Isolate specific responses of the non-host grass *Brachypodium distachyon* to the fungal pathogen *Zymoseptoria tritici*, compared to wheat.

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ABSTRACT

Septoria tritici blotch (STB) is an important foliar disease of wheat that is caused by the fungal pathogen *Zymoseptoria tritici*. The grass *Brachypodium distachyon* has been previously used as a model system for cereal-pathogen interactions. In this study we examined the non-host resistance (NHR) response of *B. distachyon* to two different *Z. tritici* isolates in comparison to wheat. These isolates vary in aggressiveness on wheat cv. Remus displaying significant differences in disease and pycnidia coverage. Using microscopy, we found that similar isolate specific responses were observed for H_2O_2 accumulation and cell death in both wheat and *B. distachyon*. Despite this, induction of isolate specific patterns of defence gene expression by *Z. tritici* did differ between *B. distachyon* and wheat. Our results suggest that *PAL (phenylalanine ammonia lyase*) expression may be important for NHR in *B. distachyon* while *PR (pathogenesis-related*) and (*ROS*) *reactive oxygen species* mediated gene expression may be important to limit disease in wheat. Future studies of the *B. distachyon- Z. tritici* interaction may allow identification of conserved plant immunity targets which are responsible for the isolate specific responses observed in both plant species.

Keywords: *Zymoseptoria tritici*, fungal, *Brachypodium distachyon*, wheat, Non-host

Introduction

Septoria Tritici Blotch (STB) is caused by the ascomycete fungus *Zymoseptoria tritici*. This disease poses a major threat to wheat production worldwide. STB is a particular concern in countries with humid, temperate climates (Orton et al. 2011; Fones and Gurr 2015).

STB control relies on the use of resistant wheat varieties together with foliar fungicide applications. However, rapid evolution of *Z. tritici* to overcome host resistance and acquire fungicide tolerance is a challenge the control of this disease (Cools and Fraaije, 2013; Dooley et al., 2016).

The infection cycle of *Z. tritici* includes: the entry of the fungus into the host leaf, colonization of plant tissue and formation of the asexual and sexual fruiting bodies (Steinberg 2015). Following leaf contact, the asexual pycnidiospores or sexual ascospores germinate to form hyphae (Dancer et al. 1999; Palmer and Skinner 2002). Recent studies have shown that *Z. tritici* may remain epiphytic for 4-10 days post inoculation and prior to infection via the stomata (Fones et al. 2017; Haueisen et al. 2018). Following infection via the stomatal openings, hyphae colonize the apoplast of the mesophyll cell layer. The infecting hyphae show no evidence of forming either haustoria or other types of specialized feeding structures (Keon et al. 2007). The wheat host remains symptomless for 7-10 days and this is dependent on the variety. Throughout this 'latent' phase there is little or no increase in fungal biomass (Kema et al., 1996; Rudd et al., 2015). It has been suggested that the fungus may utilize internal stores of nutrition such as lipids or fatty acids at this time (Rudd et al. 2015; Steinberg 2015).

The initial recognition of pathogen attack is mediated by the plant's pattern recognition receptors (PRRs) that bind conserved pathogen-associated molecular patterns (PAMPs) such as fungal chitin (Miya et al. 2007; Robatzek et al. 2016). This is termed PAMP -triggered immunity (PTI). Pathogens can attempt to suppress PTI by secreting effector proteins which

are in turn detected by plant resistance (R) gene products. However effectors that evade recognition can manipulate plant cellular and metabolic processes to aid pathogen infection (Boller and He 2009). For example, the *Z. tritici* effector protein LysM binds fungal chitin and acts to 'cloak' the invading pathogen from the plant's immune system by binding fungal chitin (Yang et al. 2013). The *Z. tritici* effector Avrstb6 is a small cysteine-rich protein recognised by Stb6, a wall-associated receptor kinase in wheat. However, the function of Avrstb6 is not yet known (Kema et al. 2018; Saintenac et al. 2018). Following the latent phase, *Z. tritici* switches to an aggressive necrotrophic growth stage (Eyal et al. 1987; Yang et al. 2015). This is characterized by an increase in fungal biomass, degradation of the host cell wall, a collapse of mesophyll tissue cells and the accumulation of H_2O_2. The loss of cell integrity during the necrotrophic phase has been suggested to share similar characteristics to that of programmed cell death (PCD) (Yang et al. 2013). While PCD may reduce the spread of a biotrophic pathogen, it may also facilitate subsequent proliferation by providing a source of nutrition for the necrotrophic phase of *Z. tritici* growth (Keon et al. 2007). The mechanisms by which the necrotrophic phase is initiated is not entirely clear, although it has been suggested that the transition may be triggered by secreted effector proteins (Yang et al. 2015).

During the final stage of its lifecycle, *Z. tritici* produces asexual and sexual fruiting bodies termed pycndia and pseudothecia respectively. The pycnidia are formed inside the leaf substomatal cavity, and release asexual pycnidiospores which are dispersed to neighbouring plants by rain splash (Steinberg 2015). After death of the host tissue, saprophytic growth begins and the pathogen forms pseudothecia which appear 25 – 30 days after infection (Sánchez-Vallet et al. 2015). These pseudothecia produce sexual ascospores dispersed by the wind (Eyal 1987).

B. distachyon is a grass species related to cereals in the Pooideae which has been previously used as a model system for cereal-pathogen interactions and its utility for the study of STB

explored (O' Driscoll et al. 2015; Omidvar et al., 2018). In this study we compare the responses of *B. distachyon* (non-host) and wheat (host) to two different *Z. tritici* isolates IPO323 (Kema and van Silfhout 1997) and 560.11 (Lynch et al. 2016) using microscopy and gene expression studies. A better understanding of NHR in *B. distachyon* against STB may provide insights into isolate aggressiveness and the future identification of the plant immunity targets. .

Materials and Methods

Plant and fungal material

Seeds of *T. aestivum* (wheat) cv Remus were kindly supplied by Prof. F. Doohan and seeds of *B. distachyon* (Bd21) were provided by Dr. C. Ng (School of Biology and Environmental Science, UCD, Dublin) (Stępień et al. 2004; O'Driscoll et al. 2015). Seeds of wild-type *B. distachyon* (Bd21) and the German spring wheat cv. Remus were stratified at 4^{o}C for 3 days and then incubated in the dark at room temperature for 4 days to allow germination. Germinated seeds were transferred to pots containing one-part vermiculite and three-part peat soil. The pots were then placed in a growth chamber under a 16:8-hour light: dark cycle at 24^{o}C:12^{o}C and watered every 2 days.

The *Z. tritici* isolates IPO323 (Kema and van Silfhout 1997) and 560.11 (Lynch et al. 2016) were used for this study. Isolate 560.11 was isolated in Ireland in 2011 (Lynch et al. 2016) and isolate IPO323 was isolated in the Netherlands in 1981 (Kema and van Silfhout 1997). Prior to use, each isolate was cultured on potato dextrose agar (PDA) and grown at 20^{o}C under white light supplemented with blue/black ultraviolet (UV-A) light under a 12:12 hour light: dark cycle for approximately 7 days (O' Driscoll et al. 2015). *Z. tritici* cultures were grown under UV to induce asexual sporulation *in vitro* (Cooke and Jones 1970; Holmes and Colhoun 1971

Kema and Annone 1991; Skinner 2001; Zelikovitch and Eyal 1989; Tiley et al. 2018). Fungal spores from the PDA cultures were harvested using deionised water and spore concentration was adjusted to 1 x $10^6 ml^{-1}$ in water containing 0.02% Tween20 solution. Each plant was sprayed with a spore suspension of 5mls using hand-held mist spray bottles. Control plants were sprayed with 5ml of 0.02% Tween20 solution each. Inoculated plants were then covered with polythene bags for 48 hours to ensure high humidity.

Microscopic analysis

For analysis of H_2O_2, leaf sections were placed in 3, 3' diaminobenzidine (DAB) (1mg per 1ml) pH 3.8 for 4 hours in the dark. Leaf samples from *Z. tritici* challenged 14 day old wheat and *Brachypodium* seedlings were placed in 90% ethanol with heat for 10 minutes to remove chlorophyll. Dead cells and fungal structures were stained using Trypan Blue (Koch and Slusarenko, 1990) while heating leaf samples for 20 minutes, before rinsing in water and de-staining in choral hydrate (2.5g/ml H_2O) overnight. Leaf samples were mounted onto slides with 80% glycerol and examined under a light microscope (Leica DM5500B). For quantitative analysis cells in the Fields of view (FoV) were examined at x40 magnification for 0, 2, 4, 7 , 9 and 21 dpi. An average of approximately 830 cells in Remus and 1600 cells in *B. distachyon* were counted per FoV surrounding a spore for DAB or Trypan blue stained guard, epidermal and mesophyll cells. The Fov for two spores per each of the two leaves (n=4) were counted. This was repeated independently twice (n=8) and averaged. Images were collected over four independent experiments where two leaves (each from different plants) with each of the isolates 560.11 and IPO323 were examined with a light microscope (Leica DM5500B). Images were recorded with a Leica DFC310FX digital camera.

Disease and pycnidia scores

Seedlings of the *Z. tritici* susceptible wheat cv. Remus and wild type *B. distachyon* (Bd21) were inoculated with *Z. tritici* isolates IPO323 or 560.11. Inoculated plants were incubated under high humidity for 48 hours and subsequently at normal humidity at 22°C under a 16:8-hour light: dark cycle. Diseased leaves were harvested at 0, 10, 14, 17 and 21 days post inoculation. The samples were boiled for 10 minutes in ethanol to remove chlorophyll and were then mounted on glass slides for scoring. High resolution images of slides were obtained using a flatbed scanner (Epson XP-620). The coverage of necrosis or pigment browning and pycnidia was calculated using ImageJ for the whole leaf area (Schneider et al., 2012). Two leaves, each from different plants were used for each time point and this was repeated over three independent experiments.

For pycnidiospore counts, four 1cm long leaf sections each from 3 leaves from individual wheat and *B. distachyon* plants inoculated with *Z. tritici* or control plants were harvested at 31 dpi. Leaves were exposed to 100% humidity for 48 hours to induce release of pycnidiospores. The leaf sections were immersed in Sterile Distilled Water and vortexed for 15 seconds. The suspension was pipetted onto a haemocytometer for a qualitative assessment of spores released from pycnidia of isolate 560.11 and IPO323 on *T. aestivum* cv Remus and *B. distachyon.* This was repeated 3 times independently.

***B. distachyon* and wheat-extract agar**

The *Z. tritici* isolates IPO323 and 560.11 were grown on Czapek Dox-V8 juice (CDV8) agar (46 g/L Czapek Dox agar, 200 ml/L V8® Original vegetable juice (Campbell's), 3 g/L calcium carbonate and 20 g/L agar) for 5 – 7 days under white light supplemented with blue/black UV (UV-A) light under 16:8-hour light: dark cycles at 20°C (Kema and Annone 1991). The vegetative, yeast-like *Z. tritici* cells were used to inoculate wheat-extract agar (WEA) (37.5g/L

homogenised *Triticum aestivum* cv. Remus leaves of 21-day old seedlings in 2% agar) and *B. distachyon*-extract agar (BEA) (37.5g/L homogenised *B. distachyon* leaves of 21-day old seedlings in 2% agar). The inoculated plates were wrapped in foil for 48 hours and then incubated as above. Petri dishes were examined every 30 days for approximately 5 months using a Leica M205 C stereo microscope.I Images were captured using Leica Application Suite software V4.4.

RNA extraction and quantitative real time PCR

For plant gene expression studies, wheat and *B. distachyon* leaves (14-day old seedlings) were sampled at 0.5, 48, 96 and 144 hours after inoculation (hpi) with mock (0.02% Tween 20) or either of the two *Z. tritici* isolates. All samples were immediately frozen in liquid nitrogen and stored at -80°C until use. Total RNA was extracted from 100mg of plant tissue using RNeasy plant Mini Kit (Qiagen, The Netherlands) and subjected to on-column DNase treatment (Sigma). Quantification of total RNA was carried out using a Nanodrop ND-1000 spectrophotometer. Reverse transcription of RNA (1µg) was carried out using Omniscript RT Kit (Qiagen) and oligo (dT) primer using manufacturer's instructions.

Six defence marker genes (*PR1*, *PR2*, *PR3*, *SOD*, *CAT*, *PAL* and *OPR3*) and 2 reference genes (*Actin* and *GAPDH*) in both wheat and *B. distachyon* were analysed by qPCR (Table 1). Real-Time quantitative PCR was carried out in 12.5 µl reactions including 1.25 µl of a 1:5 (v/v) dilution of cDNA, 0.2 µM of primers, and 1× SYBR Premix Ex Taq (Tli RNase H plus, RR420A; Takara). PCR conditions were as follows: 1 cycle of 1 min at 95°C; 40 cycles of 5 s at 95°C and 20 s at 60°C; and a final cycle of 1 min at 95°C, 30 s, at 55°C, and 30 s at 95°C for the dissociation curve. Normalised fold change was calculated for each time relative to mock using the $2^{-\Delta\Delta Ct}$ described by Livak and Schmittgen (2001) and Ct Reference = geometrical mean (Ct GAPDH: Ct Tub). Two independent experiments were carried out. Each

experiment included three leaves from three plants per isolate per time point (n=6). Average Ct and SEM were calculated from six individual Ct value per isolate per time point.

