge (magenta arrow) and to the right edge (blue arrow) (blue arrow) using ImageJ software at multiple time points. (C) Graph showing the change in gray lesion length for both lesions in (A) over time. Lesion length plateaued when the gray lesion reached the brown margin

Fig. 4 Incomplete formation of brown margin allows merging of nearby lesions on AAUFM-44 inoculated with *M. oryzae* CKF4046. Images show two gray lesions (yellow arrowheads) that developed in close proximity at 3:15 (day:hour). By 4:18, the gray lesions had expanded with brown margins beginning to form on opposite edges of the two lesions (magenta arrows) but not in the space between the two lesions. By 6:15, the brown margin had developed continuously around the now completely merged gray lesion, resulting in one large lesion. The initial positions of the developing gray lesions were visible as light brown discoloration within the fully expanded gray lesion. Bar = 2.5 mm



margins encasing isolated lesions. In contrast, inoculation with U2, a highly aggressive strain, resulted in extensively merged lesions with little or no visible brown pigmentation separating them. To determine whether brown margin formation is consistent on a resistant finger millet cultivar, we inoculated TZA1637 with the same three strains. As in AAUFM-44, TZA1637 developed isolated

lesions consistently surrounded by a dark brown margin, whereas U2 produced dense merged lesions without distinct margins. Together, these results suggest that brown margin formation is associated with isolated lesions on both susceptible and resistant finger millet cultivars, consistent with a role for the brown margin in restricting lesion expansion.

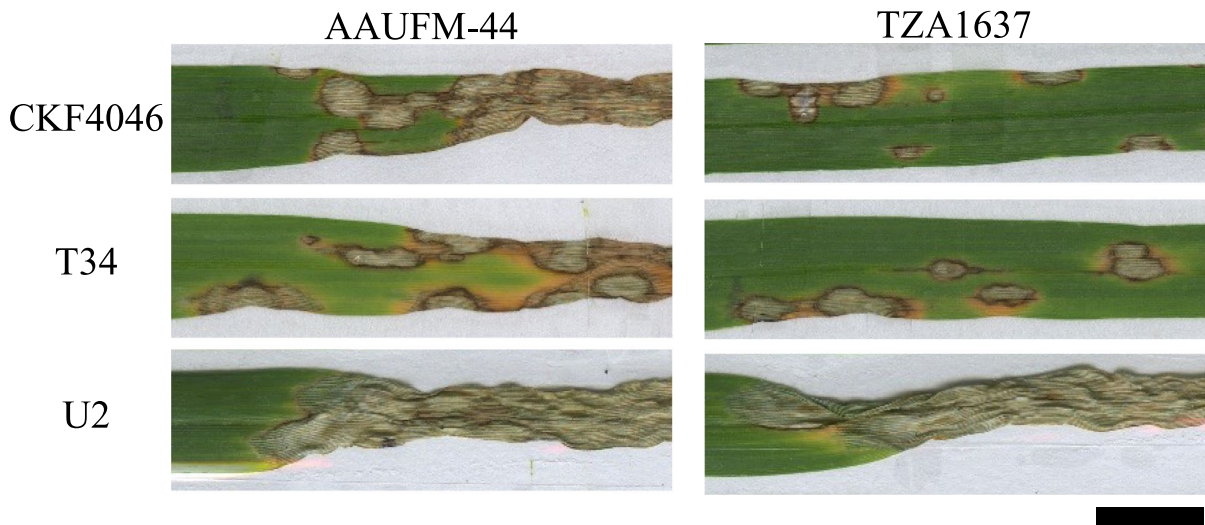


Fig. 5 Brown margins form around isolated lesions on finger millet. Leaf segments from inoculated susceptible AAUFM-44 and resistant TZA1637 finger millet leaves were imaged at 7 dpi after inoculation with each *M. oryzae* isolate: CKF4046, T34, or U2. Isolated lesions on both AAUFM-44

