Physiol Mol Biol Plants (January–February 2018) 24(1):51–59
https://doi.org/10.1007/s12298-017-0486-z

RESEARCH ARTICLE

Alternaria brassicae interactions with the model Brassicaceae member *Arabidopsis thaliana* closely resembles those with Mustard (*Brassica juncea*)

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Received: 28 March 2017 / Revised: 4 October 2017 / Accepted: 7 November 2017 / Published online: 16 November 2017

Abstract Alternaria leaf blight, a disease of oilseed Brassicas is caused by a necrotrophic phytopathogenic fungus *Alternaria brassicae*. The details of its pathogenesis and defence responses elicited in the host upon infection have not been thoroughly investigated. Here, *Arabidopsis* accession Gre-0 was identified to be highly susceptible to *A. brassicae*. A comparative histopathological analysis for disease progression and plant responses to *A. brassicae* in *Arabidopsis* and *Brassica juncea* revealed significant similarities between the two compatible pathosystems. Interestingly, in both the compatible hosts, ROS accumulation, cell death and callose deposition correlated with the development of the disease. Based on our results we propose that *Arabidopsis-Alternaria brassicae* can be an apt model pathosystem since it emulates the dynamics of the pathogen interaction with its natural host- Brassicas. The existing genetic diversity in *Arabidopsis* can be a starting point to screen for variation in responses to Alternaria leaf blight. Furthermore, several tools available for *Arabidopsis* can facilitate the dissection of genetic and molecular basis of resistance.

Keywords *Alternaria brassicae* · *Brassicae juncea* · *Arabidopsis* Gre-0 · Defense · Hydrogen peroxide (H_2O_2) · Cell death

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Introduction

Brassica juncea is one of the important oilseed crop grown extensively in the Indian sub-continent, Europe, Australia, and Canada. The yield potential of *B. juncea* is constrained by many fungal diseases among which Alternaria blight poses a major challenge worldwide. The disease lowers the grain yield and under severe incidence of infection even the oil quality is affected (Saharan et al. 2016). Alternaria leaf blight of oilseed mustard is caused by a seed borne necrotrophic fungal pathogen of *Alternaria* spp. belonging to the family Pleosporaceae. Both *A. brassicicola* and *A. brassicae* have been reported to cause the disease, yet *A. brassicae* is known to be more virulent, causing major damage to oilseed Brassicas, while *A. brassicicola* an opportunist co-inhabits the infected site. The fungus is capable of infecting the aerial plant parts at all the stages of growth. The disease symptoms are manifested as black/brown necrotic lesions on the leaves, esp. older ones, stem and siliques. The characteristic feature of these lesions is the presence of concentric rings which gives it a 'bull's eye' appearance and are seldom surrounded by a chlorotic halo (Conn et al. 1990; Sharma et al. 2002). Commercial cultivars of *B. juncea* do not possess a high level of tolerance towards *A. brassicae*. So far, histopathological studies on *A. brassicae-Brassica* interactions have focused mainly on the initial stages of infection esp. spore germination and penetration processes with little emphasis on host responses upon infection, thus leading to a gap in understanding the pathogenesis of the fungus (Giri et al. 2013; Goyal et al. 2013; Sharma et al. 2014). *Arabidopsis* has been documented as a host for a broad range of pathogens including necrotrophic fungi. The available natural genetic diversity of *Arabidopsis* has been exploited to decipher the molecular mechanisms underlying

resistance against multiple phytopathogens. *Arabidopsis* has been used for studying plant interactions with *A. brassicicola*, and most of the accessions tested show an immune response towards the pathogen (Mukherjee et al. 2009). Since, *A. brassicae* is the dominating pathogen of oilseed mustard it is imperative to investigate in depth the plant-pathogen interaction for *A. brassicae*. So far, there is no report of *Arabidopsis* being used to study *A. brassicae* pathogenesis and disease responses.

We have identified an *Arabidopsis* accession, Gre-0 (Greenville, USA), which is highly susceptible to *A. brassicae* and thus have attempted to develop *Arabidopsis* as a model host for studying plant interactions. Here, we present a comparative study of the pathobiology of *A. brassicae* on its natural host *B. juncea* and the model host *A. thaliana.* The fungal pathogenesis, disease development and host responses were quantitatively assessed both macroscopically and microscopically. Our results reveal significant parallels between the *B. juncea and Arabidopsis* pathosystems, thus making *Arabidopsis* a promising model to investigate the mechanism of *A. brassicae* pathogenesis and host resistance.

Materials and methods

Fungal strains and growth conditions

The *Alternaria brassicae* isolate J_3 used in this study was earlier isolated from the infected *B. juncea* leaf from Delhi fields and single spore culture was established (Kumar 2009). For routine culture, the isolate was maintained on radish extract medium at 20–22 °C under 12 h light/12 h dark cycle.

Plant material and growth conditions

Selfed seeds of *B. juncea* cv. Varuna were planted into 9 inch plastic pots filled with autoclaved 1:1 mix of garden soil and soilrite and grown in PGV36 at 22 °C 14 h light/10 h dark cycle and 60–80% relative humidity (RH). *Arabidopsis* accession Greenville-0 (Gre-0) seeds obtained from Nottingham Arabidopsis Stock Centre (NASC) were grown in 3 inch plastic pots containing autoclaved artificial soil mix (1 soilrite: 1 vermiculite: 1 perlite), for 5–6 weeks at 22 °C in growth chambers programmed for a 14-h-light/10-h-dark cycle.