Statistical Analysis

For gene expression studies, Relative gene expression was calculated with the equation $2^{-(Ct\ target\ gene - Ct\ housekeeping\ gene)}$ using the qRT-PCR threshold cycle (Ct) values. Statistical analysis was conducted using ANOVA incorporating Tukey's significant difference test at $P \leq 0.05$ in Graph Pad Prism (version 5.03 for windows; GraphPad software, San Diego, CA, United States).

All other statistical analysis was performed using the R statistical package (R Core Team, 2016). The proportion of disease symptoms and pycnidia per leaf area, the proportion of dead cells per FoV and proportion of cells showing the presence of H_2O_2 per FoV, were analysed using a generalised linear model with a quasi-binomial distribution (Crawley 2012). Multiple comparison tests were performed using the *emmeans* function of the *emmeans* package.

Results

T. aestivum (cv. Remus) and *B. distachyon* were compared for their response to infection with an Irish *Z. tritici* isolate (560.11) and Dutch isolate (IPO323).

Pathogenicity of the *Z. tritici* 560.11 and IPO323 isolates on wheat

Disease symptoms caused by the *Z. tritici* isolate 560.11 on wheat were more severe compared to those caused by IPO323 (Fig. 1A). At 21 days-post inoculation (dpi), disease coverage on cv. Remus was significantly ($p<0.05$) higher on plants inoculated with isolate 560.11 (74 ± 9%) than with IPO323 (56 ± 10%) (Fig. 1C). Pycnidia coverage on wheat infected with isolate 560.11 was also found to be significantly ($p<0.05$) higher at 14 dpi (23 ± 6%) and 21 dpi (74 ± 9%) compared to with IPO323 (2 ± 1% and 45 ± 9% respectively) (Fig. 1E).

B. distachyon non-host response to *Z. tritici* isolates 560.11 and IPO323

A macroscopic response was observed in *B. distachyon* to infection with both of the *Z. tritici* isolates (Fig. 1B). *Z. tritici* isolates on *B. distachyon* caused the production of brown pigments (polyphenols) (Fig. 1B, Supplementary Fig. 1). Levels of pigment accumulation on *B. distachyon* plants inoculated with the 560.11 isolate were significantly ($p<0.05$) higher at 14 (58 ± 9%) and 21 dpi (70 ± 5%) compared to leaves inoculated with the IPO323 at 14 dpi (32 ± 6) and 21 dpi (44 ± 8) (Fig. 1D)

Z. tritici isolate interactions with the non-host grass *B. distachyon*

Infected leaves were examined microscopically to determine if the symptoms observed on *B. distachyon* with *Z. tritici* isolate 560.11 were associated with any fungal colonisation. *Z. tritici* isolate 560.11 formed intracellular hyphae within the mesophyll cell layer (Fig. 2 F-J) comparable to that found in the wheat *cv*. Remus (Fig. 2 A-E). Rarely, a few immature pycnidia-like structures were observed on *B. distachyon* inoculated with *Z. tritici* isolate 560.11

after 28 dpi (Fig. 2 H-J) which were similar to those observed on the wheat *cv*. Remus 28 dpi (Fig 2 C-E). However, no pycnidiospores were released from the immature pycnidia in *B. distachyon*. Whereas Remus pycnidia released pycnidiospores via oozing cirrus, which were most abundant per cm^2 with isolate 560.11 (Supplementary Fig. 2).

Z. tritici pycnidia production on *B. distachyon* leaf extract

The ability of *Z. tritici* to produce pycnidia in *B. distachyon* was investigated further by inducing asexual sporulation *in vitro* on wheat extract agar (WEA) and *B. distachyon* extract agar (BEA). This enables the uncoupling of the *B. distachyon* non-host response with the ability of this grass to support *Z. tritici* asexual sporulation.

Previous studies have shown that asexual sporulation can be established in *Z. tritici* by incubating the fungus on WEA under white light supplemented with fluorescent blue/black UV-A light but not on water agar alone (Cooke and Jones 1970; Kema and Annone 1991; Skinner 2001; Zelikovitch and Eyal 1989; Tiley et al. 2018).

Both the *Z. tritici* IPO323 and 560.11 isolates produce hyphal knots and pycnidia on WEA and BEA (Fig. 3). By 60 days post-inoculation, both *Z. tritici* isolates produced white lateral hyphae radiated across the surface of the agar, away from the point of inoculation. These hyphae formed white hyphal knots, the initial stages of pycnidia development. By 5 months post-inoculation, the hyphal knots had developed into dark-brown, globose pycnidia which were approximately 50 – 100 µm in diameter (Fig. 3). The structures resembled pycnidia observed *in planta* on wheat cv. Remus isolates, and some oozed a cirrus-like substance. Only the *Z. tritici* 560.11 isolate consistently formed pycnidia on BEA in two independent experiments (Fig. 3).

Hydrogen peroxide accumulation and cell death during the host wheat-*Z. tritici* interaction

Wheat leaves inoculated with the two *Z. tritici* strains were stained with 3, 3'-Diaminobenzidine (DAB) and Trypan blue to assess H_2O_2 accumulation and PCD respectively at 0, 2, 7, 9, and 21 dpi (Fig. 4) (Supplementary Fig. 3A & 4A).

Following inoculation, the cells in the field of view surrounding the site of *Z. tritici* spores were assessed for H_2O_2 accumulation. The percentage of Remus cells with H_2O_2 significantly increased between 0 dpi (1 ± 0.2 %) and 2 dpi (12 ± 2%) before peaking at 9 dpi (25 ± 6%) following infection with isolate IPO323. A similar pattern of H_2O_2 accumulation was observed with isolate 560.11 increasing to 29 ± 6% at 2 dpi and peaking at 9dpi (44 ± 7%) (Fig. 4).

The different cell types were assessed for the percentage of H_2O_2 to determine responses in epidermal, guard and mesophyll cells. No significant differences in the percentage of guard or epidermal cells accumulating H_2O_2 were found between the isolates, at any of the time points. However, a significantly higher percentage of mesophyll cells were found to accumulate H_2O_2 in response to isolate 560.11 (56 ± 7%) compared to IPO323 (16 ± 3%) at 2dpi. The highest percentage H_2O_2 accumulation in wheat was at 9 dpi in response to 560.11 (73 ± 4%) compared to IPO323 (32 ± 4) (Fig.4).

Following challenge with isolate IPO323 the percentage of dead cells in wheat significantly increased between 0 (0.3 ± 0.2%) and 9 dpi (17 ± 5%) before levelling off. A significant increase in the percentage of dead cells was observed from 0 dpi (0.2 ± 0.2%) to 7 dpi (5 ± 2%) , 9 (19 ± 6%) and 21 dpi (26 ± 5%) following challenge with isolate 560.11 (Fig. 4).

No significant differences in the percentage of cell death in guard or epidermal cells were found when comparing between the isolates. However, at 21 dpi a significantly higher percentage of dead mesophyll cells were found in response to 560.11 (20 ± 5%) compared to IPO323 (7% ± 1) (Fig. 4).

Hydrogen peroxide accumulation and cell death during the *B. distachyon* non-host-*Z. tritici* interaction

B. distachyon leaves were stained for H_2O_2 and PCD induction, using 3,3'-Diaminobenzidine (DAB) and Trypan blue staining at 0, 2, 7, 9 and 21 dpi with isolate 560.11 and IPO323 (Fig. 5) (Supplementary Fig. 3B & 4B).

Following inoculation, *B. distachyon* cells in the field of view surrounding the site of *Z. tritici* spores were assessed for H_2O_2 accumulation. After infection with isolate IPO323 the percentage of *B. distachyon* cells with H_2O_2 increased significantly between 0 dpi (0.2 ± 0.1%) and 7 dpi (22 ± 4%) before peaking at 9 dpi (30 ± 5%). Whereas, a significant accumulation of H_2O_2 in cells following 560.11 infection was observed earlier at 2 dpi (28 ± 4%) before peaking at 9dpi (48 ± 5%) (Fig. 5)

In *B. distachyon* the percentage of epidermal cells accumulating H_2O_2 in response to isolate 560.11 (21 ± 4%) was found to be significantly higher than in response to IPO323 (2 ± 1%) at 2 dpi (Fig. 5). Whereas such a significant difference was not observed in wheat (Fig. 4). *B. distachyon* also had a significantly higher percentage of mesophyll cells accumulating H_2O_2 in response to isolate 560.11 compared to IPO323 at 2, 7, 9 and 21 dpi (Fig. 5). The highest percentage was observed in response to 560.1 (77 ± 5%) at 9 dpi compared to (47% ± 7%) for IPO323.

Following challenge with isolate IPO323 the percentage of dead cells significantly increased between 0 dpi (0.6 ± 0.3%) and 7 dpi (6 ± 2%) with a further increase not observed until 21 dpi (14 ± 4 %) (Fig. 5). Following challenge with 560.11 a significant increase in the percentage of dead cells was observed between 0 dpi (0.5 ± 0.3%) and 7 dpi (14 ± 5%) before peaking at 21 dpi (22 ± 6%).

No significant differences were observed in *B. distachyon* for epidermal or guard cell death with the exception of 7 dpi, where a significantly higher percentage of dead guard cells were

found in response to 560.11 (41 ± 11%) compared to IPO323 (13 ± 4%) (Fig. 5). In mesophyll cells a significantly higher percentage of dead cells were found in response to 560.11 at 9 dpi (14 ± 2%) and 21 dpi (17 ± 4%) compared to IPO323 at 14 dpi (3 ± 1%) and 21 dpi (7 ± 1%) (Fig. 5).

Defence gene expression following challenge of wheat and *B. distachyon* with the two *Z. tritici* isolates

Quantitative real-time PCR (qRT-PCR) was performed to assess the relative expression of defence gene induction in *B. distachyon* and wheat following inoculation with the two *Z. tritici* isolates during the early stages of infection (0.5, 48, 96, 144 hours post inoculation (hpi).

SA signalling pathway marker gene expression

To explore a potential role for the SA signalling pathway the expression of *PR1* (*Pathogenesis-Related*), *PR2, NPR1 (Non-expresser of PR genes 1)*, and *PAL* (*Phenylalanine Ammonia Lyase*) were investigated.

TaPR1 transcripts were significantly induced in wheat inoculated with IPO323 compared to mock controls at all time points analysed. Whereas, wheat seedlings inoculated with 560.11 only had significantly induced *TaPR1* levels at 144 hpi. Significant differences were observed between seedlings treated with different isolates at 96 hpi where *TaPR1* levels had increased 28-fold for IPO323 compared to a 4- fold induction for 560.11. Conversely at 144 hpi *TaPR1* levels were significantly higher with 560.11 (34-fold induction) compared to IPO323 (18-fold induction) (Fig. 6A).

Both *TaPR2* and *TaNPR1* transcripts were significantly higher in wheat plants infected with IPO323 at 0.5 hpi compared to those inoculated with 560.11 (Fig. 6C, 6E). *TaPR2* also had significantly higher levels following IPO323 inoculation than 560.11 at 144hpi .

BdPR1 transcripts were only found to be significantly induced at 144 hpi with isolate IPO323 (Fig. 6B). No significant difference in relative transcript levels of *BdPR2* and *BdNPR1* were found in *B. distachyon* plants inoculated with either of the two *Z. tritici* isolates (Fig. 6D, 6G).

No significant induction of *TaPAL* in wheat was observed compared to mock control with both isolates (7F). However, a significant induction of *BdPAL* was observed in *B. distachyon* relative to the mock control at 48 hpi, 96 hpi and was significantly different when challenged with 560.11 compared to IPO323 at 48 hpi, 96 hpi and 144 hpi (Fig. 7H).

JA signalling pathway marker gene expression

To explore the role of the JA signalling pathway during *Z. tritici* infection, the expression of *PR3* and *Oxo phytodienoate reductase 3 (OPR3*) was investigated.

TaPR3 transcript accumulation was significantly induced at 0.5, 48 and 96 hpi in wheat plants infected with IPO323 and this was significantly different to induction by 560.11 at 0.5 hpi(Fig 7A). In infected *B. distachyon* plants, *BdPR3* transcripts were induced relative to mock control at 48 and 144hpi with IPO323. There was a significant difference in the transcript accumulation of *BdPR3* at 48 and 96 hpi between the two *Z. tritici* isolates where 560.11 had highertranscript levels (Fig. 7B). Levels of *TaOPR3* and *BdOPR3* transcripts were not found to be induced following infection with either *Z. tritici* isolate (Fig. 7C and 7D).

ROS production and catalase expression

To further explore the role of the ROS production and turnover the expression of Super Oxide Dismutase (SOD) and Catalase (CAT) was investigated.

Significant induction of *TaSOD* was observed at all timepoints infected with IPO323 and the transcript levels were significantly higher compared to with isolate 560.11 (Fig. 8A). *TaCAT* was significantly induced by IPO323 at 48 hpi compared to the mock control and was significantly higher compared to inoculation with 560.11 (Fig. 8C). In *B. distachyon*, *BdSOD* was induced relative to the control at 48 hpi and 144 hpi but there was no significant difference between isolates(Fig. 8B). In contrast, *BdCAT* was induced and differentially expressed between isolates in *B. distachyon* plants. IPO323 had significantly higher transcript levels at 0.5 hpi while 560.11 induced higher transcript levels at 48 and 96 hpi (Fig. 8D).