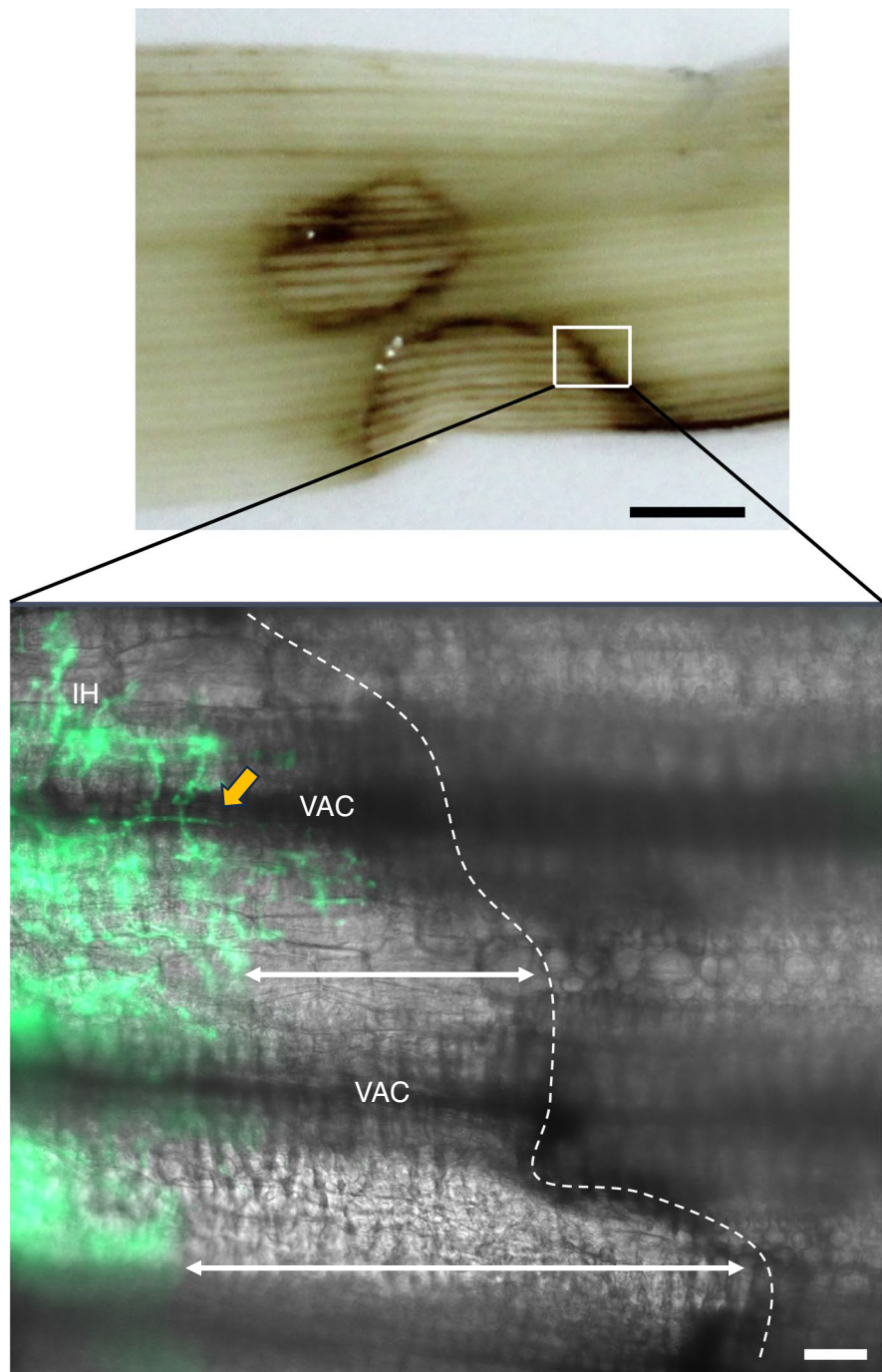
and TZA1637 showed distinct brown margins when inoculated with CKF4046 and T34. In contrast, highly coalesced lesions, such as those resulting from U2 infection on both cultivars, lacked brown margins. Scale bar=0.5 cm