DISCUSSION

Non-host resistance has been proposed as a durable source of disease control (Lee et al. 2013; Gill et al. 2015). In this study we found isolate specific responses when comparing the interaction between the wheat pathogen *Z. tritici*, the wheat host and the non-host grass *B. distachyon*.

In previous studies, symptoms caused by *Z. tritici* on *B. distachyon* were found to be similar to an incompatible interaction with the natural wheat host. The *Z. tritici* isolate IPO323 showed no intracellular infection and no stomatal penetration was detected (O' Driscoll et al. 2015). In this present study, isolate 560.11 (Lynch et al., 2016) was used for comparison to IPO323 (Kema and van Silfhout 1997). Early in infection both isolates showed an increase in biomass, suggested via an increase in *ZtTUB* transcripts on *B. distachyon* between 0.5 and 48 hpi (Supplementary Fig. 5). The 560.11 isolate was able to achieve stomatal penetration and intracellular growth in *B. distachyon* and there was possible immature pycnidia-like formation (Fig. 2). Evidence that *B. distachyon* has the nutrients for *Z. tritici* pycnidia formation was supported by growth on BEA (Fig. 3). It was rare to find these pycnidia-like structures on *B. distachyon* and when found, they were of an immature form. However, *B. distachyon* were not observed post 31 days and it is therefore possible that these pycnidia develop further and eventually mature. The immature pycnidia were comparable to those found in *Z. tritici* transcription mutants (Mohammidi et al. 2017). Our results show that the *Z. tritici* isolate 560.11 was more aggressive on wheat than IPO323 displaying a higher disease and pycnidia coverage (Fig.1.) This isolate was able to penetrate and intracellularly colonise *B. distachyon* leaves, suggesting that some aggressive isolates of *Z. tritici* may be able to partially overcome pre-invasive and initial PTI responses. This result is similar to findings with stem rust (*Puccinia graminis*), where occasional sporulation was observed with some isolates (Figueroa et al. 2013; Ayliffe et al. 2013). Although here. we were unable to obtain pycnidiospores from the immature pycnidia in *B. distachyon* (Supplementary Fig. 2). The uncoupling of pycnidia

formation from pycnidiospore formation has also been reported for *Z. tritici* in chlorophyll-deficient wheat leaves silenced for wheat phytoene desaturase (PDS) and magnesium chelatase subunit H (CHlH) (Lee et al. 2015). Another possibility is that the smaller sub-stomatal cavities in *B. distachyon* prevent mature pycnidia formation.

It is hypothesised that in distantly related plants NHR is mediated by PTI since non-host pathogen effectors are likely to be unable to supress PTI. This may be due to a lack of effector targets or an inability to manipulate the non-host plant targets (Stam et al., 2014). Therefore, differences in NHR may reflect differences in the effector complement or effector expression between isolates. However, there is evidence that effectors can act in distantly related species. For example, RXLR effectors from *P. infestans* were found to suppress PTI responses in the tomato host and in the nonhost *Arabidopsis* (Stam et al., 2014; Zheng et al., 2014). Previously it was proposed that ETI may contribute to NHR of *B. distachyon* to isolates of the crown rust oat pathogen *P. coronata f. sp. avenae*, where isolate specific responses were found *B. distachyon* and oat (Omidvar et al., 2018).

Despite both *Z. tritici* isolates accumulating brown pigmentation in *B. distachyon* this was significantly higher with 560.11. Phenylalanine ammonia-lyase (PAL) catalyses the production of cinnamic acid from phenylalanine which can be converted into phenolic compounds. These compounds are in turn oxidised and polymerized into brown pigments (Ngadze et al. 2012). This is consistent with the increased levels of brown pigment observed in *B. distachyon* following challenge with isolate 560.11. In addition, higher transcript levels of *BdPAL* were found in *B. distachyon* infected with 560.11 isolate from 48 to 144 hpi (Fig. 6H). We found no induction of *TaPAL* by either *Z. tritici* isolate in the susceptible wheat cv. Remus (Fig. 6F). However, expression of wheat *TaPAL* has been previously reported to be induced by *Z. tritici* and was higher in the STB semi-resistant wheat cv. Premio (Ors et al. 2017). PAL is a key enzyme in biosynthesis of polyphenolic compounds and lignin precursors. Furthermore,

production of phenylpropanoid radicals by H_2O_2 and peroxidase may also form a chemical and or physical barrier to *Z. tritici* in *B. distachyon* (Ors et al. 2017).

The role of reactive oxygen species has been studied in both host and non-host defence responses (Hückelhoven and Kogel 2003; Nimchuk et al. 2003). H_2O_2 accumulation at the site of *Z. tritici* penetration has been associated with resistance (Shetty et al. 2003; 2007). Despite this, significant epidermal H_2O_2 accumulation and cell death also took place from 13 dpi in a compatible reaction with the wheat cv. Sevin (Shetty et al., 2003). *Z. tritici* secretes catalase-like proteins at the stage of pycnidia development to detoxify ROS accumulation in the host (Haueisen et al., 2018). Our results are in agreement with an accumulation of H_2O_2 promoting infection since we observed a peak of H_2O_2 accumulation at 9 dpi and this was higher for the aggressive isolate 560.11 (Figure 4). *Superoxide dismutase* (*SOD*) and *catalase* are known to break down superoxide radicals (O_2^-) to H_2O_2 and breakdown H_2O_2 respectively. These enzymes play a key role in antioxidant defence against pathogens (Zhang et al. 2008; Bednarski et al. 2013). Wheat SOD (*TaSOD*) expression was significantly lower in 560.11 infected leaves over the majority of timepoints and *TaCAT* lower at 48 hpi. This may explain the accumulation of H_2O_2 observed during infection by isolate 560.11 (Fig. 4). Isolate specific difference were found for H_2O_2 accumulation in epidermal cells at 2 dpi and in mesophyll cells at all timepoints assessed. Significantly lower expression levels of the antioxidant *BdCAT* were observed at 0.5 hpi on plants inoculated with the *Z. tritici* 560.11 isolate compared to IPO323. This may promote H_2O_2 accumulation in response to 560.11. Together our data suggests that H_2O_2 accumulation occurs from 2 dpi in both a compatible host and non-host response and that this is promoted by aggressive isolates. This suggests that *Z. tritici* recognition, possibly via PTI may be conserved between *B. distachyon* and wheat.

Previous observations during an incompatible interaction in the resistant cv. Stakado showed no cell death until 15 dpi (Shetty et al. 2003). Studies on susceptible and resistant wheat cultivars have suggested that cell death might induce the necrotrophic phase in *Z. tritici*, by

458 providing nutrients and energy for the fungus to sporulate (Shetty et al. 2003; Lee et al. 2015).
459 While cell death may trigger the switch to the necrotrophic phase, early cell death may limit
460 pycnidia formation (Lee et al. 2015). We recorded increases in cell death from 2 dpi in the non-
461 host *B. distachyon* and from 7 dpi in the wheat host (predominantly in guard cells) (Fig. 5). It
462 is conceivable that cell death at 2 dpi in guard cells may prevent stomatal penetration. It would
463 be interesting to study the effects of guard cell death on attempted *Z. tritici* entry in *B.*
464 *distachyon*. The only difference in cell death accumulation in wheat was at 21 dpi where cell
465 death was higher with 560.11 compared to IPO323 (Fig. 1 & Fig.5). The similar levels of cell
466 death at earlier time points is unexpected given that pycnidia formation was higher in 560.11
467 at 14 dpi as well as 21 dpi (Fig. 1). Nonetheless, it has been found that necrosis and pycnidia
468 formation are largely independent (Karisto et al., 2018).

469 The role of plant hormones in defence against pathogen infection has been well characterised
470 (Baris and Jones 2009; Pieterse et al. 2009) but is unclear with respect to STB. Higher induction
471 of SA- related *PR* genes against *Z. tritici* have been observed in resistant cultivars, while little
472 induction of these genes was observed in susceptible cultivars at initial stages of infection
473 (Shetty et al. 2003; Ray et al. 2003; Adhikari et al. 2007). *TaPR1*, *TaPR2* and *TaNPR1* were
474 induced in wheat following *Z. tritici* infection and this was earlier and transcript levels were
475 higher with IPO323 (Fig. 6A, C, and E). This suggests that SA-mediated signalling is triggered
476 in wheat possibly as a defence response against *Z. tritici*. This result is in agreement with
477 previous research where higher expression of *PR1* was observed in the resistant cultivar Atigo
478 compared to the susceptible cultivar Alixan (Ors et al. 2017).

479 The JA signalling pathway mediates defence against necrotrophic and hemi-biotrophic
480 pathogens (Li and Yen 2008). No differential expression of *TaPR3* and *TaOPR3* between
481 isolates was observed with wheat, with the exception of *TaPR3* induction by IPO323 at 0.5 hpi
482 (Fig. 7A). *BdPR3* transcripts were found to be induced in *B. distachyon* following *Z. tritici*
483 inoculation but this was higher at 48 hpi and 96 hpi with 560.11 isolate than IPO323 (Fig. 7B).

Taken together, the expression profiles of these defence marker genes suggest that induction of SA mediated *PR* genes may inhibit STB disease progression in wheat but that aggressive isolates such as 560.11 may be able to interfere with this. While the phenylpropanoid pathway appears important for the *B. distachyon* NHR response.

The aim of this study was to investigate host and non-host interactions using different isolates of *Z. tritici*. The limited growth of the *Z. tritici* isolate 560.11 may suggest that homologous susceptibility factors are conserved between *B. distachyon* and wheat. These plant targets may be differentially manipulated by isolates, perhaps due to different effector repertoires. Future, *transcriptomic* and genomic studies of the *B. distachyon* interaction with these isolates would reveal such effectors.

These findings may provide a basis to identify the genes responsible for *Z. tritici* recognition in this non-host and the targets responsible for isolate specific responses shared in both plant species. It may be possible to use genome wide association studies to identify the effector targets of aggressive isolates or "susceptibility factors" using *B. distachyon*.

ABBREVIATIONS

STB: *Septoria tritici* blotch

NHR: non-host resistance

PTI: PAMP triggered immunity

ETI: Effector triggered immunity

PCD: Programmed cell death

BEA: *Brachypodium* distachyon extract agar

WEA: Wheat extract agar

DAB: Diaminobenzidine

CAT: Catalase

PAL: Phenylalanine ammonia lyase

SOD: Superoxide dismutase

PR: Pathogenesis-related

NPR1: Non-expresser of PR1

OPR3: Oxophytodienoate reductase 3

SDHI: Succinate dehydrogenase inhibitor

PDS: Phytoene desaturase

CHlH: magnesium chelatase subunit H

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532

533 **Authors contributions**

534 A.F designed the research. A.R, S.J.K, A.T and A.M.M.T performed the experiments. A.F, A.R
535 and S.J.K wrote the manuscript. A.F and S.K critically read the manuscript.

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Figure legends

Fig. 1. Progression of symptoms on *T. aestivum* cv. Remus (**A**) and *B. distachyon* (**B**) induced by *Z. tritici* isolate IPO323 and 560.11. Infected leaves were cleared of chlorophyll and symptoms were recorded at 0, 10, 14, 17 and 21dpi. Two leaves each from a different plant were examined per timepoint. Images are representative of three independent experiments . Scale bar, 2mm. At 0, 10, 14, 17 and 21 days post inoculation, disease coverage on *T. aestivum* cv. Remus (**C**) and pigment coverage on *B. distachyon* (**D**). Pycnidia coverage on Remus (**E**). High resolution images of diseased leaves (one per seedling of two seedlings) were obtained and coverage of symptoms and coverage of pycnidia were measured using ImageJ. Each data point represents them mean ± SEM of three independent experiments. The p-values shown above bars are differences between isolates using Tukey's significant difference test at $P \leq 0.05$.

Fig. 2. Images of *Z. tritici* growth (isolate 560.11) in wheat cv. Remus and *B. distachyon*. (A) Hyphae interacting with a stomata in cv. Remus at 2 dpi; (B) Hyphae growing along cv. Remus mesophyll cells at 7dpi; (C) Pycnidia forming and mesophyll cell death at 21 dpi; (D) and (E) Pycnidia formation in cv. Remus stomata at 28 dpi. (F) and (G) Hypha interacting with a stomata in *B. distachyon* at 9 dpi (H) Hyphae growing along mesophyll cells in *B. distachyon* at 9 dpi; (I) immature pycnidia formation in *B. distachyon* at 28 dpi. (J) same FoV as I but different field of plane, focus on hyphae growing along mesophyll cells in *B. distachyon*. Black arrowhead indicates hyphae interacting with a stomata, black arrows indicate hyphae, white asterisks indicates pycnidia. Scale bar = 25µm

Fig. 3. *Zymoseptoria tritici* IPO323 and 560.11 isolate pycnidia formation on *Triticum aestivum* (cv. Remus)-extract agar (WEA) *Brachypodium distachyon*-extract agar (BEA) at 5 months post-inoculation. Incubated under white light supplemented with blue/black UV (UV-

757 A) light on 16:8 hour light:dark cycles at 20°C. Images are representative of two independent
758 experiments, and two technical repeats (plates). Arrows indicate pycnidia and white arrows
759 indicate cirrhus.

760

761 **Fig. 4.** (A) Proportion of infected Remus and *B. distachyon* cells with H_2O_2 accumulation. (B)
762 Proportion of Remus and *B. distachyon* guard, epidermal and mesophyll cells with H_2O_2
763 accumulation. Leaves infected with IPO323 and 560.11 were stained with DAB at 0, 2, 7, 9
764 and 21 dpi, mounted onto microscope slides with 80% glycerol. For each experiment FoV's
765 for *Z. tritici* spores were examined, two per each of two leaves (independent plants). Bars
766 represent the mean of two independent experiments (n=8) and error bars indicate ±SEM.
767 Different letters denote significant differences between Remus/*B. distachyon* and
768 IPO323/560.11 at each time point ($p \leq 0.05$) using a generalised linear model with a quasi-
769 binomial distribution (Crawley 2012).

770

771 **Fig. 5.** (A) Proportion of dead cells following infection with Remus and *B. distachyon*. (B)
772 Proportion of Remus and *B. distachyon* guard, epidermal and mesophyll dead cells. Leaves
773 infected with IPO323 and 560.11 were stained with DAB at 2, 7, 9 and 21 dpi, mounted onto
774 microscope slides with 80% glycerol. For each experiment FoV's for *Z. tritici* spores were
775 examined, two per each of two leaves (independent plants). Bars represent the mean of two
776 independent experiments (n=8) and error bars indicate ±SEM. Different letters denote
777 significant differences between Remus/*B. distachyon* and IPO323/560.11 at each time point
778 ($p \leq 0.05$) a generalised linear model with a quasi-binomial distribution (Crawley 2012).

779

Fig. 6. Expression profile of *PR-1* (**A**), (**B**), *PR-2* (**C**), (**D**), *NPR-1* (**E**), (**G**), and *PAL* (**F**), (**H**) genes in *T. aestivum* (**A**), (**C**), (**E**) and (**F**), and *B. distachyon* (**B**), (**D**), (**G**) and (**H**) after infection by *Z. tritici*. qRT-PCR was used to assess gene expression (fold change) in leaves infected with *Z. tritici* isolates IPO323 and 560.11 relative to mock inoculations. Normalisation was carried out using *Actin* and *GAPDH* of *T. aestivum* and *B. distachyon* respectively. Results represent mean of three leaves (each from individual plants) per isolate per time point over two independent experiments (n=6), (error bars indicate ±SEM). Means with the same letters are not significantly different while means with asterisk are significantly different from mock treated control determined using Tukey test at $P \leq 0.05$.

Fig. 7. Expression profile of *PR-3* (**A**), (**B**) *and OPR-3* (**C**), (**D**) genes in *T. aestivum* (**A**), (**C**) and *B. distachyon* (**B**), (**D**) after infection by *Z. tritici*. qRT-PCR was used to assess gene expression (fold change) in leaves infected with *Z. tritici* isolates IPO323 and 560.11 relative to mock inoculations. Normalisation was carried out using *Actin* and *GAPDH* of respective *T. aestivum* and *B. distachyon.* Results represent mean of three leaves (each from individual plants) per isolate per time point over two independent experiments (n=6), (error bars indicate ±SEM). Means with the same letters are not significantly different while means with asterisk are significantly different from mock treated control determined using Tukey test at $P \leq 0.05$.

Fig. 8. Expression of *Superoxide dismutase (SOD)* (**A**), (**B**) and *Catalase (CAT)* (**C**), (**D**) gene in *T. aestivum* (**A**), (**C**) and *B. distachyon* (**B**), (**D**) after infection by *Z. tritici*. qRT-PCR was used to assess gene expression (fold change) in leaves infected with *Z. tritici* isolates IPO323 and 560.11 relative to mock inoculations. Normalisation was carried out using *Actin* and *GAPDH* of respective *T. aestivum* and *B. distachyon.* Results represent mean of three leaves (each from individual plants) per isolate per time point over two independent experiments.

(n=6), ((error bars indicate ±SEM). Means with the same letters are not significantly different while means with asterisk are significantly different from mock treated control determined using Tukey test at $P \leq 0.05$.

Supplementary files

Supplementary Fig. 1 *B. distachyon* pigmented cells with no evidence of necrosis or cell death on (**A**) IPO323 inoculated (Left) or (**B**) 560.11 inoculated (Right) at 21 dpi. Black arrows show example cells with deposited polyphenols. Two leaves were examined (each from different plants). Images are representative from three independent experiments. Chlorophyll was removed from leaves prior to microscopy.

Supplementary Fig. 2 Four cm long leaf sections from wheat and *B. distachyon* plants inoculated with *Z. tritici*, and control plants were harvested at 31 dpi, exposed to 100% humidity for 48 hours to induce release of pycnidiospores. The leaf sections were then immersed in Sterile Distilled Water and vortexed for 15 seconds. The suspension was pipetted onto a haemocytometer for a qualitative assessment of spores released from the pycnidia by (**B**) 560.11, (**C**) IPO323 compared to (**A**) uninoculated controls on *T. aestivum* cv Remus and *B. distachyon.* Each experiment included 3 leaves (each from 3 plants) per isolate. Representative of three independent experiments.

Supplementary Fig. 3 DAB staining for H_2O_2 accumulation in (A) *B. distachyon* and (B) *T. aestivum* cv. Remus following infection with *Z. tritici* isolates IPO323 and 560.11 at 2, 7, 9 and 21 dpi or uninfected. The top panel for each isolate is focused on the epidermal cell layer and the bottom on the mesophyll cell layer. Images are representative of four independent

experiments with two leaves (each from different plants). Asterisks indicate site of *Z. tritici* spore. Scale bar = 50µm.

Supplementary Fig. 4 Trypan blue staining for cell death in (A) *B. distachyon* and (B) *T. aestivum* cv. Remus following infection with *Z. tritici* isolates IPO323 and 560.11 at 2, 7, 9 and 21 dpi or uninfected. The top panel for each isolate is focused on the epidermal cell layer and the bottom on the mesophyll cell layer. Images are representative of four independent experiments with two leaves (each from different plants). Asterisks indicate site of *Z. tritici* spore. Scale bar = 50µm.

Supplementary Fig. 5 Semi-quantitative RT-PCR in *B. distachyon* (Bd-21) and *T. aestivum* (Cv. Remus) leaves for *BdGAPDH* and *TaGAPDH* respectively and *Z. tritici Tubulin* (*ZtTUB*) at 0.5, 48, 96 and 144 hpi after inoculation with *Z. tritici* isolates (A) IPO323 and (B) 560.11. Three leaves (each from a different plant) per isolate per timepoint. Representative of two independent experiments.

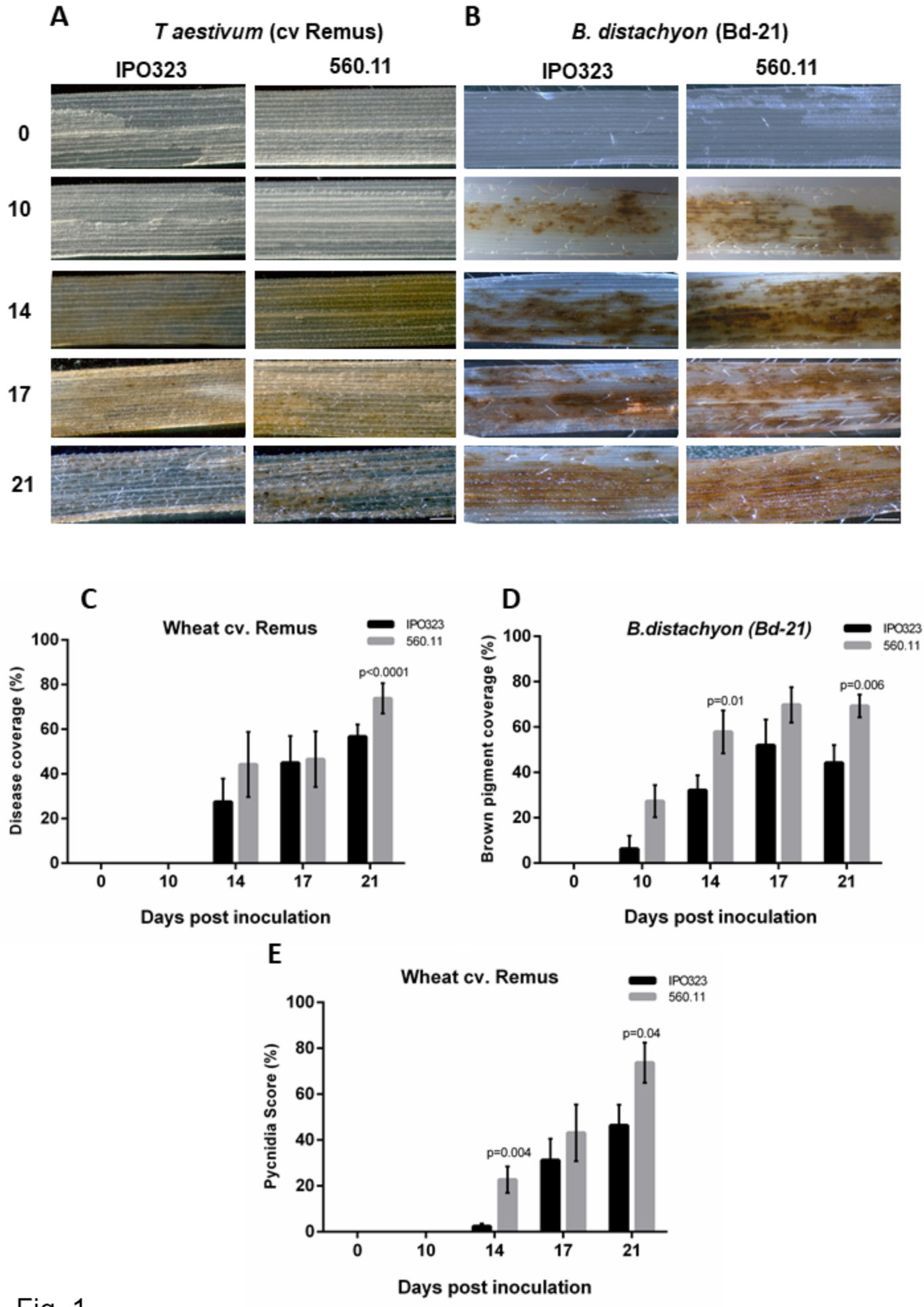

A
T aestivum (cv Remus)
IPO323
560.11
B
B. distachyon (Bd-21)
IPO323
560.11
0
10
14
17
21
C
Wheat cv. Remus
IPO323
560.11
p<0.0001
Disease coverage (%)
Days post inoculation
D
B.distachyon (Bd-21)
IPO323
560.11
p=0.01
p=0.006
Brown pigment coverage (%)
Days post inoculation
E
Wheat cv. Remus
IPO323
560.11
p=0.004
p=0.04
Pycnidia Score (%)
Days post inoculation

Fig. 1

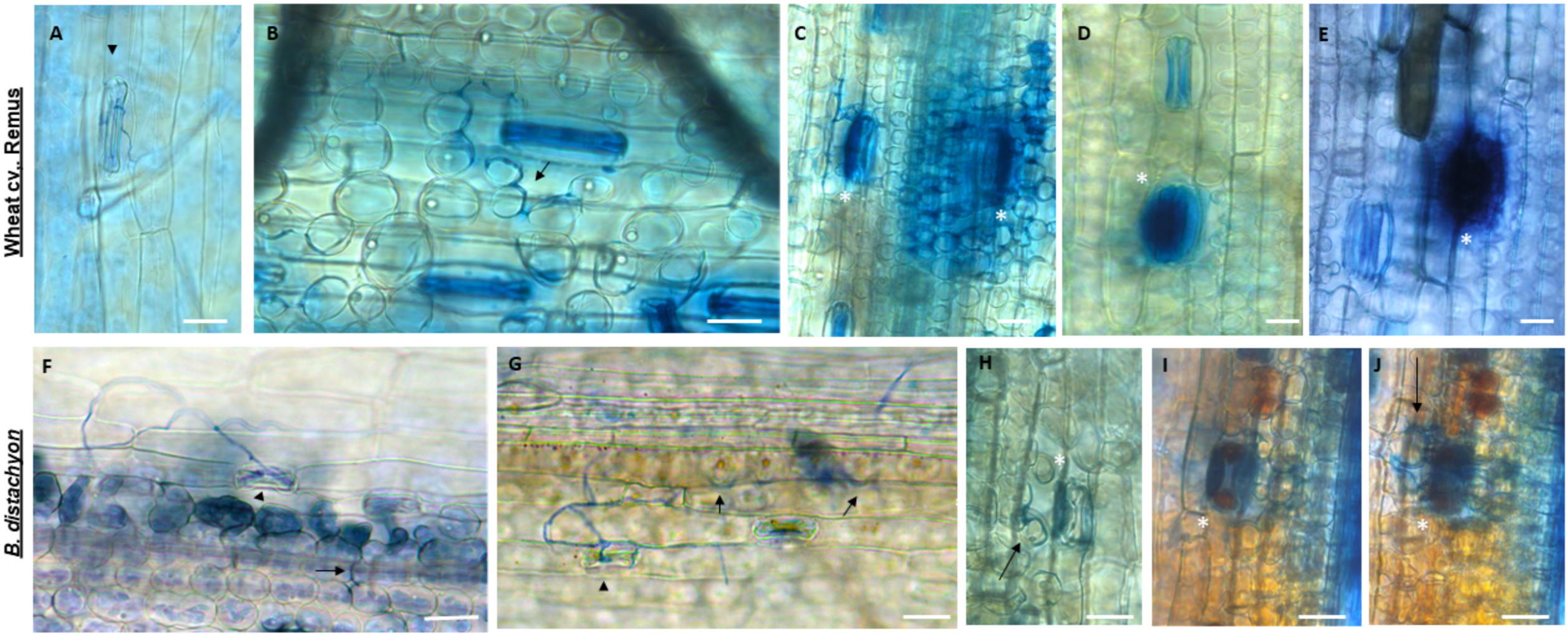


Fig. 2.