Invasive hyphal colonization is contained within the brown margin

To test the hypothesis that the brown margin restricts hyphal growth, we determined the cellular localization of invasive hyphae relative to the brown margin. We prepared inoculated AAUFM-44 leaf samples (6–9 dpi) by quickly clearing the leaf to be transparent. To visualize the invasive hyphae, we used Wheat Germ Agglutinin-Alexa Fluor 488 conjugate, which binds to chitin in the cell wall of fungal cells and fluoresces green. Lesions with prominent brown margins were observed using a fluorescence microscope, and multi-focal plane (z-stack) images were collected to map the hyphal colonization throughout the tissue cell layers (Fig. 6). We found that the majority of the area associated with the gray lesion at the macroscopic level represented mass colonization and disorganization of plant cells. The brown margin observed on the leaf surface correlated with darkened cells, with the darkest cells often being those associated with veins. Near the hyphal colony edge, we observed a trend of spatial separation between the brown margin-associated cells and the hyphal growth (Fig. 6, $n = 7$ out of 13 brown margin sections). This

separation ranged in length from 140 to 500 μm , equivalent to about 1–5 epidermal cells. However, we observed instances where the invasive hyphae had colonized up to the brown margin-associated cells but had not infiltrated the margin ($n = 6$ out of 13). In some cases, we found the colonizing hyphae had infiltrated the brown margin as determined by the detection of stained hyphae within the brown margin-associated cells ($n = 3$ out of 13). Furthermore, we observed that at two sections of the brown margin, invasive hyphae breached the brown margin and grew to a limited extent past the margin-associated cells. Interestingly, at both sites, the breach points were correlated with vein-associated cells. Notably, we observed that the invasive hyphae appeared to use the vein-associated cells as a highway to move through the plant tissue and brown margin-associated cells. Then, the hyphae branched out laterally into the epidermal cells. The extent of hyphal growth beyond the margin was limited, reaching a distance of about 250 μm or about two epidermal cells in length. Even so, our results suggest that the brown margin, as seen macroscopically, acts as a barrier that contains the majority of invasive hyphae and inhibits lesion expansion at the cellular level.

Fig. 6 Invasive fungal hyphae are contained within the brown margin. The top panel shows a photograph of a chemically cleared leaf segment of AAUFM-44 at 6 dpi with *M. oryzae* CKF4046. The boxed area indicates the lesion section observed under a fluorescence microscope. In the lower panel, the brown margin appeared as darkened cells indicated with the white dashed line. Invasive hyphae (IH) were stained with WGA-Alexa Fluor 488, resulting in green fluorescence. White double-headed arrows indicate the separation between the green-labeled invasive hyphae and the brown margin-associated cells. Some invasive hyphae (yellow arrow) can be seen extending through vein associated cells (VAC). Black scale bar = 2.5 mm. White scale bar = 50 μ m



ROS accumulation precedes brown margin formation

Reactive oxygen species (ROS) play key roles in plant defense by inducing downstream defense responses (Haghpanah et al., 2025; Kumar et al., 2014; Sood, 2025; Wu et al., 2023). Additionally, ROS

accumulation has been shown to enhance browning in resistance lesions on rice (Hayashi et al., 2016). Given the reported roles of ROS in plant defense and browning, and our observation that brown margins are associated with lesion restriction, we hypothesized that ROS accumulation precedes brown margin

formation during lesion development in a susceptible host. To test this, we used DAB staining to detect ROS in AAUFM-44 leaves inoculated with CKF4046 (Fig. 7). At 3 dpi, when gray lesions had appeared, but brown margin had not yet developed, ROS had already accumulated in segments separated from the gray lesion at the anticipated sites where the brown margin would later form ($n=30$; Fig. 7b). These ROS segments were located on average 1.60 ± 0.45 mm from the edge of the gray lesion ($n=4$). Further, even in lesions at later time points with partial brown margin formation, DAB staining revealed a ROS ring that colocalized with the existing brown margin segments and filled the intervening spaces between them, where we would expect the brown margin to later develop to form a continuous ring enclosing the gray lesion (Fig. 7c). ROS also accumulated in a pattern

that overlapped the gray lesion and extended beyond it (Fig. 7b). The area of DAB staining (0.26 mm^2) at 3dpi was on average 7.5 ± 2.3 times larger than that of the gray lesion (0.04 mm^2) ($n=6$; Fig. 7b). This indicates that host cells beyond the visible gray lesion had accumulated ROS, likely as part of a response to the infection. Together, these results indicate that ROS accumulation extends beyond the gray lesion and appears at sites where the brown margin later forms, preceding visible brown margin formation.

Discussion

In this report, we detailed the progression of blast disease in susceptible finger millet by using our innovative high-resolution imaging platform. Our platform,

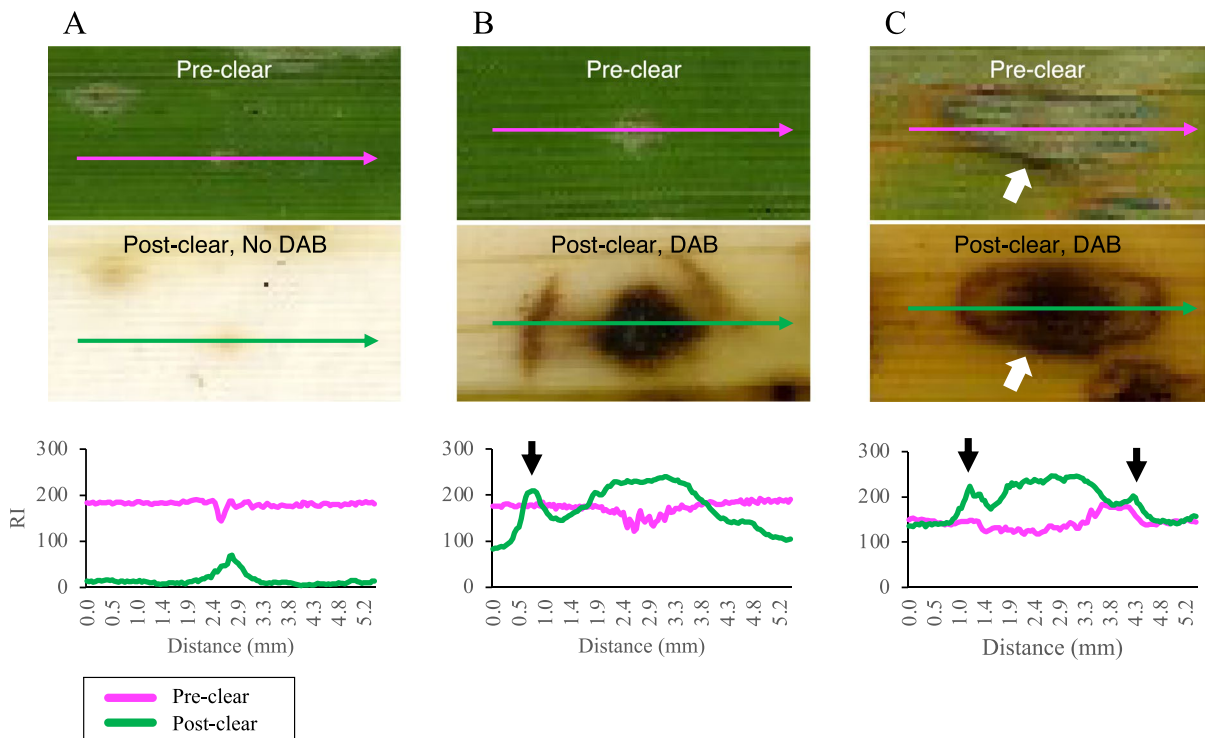


Fig. 7 DAB staining reveals that ROS accumulation precedes visible brown margin formation in AAUFM-44 leaves infected with *M. oryzae* CKF4046. **(A)** A representative lesion imaged before leaf clearing (top) and after clearing without DAB staining (bottom). **(B, C)** Representative lesions imaged before clearing (top) and after DAB staining and clearing (bottom) at 3 dpi **(B)** and 5 dpi **(C)**. In **(C)**, white arrows indicate one segment of the partially formed brown margin, while additional brown margin segments are also present. Line scan analysis