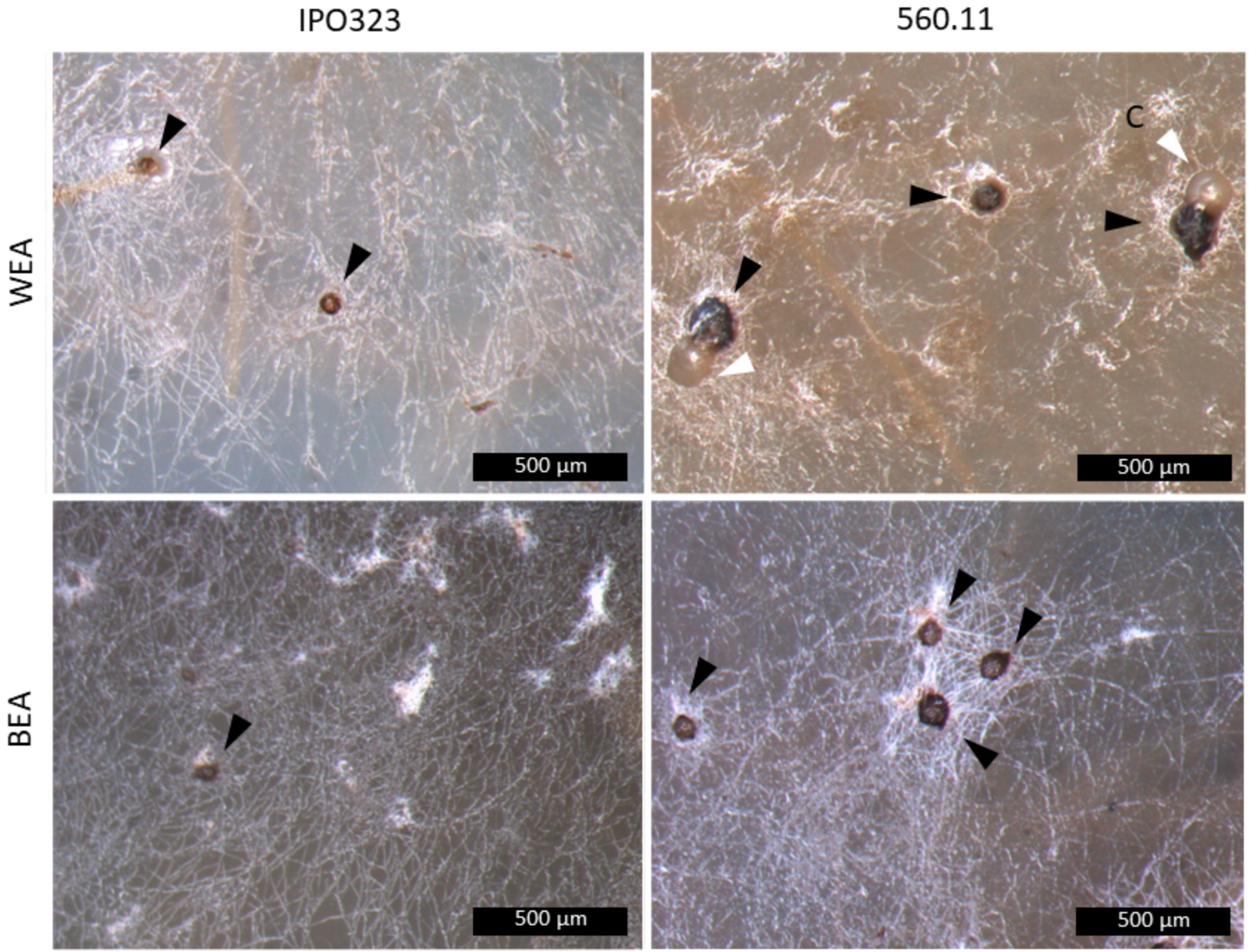


Fig. 3.

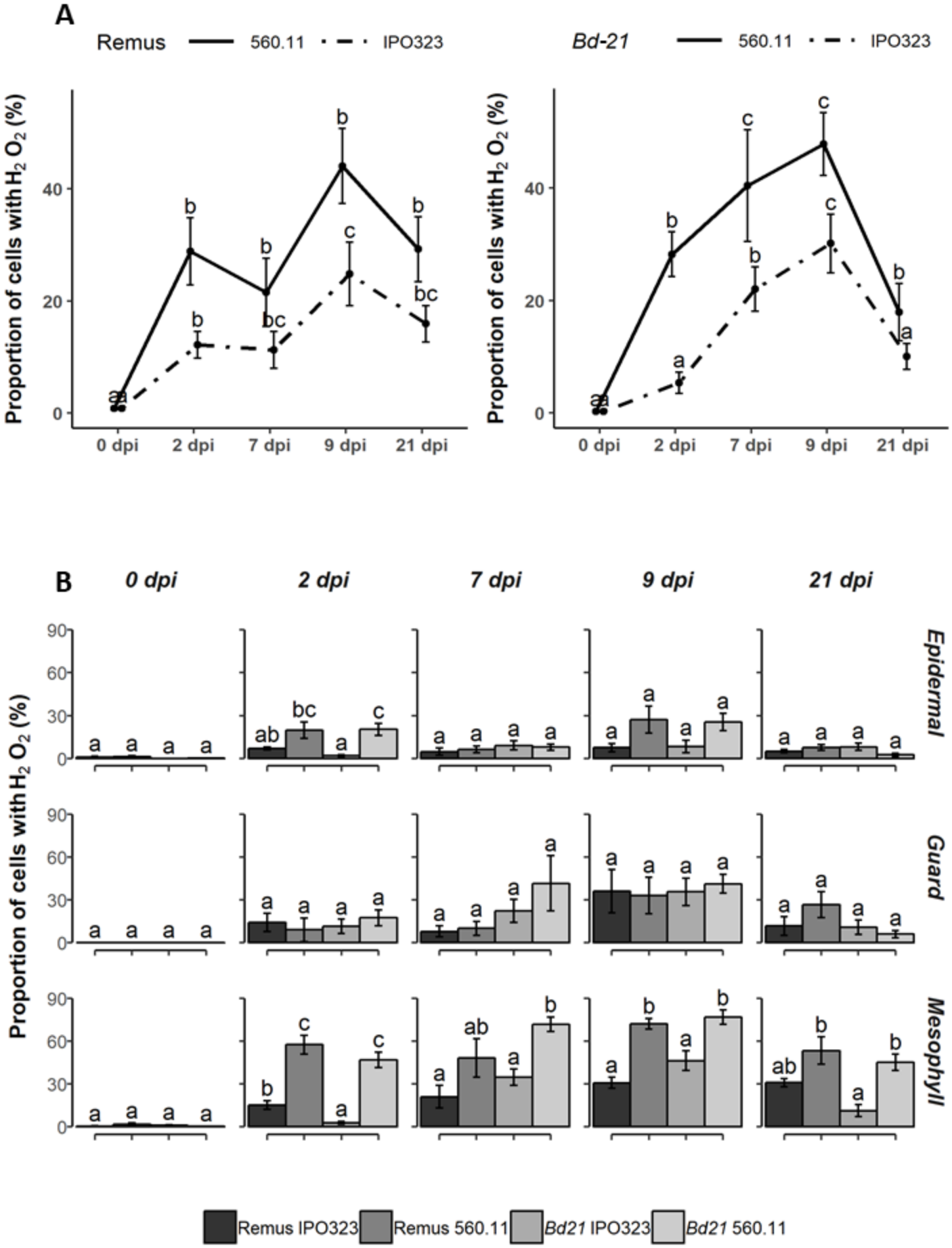

A
Remus
560.11
IPO323
Bd-21
560.11
IPO323
Proportion of cells with H2 O2 (%)
0 dpi
2 dpi
7 dpi
9 dpi
21 dpi
B
0 dpi
2 dpi
7 dpi
9 dpi
21 dpi
Epidermal
Guard
Mesophyll
Proportion of cells with H2 O2 (%)
Remus IPO323
Remus 560.11
Bd21 IPO323
Bd21 560.11

Fig. 4.

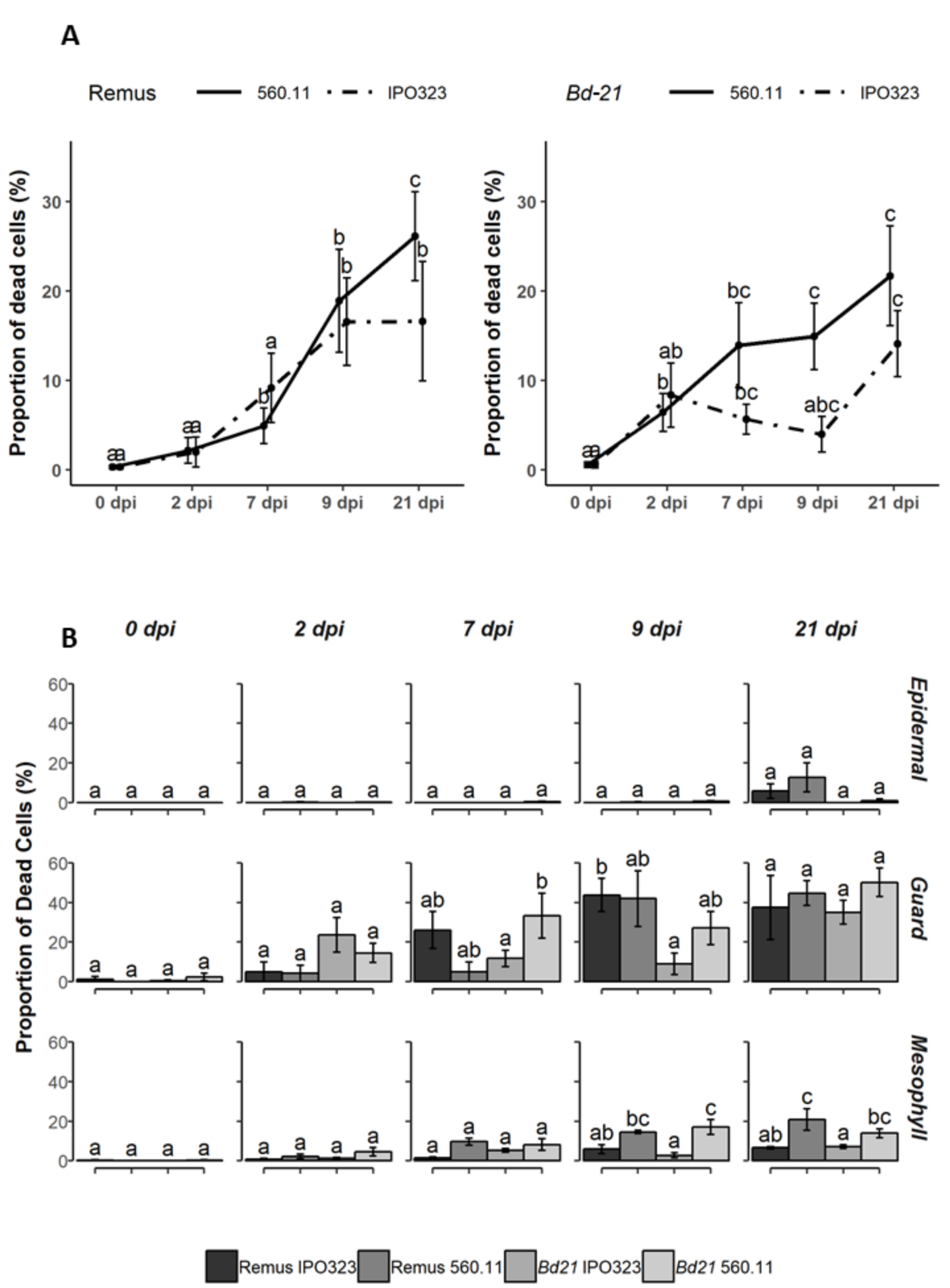

A
Remus
560.11
IPO323
Bd-21
560.11
IPO323
Proportion of dead cells (%)
0 dpi
2 dpi
7 dpi
9 dpi
21 dpi
B
0 dpi
2 dpi
7 dpi
9 dpi
21 dpi
Epidermal
Guard
Mesophyll
Proportion of Dead Cells (%)
Remus IPO323
Remus 560.11
Bd21 IPO323
Bd21 560.11

Fig. 5.