was performed before clearing (pink) and after clearing (green) for each representative lesion shown. Images were converted to grayscale in ImageJ, and relative intensity (RI) was measured along the line indicated by the arrows. Each line scan was 5.2 mm long. The graphs show the relative intensity across each lesion along the indicated line. Black arrows in the line scan graphs indicate peaks corresponding to the DAB-stained regions in the post-cleared images in **(B)** and **(C)**

capable of photographing inoculated leaves at customizable intervals for multiple days, along with efficient leaf clearing and visualization of invasive hyphae, revealed previously unrecognized dynamics of blast lesion development in finger millet. Notably, our daily and hourly photographing consistently showed that lesions were not visible on the leaf surface until approximately three days after inoculation. This initial symptomless period suggests that the fungus colonizes host cells while maintaining their viability at first, but then the transition to visible lesion development and tissue collapse demonstrates that viability of the host cells is lost. This disease progression in finger millet resembles blast disease observed in other hosts such as rice, weeping lovegrass, and goosegrass, in which each host-adapted *M. oryzae* isolate exhibits a hemibiotrophic infection pattern characterized by an initial biotrophic phase followed by a necrotrophic phase (Heath et al., 1990; Jones et al., 2021; Kankanala et al., 2007; Khang & Valent, 2010; Martínez & Perelló, 2024). Given the roles of fungal effectors in infection phases, the similar blast infection pattern suggests the presence of conserved effectors that facilitate hemibiotrophic host colonization across diverse *M. oryzae* isolates adapted to different hosts (Mosquera et al., 2009; Oliveira-Garcia et al., 2024; Sharpee et al., 2017; Yan et al., 2023). In rice-adapted *M. oryzae*, effector biology has been extensively studied, including biotrophy-associated effector genes (e.g., *BAS* genes encoding Biotrophy-Associated Secreted proteins) and host delivery process and structures (e.g., Biotrophic Interfacial Complexes), which are yet to be determined in finger millet and other blast hosts (Berruyer et al., 2006; Giraldo et al., 2013; Jones et al., 2021; Khang et al., 2010; Mosquera et al., 2009; Shipman et al., 2017). Future comparative studies of blast biotrophy and effector biology will provide strategic opportunities to disrupt early fungal establishment before extensive tissue damage occurs in finger millet and other blast-susceptible hosts.

Furthermore, our study explains how blast lesions in susceptible finger millet typically end up with a gray center bordered by brown pigmentation, as previously documented when observed in endpoint assessments at around 7 dpi (Masaki et al., 2023; Qi et al., 2025). The hourly photographs revealed that on average 20 h after gray lesion centers became visible, the brown margin appeared initially as discontinuous

segments spatially separated from the gray lesion and, over time, expanded to form a rim of brown pigmentation around the gray lesion. The brown pigmentation that we observed as brown margins of blast lesions in the susceptible finger millet is intriguing, given the well-established association of brown pigmentation with host resistance. In resistant hosts such as rice, barley, and weeping lovegrass, pinpoint brown pigmentations, homogeneously distributed and typically less than 0.5 mm in diameter, are characteristic of hypersensitive response (HR)-mediated defenses in response to *M. oryzae* infections (Heath et al., 1990; Hyon et al., 2012; Valent et al., 1991). In susceptible finger millet, brown pigmentation shows a different architecture, first forming as an extended zone approximately 0.6 mm from the edge of the expanding gray lesion and then subsequently forming the brown margin. Brown pigmentation has been observed in other blast susceptible host species but remains largely unexplored. In susceptible rice cultivars, such as CO39, isolated blast lesions are accompanied by brown margins, though limited information has been reported about these lesion-associated brown margins (Valent et al., 1991). Similarly, the presence of brown pigmentation in lesions on wheat and barley has been used as a metric to define resistant hosts, yet the basis of this resistant-associated browning has not been further investigated (Hyon et al., 2012; Murakami et al., 2000). Our study confirmed that brown margin formation was consistent in susceptible and resistant finger millet cultivars in response to infection by different *M. oryzae* strains. Isolated lesions on both susceptible and resistant cultivars were encased in brown margins. Together, these reports and our findings demonstrate that brown pigmentation is a conserved feature of blast lesions across multiple host species in both susceptible and resistant interactions. As such, our findings help bridge the gap in characterizing the temporal and spatial development of brown margins in response to *M. oryzae* infection and pave the way for future studies on its biological significance in finger millet and other hosts.