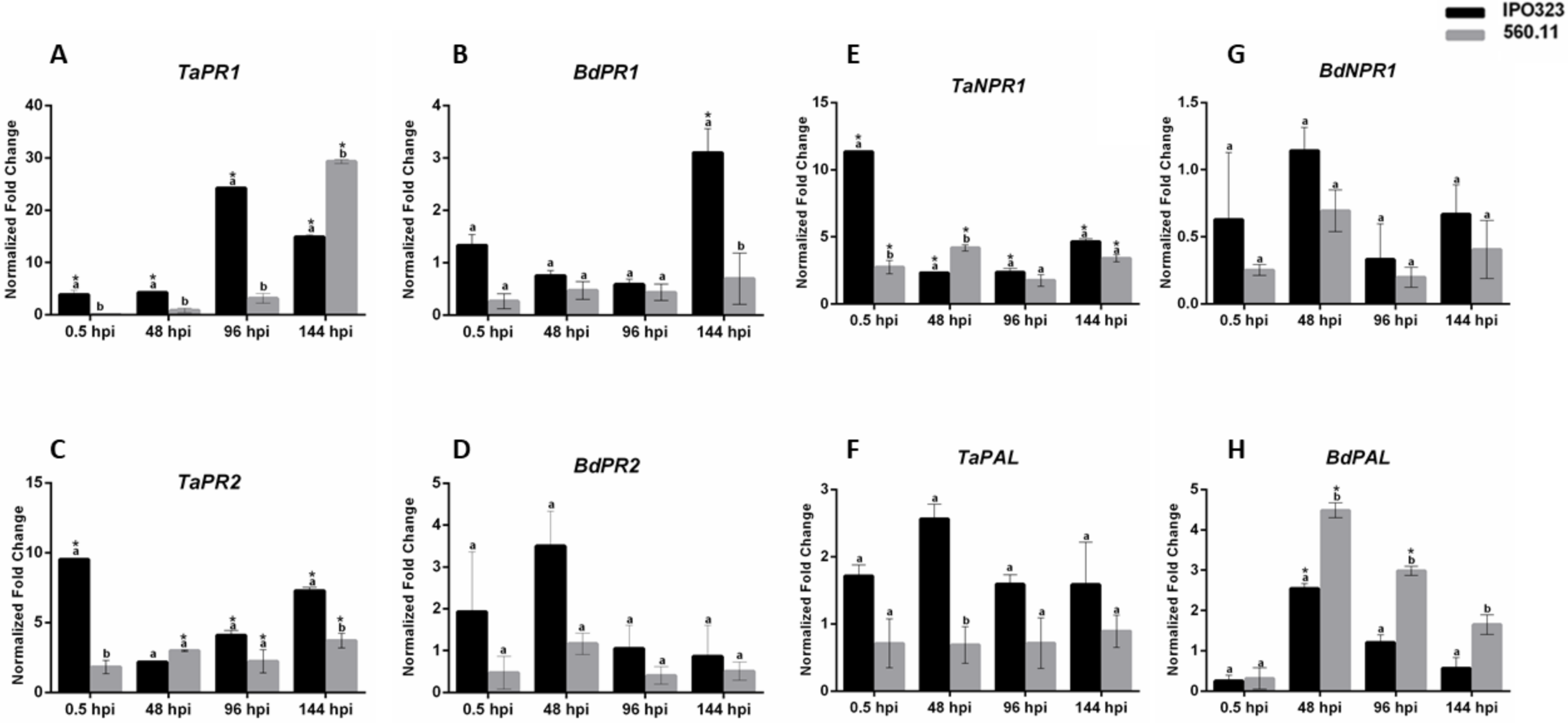

IPO323
560.11
A
TaPR1
B
BdPR1
E
TaNPR1
G
BdNPR1
C
TaPR2
D
BdPR2
F
TaPAL
H
BdPAL
Normalized Fold Change
0.5 hpi
48 hpi
96 hpi
144 hpi

Fig. 6.

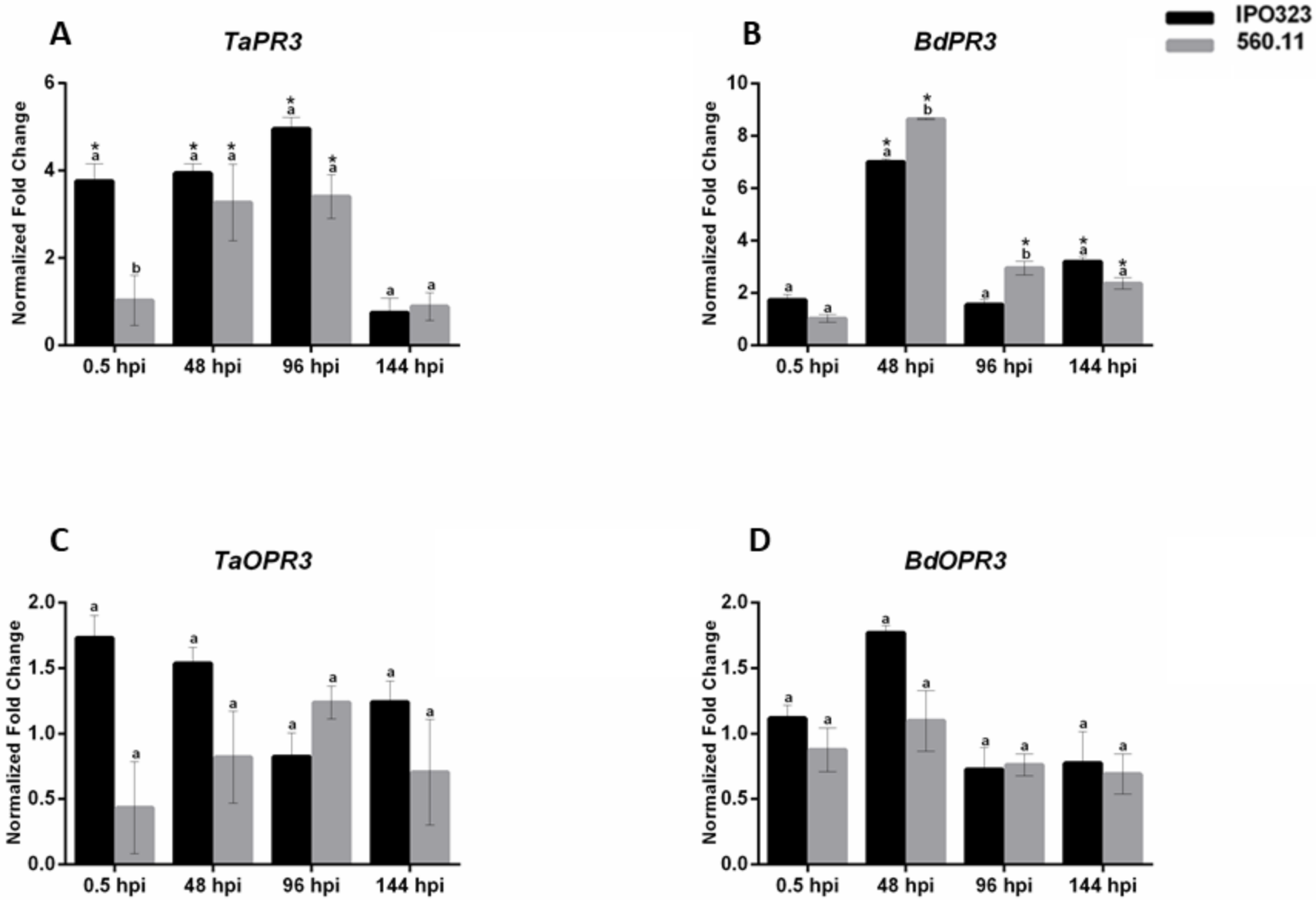

A
TaPR3
Normalized Fold Change
0.5 hpi
48 hpi
96 hpi
144 hpi
B
BdPR3
IPO323
560.11
C
TaOPR3
D
BdOPR3

Fig. 7.

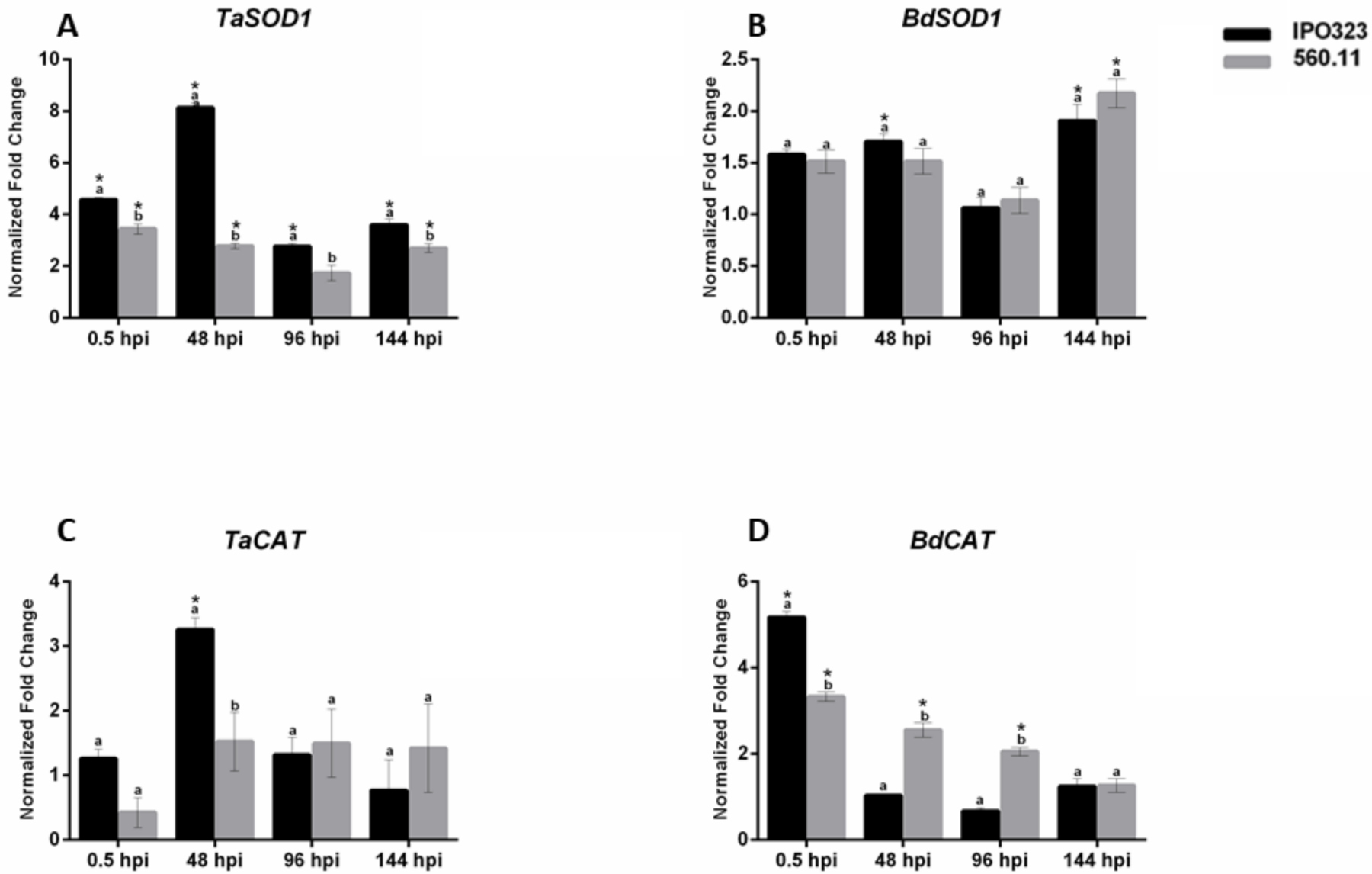
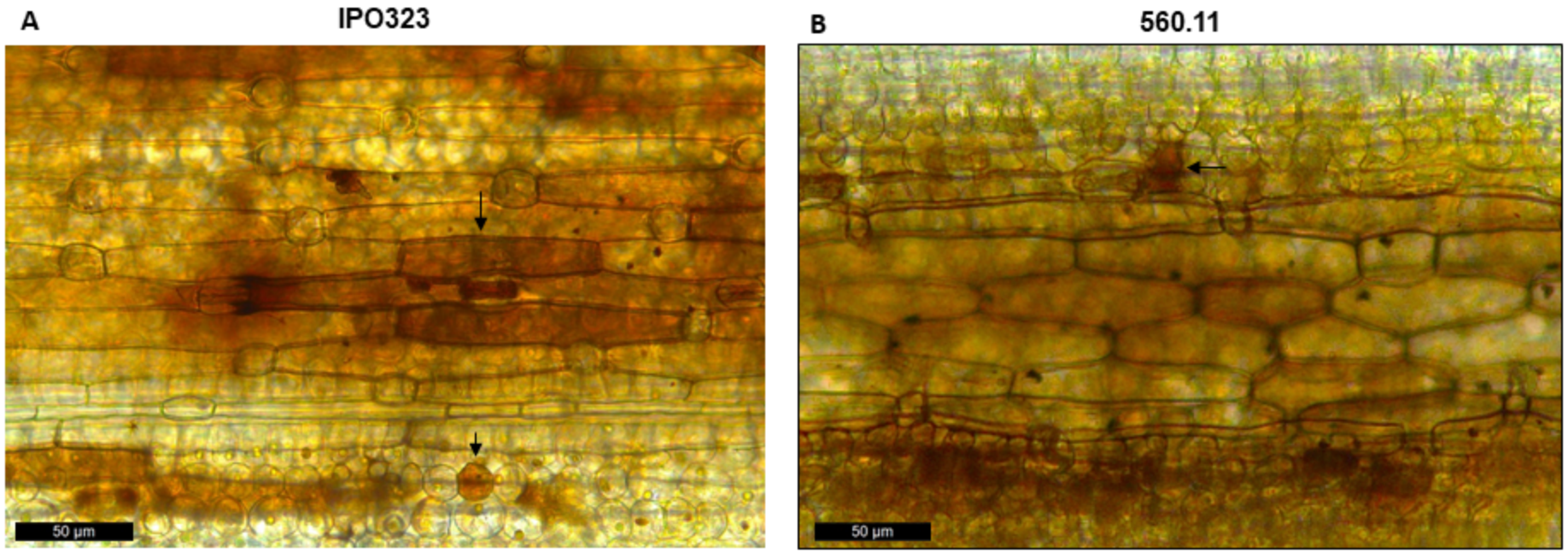
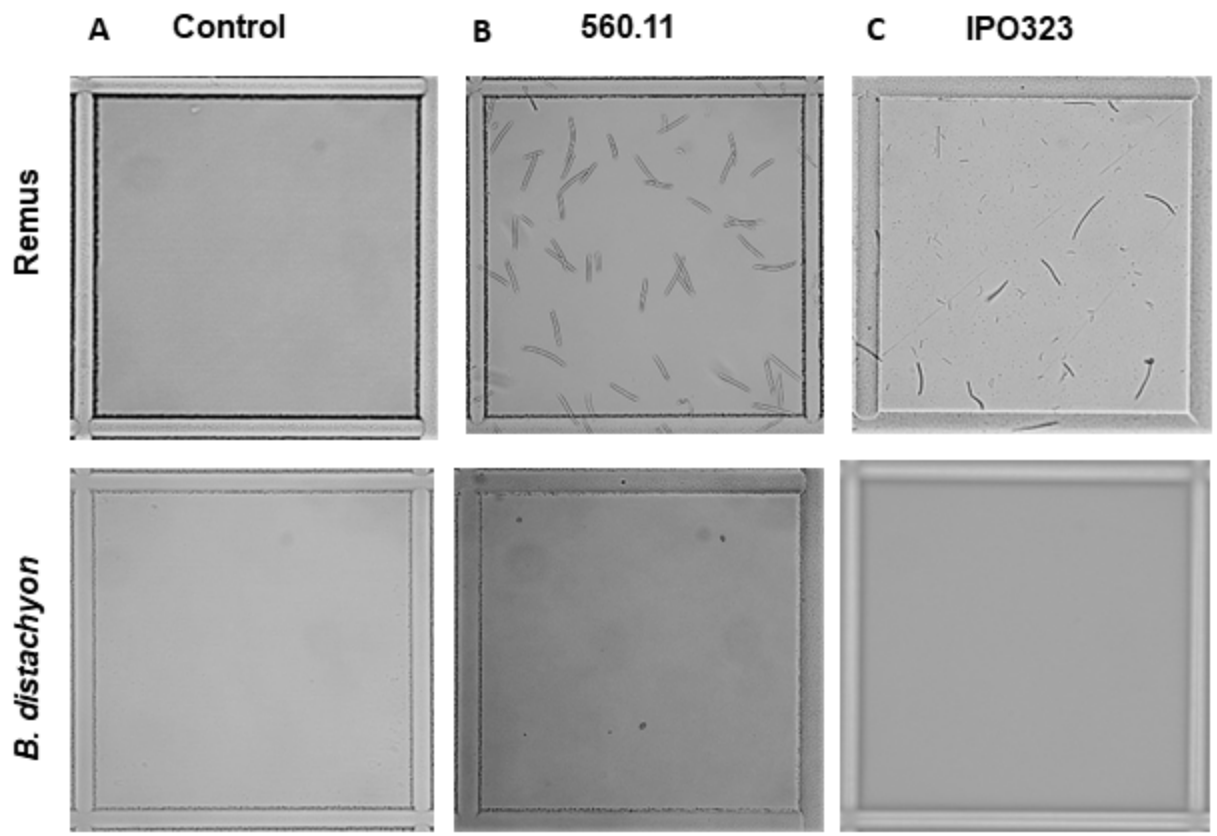

A
TaSOD1
Normalized Fold Change
0.5 hpi
48 hpi
96 hpi
144 hpi
B
BdSOD1
IPO323
560.11
C
TaCAT
D
BdCAT

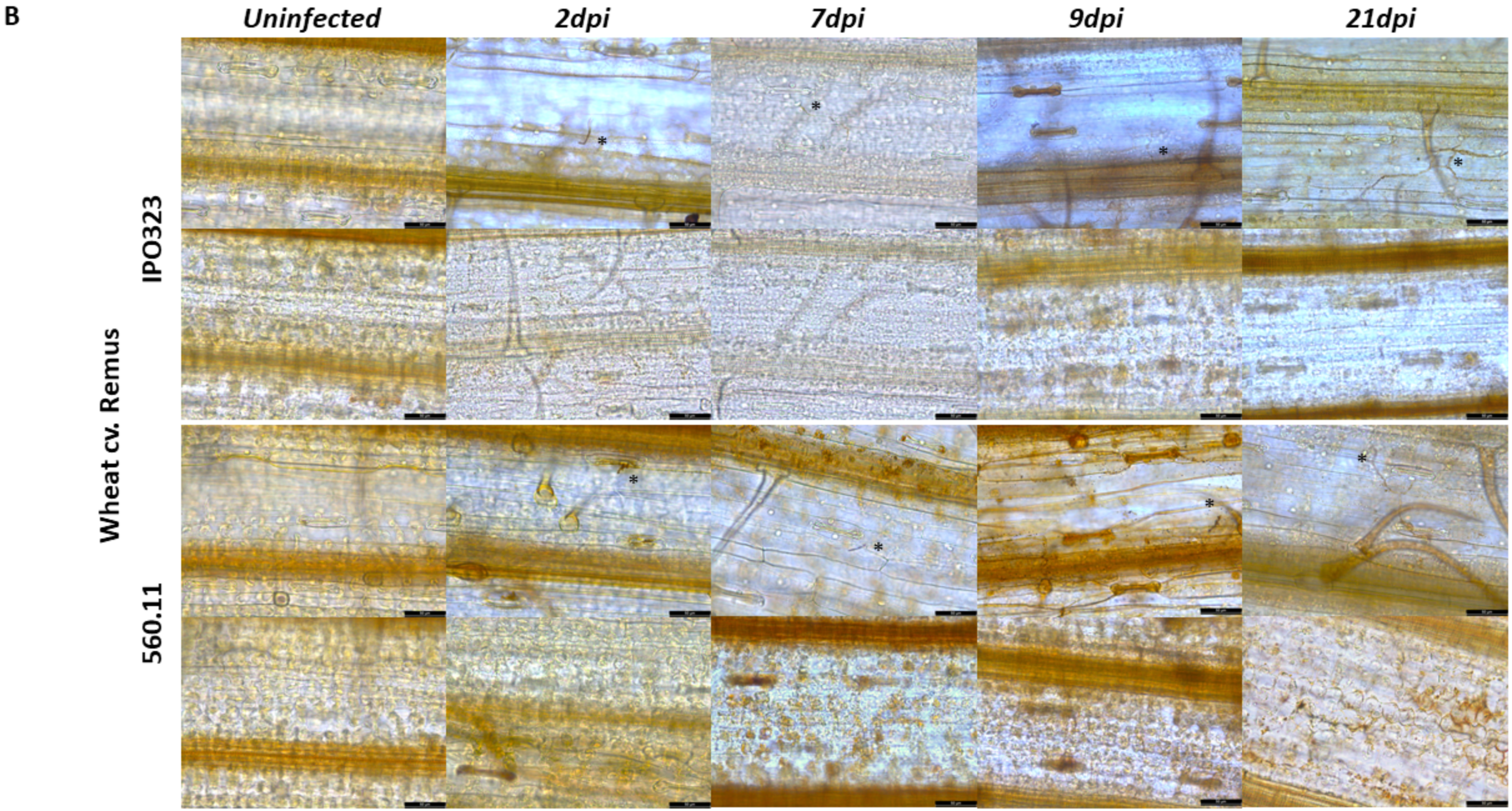
Fig. 8.