Our study provides several lines of evidence suggesting a role for the finger millet brown margin in limiting fungal spread at the advancing front of colonization. Quantitative analysis of hourly photographs showed that gray lesions ceased expansion when they reached the brown margin that was induced

in response to initial gray lesion formation. In the absence of the brown margin, gray lesions continued to expand and coalesce, leading to extensive degradation of the leaf tissue. Cytological observations further confirmed that invasive hyphae were largely confined within areas demarcated by brown margins. Interestingly, this suggests that the discoloration and eventual collapse of leaf tissue outside of the brown margins observed at 4–7 dpi may not be directly caused by fungal hyphal presence in that tissue, but rather by another cause, such as wilting due to blockage of the leaf veins, which will require further investigation. Studies in rice revealed that brown pigmentation in resistance lesions is due to the deposition of oxidized serotonin into the rice cell walls (Hayashi et al., 2016; Ishihara et al., 2008; Yang et al., 2025). These deposits act as a structural barrier, blocking invasive hyphae from further colonizing the plant tissue. Analysis of the rice cultivar Sekiguchi, which has a mutation in the *sl* gene encoding an enzyme that converts tryptamine to serotonin, resulted in pale yellow lesions that were unable to block hyphal spread (Ishihara et al., 2008). Whether brown margin formation on finger millet also results from serotonin oxidation remains to be investigated. However, based on our data, we hypothesize that serotonin is involved in finger millet brown margin development. ROS has been shown to enhance serotonin oxidation as well as intensify brown pigmentation on rice (Hayashi et al., 2016). ROS is also well known to function in plant defense taking part in signaling and facilitating other plant cell modifications such as lignification, in response to pathogen invasion (Haghpanah et al., 2025; Sood, 2025; Thordal-Christensen et al., 1997; Wu et al., 2023). Our study showed that prominent ROS accumulation occurred prior to brown margin formation, and the pattern of ROS accumulation overlapped with where we would expect the brown margin to form. In all these results indicate that ROS may have a role in brown margin formation by facilitating brown pigment deposition in finger millet, and future assessment of serotonin and ROS role in brown margin formation would provide further insight. Thus, our data support the hypothesis that even susceptible finger millet is capable of inducing brown margins as a barrier against pathogen colonization. As shown in the literature, susceptible hosts are able to initiate immune responses to pathogen infection at a single-cell level in an attempt to mitigate the damage from

the pathogen (Tang et al., 2023; Zhu et al., 2023). This presumed defense provided by the brown margin, at its current level, would be insufficient to disrupt the finger millet blast disease cycle because the fungus could still produce conidia from the gray central region to continue the infection cycle. However, our hypothesis remains exciting because, if validated, it could reveal strategies to harness the brown margin response as a more robust resistance mechanism. If serotonin is involved in brown margin formation in finger millet, strategies to induce serotonin oxidation at earlier infection stages could lead to more effective fungal restriction. Achieving this potential will require future studies that directly demonstrate the role of brown pigmentation and margin formation in limiting pathogen colonization, characterize the biochemical nature of the brown margin, identify the underlying genes and cellular processes, and manipulate their activity. Comparative studies between resistant and susceptible cultivars of finger millet as well as cross species comparison with rice may provide insight into the mechanisms underlying browning-related plant defense strategies. This work thus opens a new direction of research and provides a valuable tool for comparative analyses of disease progression and resistance responses across diverse blast host–pathogen systems.

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Authors contribution **Prado:** Conceptualization, Methodology, Investigation, Formal analysis, Visualization, Writing-Original draft preparation. **Khang:** Conceptualization, Methodology, Writing- Reviewing and Editing.

Data availability Data will be made available on request.

Declarations

Ethics approval The manuscript does not contain experiments using animals, and it does not contain human studies.

Competing interest The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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