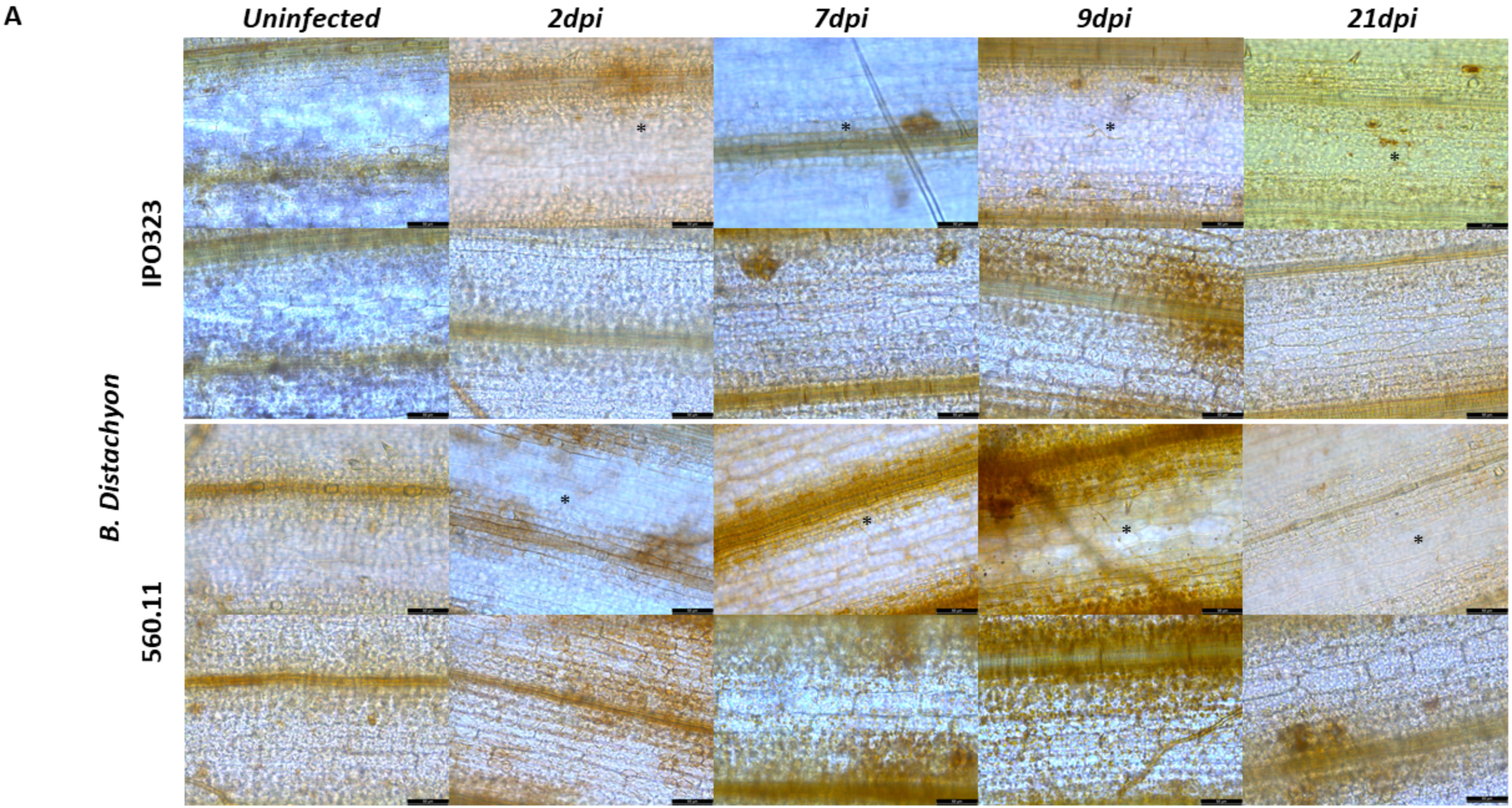


Supp. Fig. 1

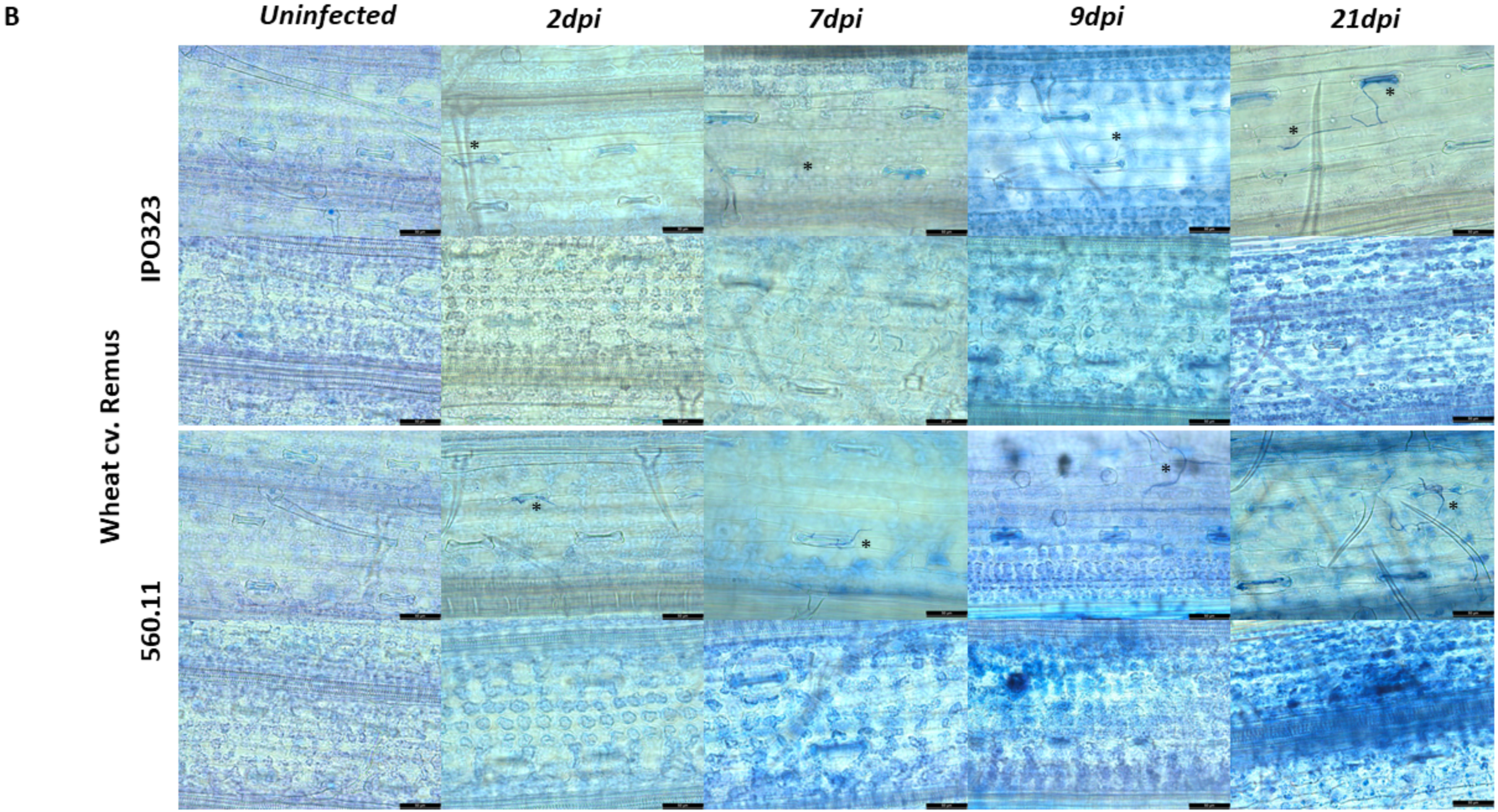


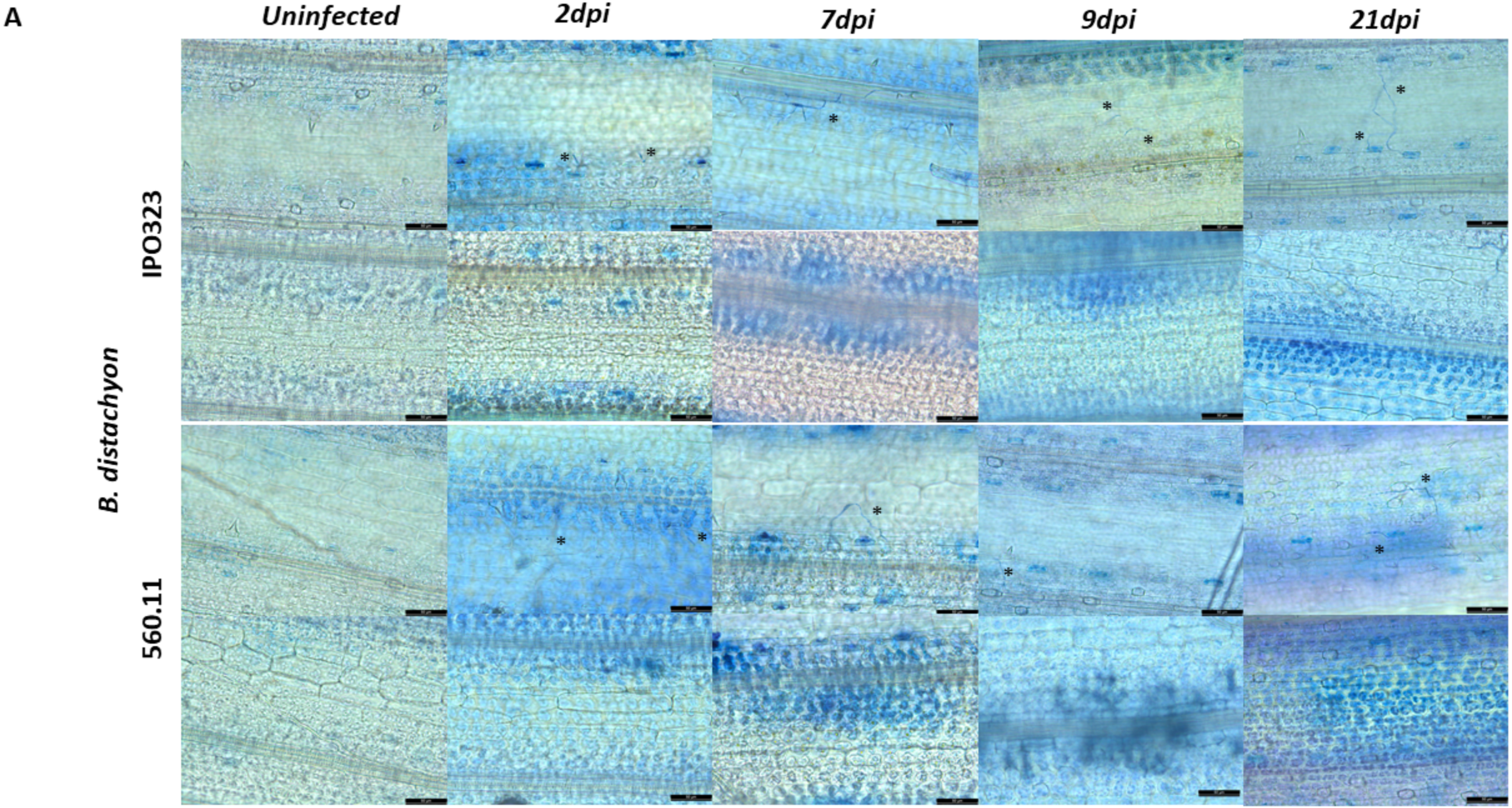
Supp. Fig. 2.


B
Uninfected
2dpi
7dpi
9dpi
21dpi
Wheat cv. Remus
IPO323
560.11

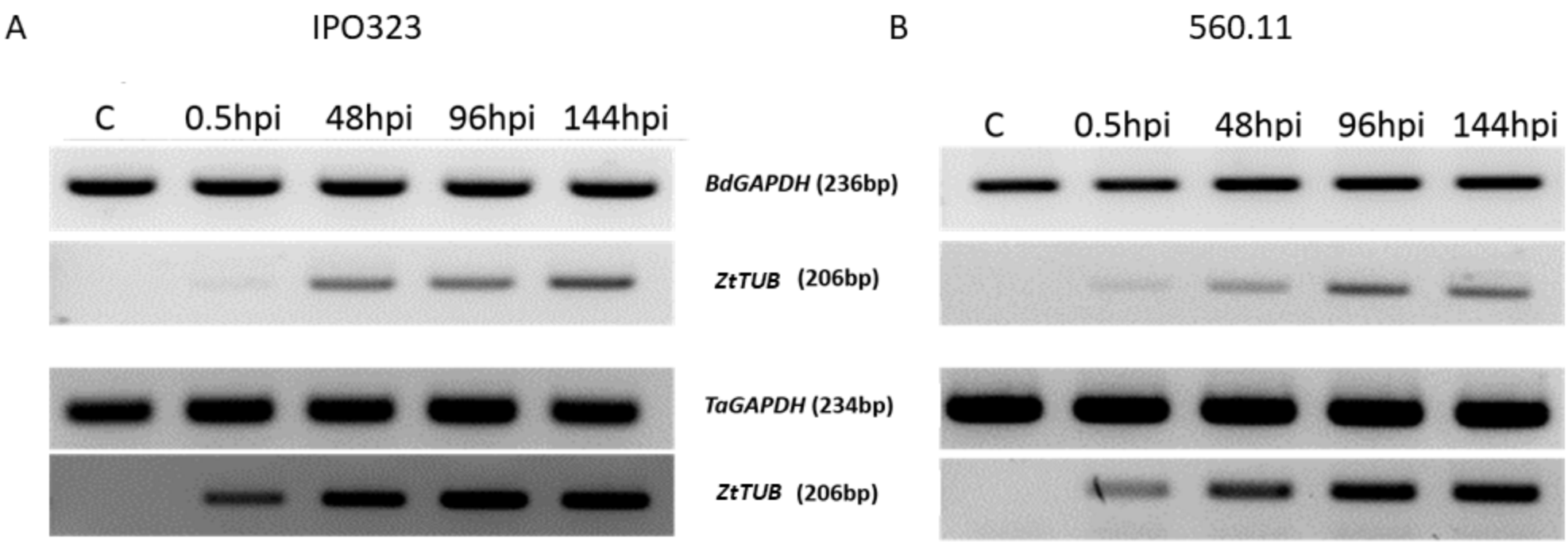

A
Uninfected
2dpi
7dpi
9dpi
21dpi
B. Distachyon
IPO323
560.11

Supp. Fig. 3


B
Uninfected
2dpi
7dpi
9dpi
21dpi
Wheat cv. Remus
IPO323
560.11


A
Uninfected
2dpi
7dpi
9dpi
21dpi
B. distachyon
IPO323
560.11

Supp. Fig. 4


A
IPO323
C
0.5hpi
48hpi
96hpi
144hpi
BdGAPDH (236bp)
ZtTUB (206bp)
TaGAPDH (234bp)
ZtTUB (206bp)
B
560.11
C
0.5hpi
48hpi
96hpi
144hpi

Supp. Fig. 5.

Table 1. Primers and references used for the analysis of transcript profiles from *Z. tritici* infected leaves.

Gene	Forward Primer	Reverse Primer	Study
TaPR1	CAATAACCTCGGCGTCTTCATCAC	TTATTTACTCGCTCGGTCCCTCTG	Casassola *et al* 2015
TaGAPDH	GGCCGGGATTGCTCTGAACG	TGGTGCTGTGCATGTGACGG	Guo *et al* 2011
Ta-ATub	ATCTCCAACTCCACCAGTGTCG	TCATCGCCCTCATCACCGTC	Ding *et al* 2016
TaCAT	CCATGAGATCAAGGCCATCT	ATCTTACATGCTCGGCTTGG	Sheoran *et al* 2015
TaMnSOD	CAGAGGGTGCTGCTTTACAA	GGTCACAAGAGGGTCCTGAT	Sheoran *et al* 2015
TaPAL	CAAGATGGTCGAGGCTTACC	CGAAGTCGATCATGAAGCAA	Casassola *et al* 2015
TaOPR3	TCGCCCTTCATGGACTACATG	TAGAGGATGCCGTGGTCGTT	This study
TaPR3	CCTCCATTATCTCGCAGTCGCTC	CGCCGTAGTTGTAGAACCCCTTG	Ding *et al* 2016
TaPR2	CGCCAACGTGTACCCCTACTT	TCTCGGAAATCACCACCTTCAC	Ding *et al* 2016
BdTUA6	ACCAACCTTGTGCCCTATCC	GGGCACCAGTCAACAAACTG	Hong *et al* 2008
BdPAL	ATTCAGGCTATCCTTGCTGAGG	AGGAGCTTCCTTCCAAGATGTG	Gill *et al* 2015
BdNPR1	AGCTTCAACTCGACCAGCAT	CGATCACCACATCATTGAGC	Kouzai *et al* 2016
BdPR2	CATCAACTCCATGCGGATCTAC	GGCGATGTACTTGATGTTGACC	Gill *et al* 2015
BdGADPH	TTGCTCTCCAGAGCGATGAC	CTCCACGACATAATCGGCAC	Hong *et al* 2008
BdPR1	AGCTCTGGCATCATCAGCATCC	CGTTGTGTGGGTCCAGGAAATC	Mandadi & Scholthof 2012
BdPR-3	GCTCGGCTGATTGTTCAACACG	TTGCCCGAACCACAAATATGCC	Mandadi & Scholthof 2012
BdOPR3	ACCCATTTCTTCTCGAATGATCCC	ACACGTGCAAGTACGGAAAGAAAG	Mandadi & Scholthof 2012
BdCAT	GACTATTTCCCGTCCAGGTTC	CCTTCTCGATCACCATCTTCTC	Wang *et al* 2017
BdMnSOD	GCGCAATCAAGTTCAACGG	TCACCACCACCCTCACTG	Glover *et al* 2014
ZtTUB	ATCTACCGCGGAAAGGTGTCCA	TGGTCACCGACACGCTTAAAGAG	Rudd *et al* 2015