Infection assays

Conidial suspensions for drop inoculation were prepared from 15-days old sporulating plates. To collect conidia, the plates were flooded with sterile water, gently scrapped and the resulting suspension was passed through three layers of muslin cloth. The concentration of spores was adjusted to 3×10^3 conidia ml^{-1}. Leaves of one month old Varuna plants were inoculated with four to six droplets of 5 µl of *A. brassicae* conidial suspension. For *Arabidopsis*, leaves of 6 week old plants were inoculated with four droplets (5 µl) of spore suspension. During the first 3 days post inoculation (dpi), the RH was maintained at 90% and thereafter humidity was reduced to 70% till symptoms developed i.e. 7 days of post infection (dpi). For histopathological studies, the tissue samples were collected at 6, 24, 48, 72 and 96 hpi.

Histochemical assay and microscopy

A total of at least 36 infection sites for *B. juncea* and Gre-0 each, from different biological replicates were examined for recording various host responses upon fungal penetration. For initial fungal growth, like spore germination, hyphal structure and spread on the leaf surface, the infected leaf samples were decolorized in ethanol: acetic acid (3:1 vol/vol) and stained with 0.25% coomassie blue solution (R250) (Qualigens) for 1 min, washed in water and mounted in 50% glycerol for observation (Göllner et al. 2008). For detection of H_2O_2, endogenous peroxidase-dependent in situ histochemical staining employing 3-3′-Diaminobenzidine (DAB) (Sigma-D5637-1G) was used (Thordal-Christensen et al. 1997). To visualize fungal structures like ALS (appressoria like swollen, globular structures), and dead plant cells, infected leaf samples were harvested and boiled for 2 min in lactophenol trypan blue. Stained leaves were then cleared in chloral hydrate (1 g/ml in water) for 24 h at room temperature. Cleared leaves were mounted in 50% glycerol for microscopic observation (Bartsch et al. 2006). To detect callose deposition, infected leaves were cleared in ethanol: acetic acid solution (3:1 vol/vol), stained for 30 min in 150 mM K_2HPO_4 (pH 9.5) containing 0.01% aniline blue (Sigma: 415049-25G) in dark, and examined for fluorescence using Olympus BX51 microscope (Olympus Corporation, Japan) DAPI filters (λexc 340–380 nm, barrier filter 435–485 nm) (Vogel and Somerville 2000).

The fungal penetration events triggering defense responses (H_2O_2 accumulation, cell death and callose deposition) were calculated using the formula:

$$\text{Percentage of penetration events triggering host responses (ROS, cell death and callose deposition)} = \frac{\text{No. of penetration events associated with defense respose (ROS, cell death and callose deposition)}}{\text{Total no. of penetration events}} \times 100$$

Quantitative reverse transcriptase (qRT)-PCR assays

Total RNA was extracted from the leaves collected from six plants in each experiment using RNeasy plant mini kit according to manufacturer's recommendation (Qiagen, Gaithersburg, MD, USA). First-strand cDNA was synthesized from 1 µg of total RNA using MMLV Reverse Transcriptase 1st-Strand cDNA Synthesis Kit (Epicentre Biotechnologies, Madison, USA) as per manufacturer's protocol. PCRs were carried out using the standard setting in a QuantStudio 6 Flex Real-Time PCR System (ThermoFisher Scientific, Waltham, MA, USA) with 2X SYBR Select Master Mix (ThermoFisher Scientific, Waltham, MA, USA). Gene expression levels were standardized using UBC8 (At5g41700) as internal control. Relative expression levels were calculated and represented (Willems et al. 2008).

Results

Leaves of *Arabidopsis* accession Gre-0 and *B. juncea* cv. Varuna were challenged with *A. brassicae* spore (3×10^3 spores ml^{-1}) by drop inoculations. Under conducive conditions macroscopic disease symptoms in both the hosts were visible as early as 72 hpi as water-soaked lesions and by 7 dpi prominent nectroic lesion were observed (Fig. 1a–c). A comparative disease progression and induced plant defense responses for the Gre-0 and Varuna were studied.

Microscopic studies of *Arabidopsis*-*A. brassicae* interactions

The conidia were able to germinate on the *Arabidopsis* leaf surface by 12–24 hpi. Microscopic examination of cleared inoculated leaves revealed initiation of spore germination, within 6 hpi, as a small swelling either at the ends of the conidia or from intercalary spores (Fig. 1d, e). By 24 hpi almost 99% conidial germination was observed on both *Arabidopsis* and Varuna leaf with multiple branched hyphae emerging from conidiospore (Fig. 1f, Table 1).

Post spore germination fungal entry into the plant is the most critical step towards successful infection. At 24 hpi, branched -primary and -secondary hyphae with several penetration attempts were observed in both the hosts. The successful penetration events increased over time from 341 sites at 24 hpi to 884 sites at 48 hpi in Gre-0 and a similar trend was observed for Varuna (Table 1). Similarly, for both the pathosystems more than 90% of the fungal penetration attempts were recorded through stomatal openings although some hyphae were seen growing over the stomata without entering them (Fig. 1h, i, k). The fungus also penetrated directly at the junctions between epidermal cells albeit at a low frequency ($\sim$ 8.3%) (Table 1; Fig. 1l). In several instances, the hyphal apices appressed to leaf surface were swollen, similar to simple appressoria like structures, and stained more intensely with trypan blue than the rest of the hyphae. In both the host plant, fungal penetration via stomata was mostly associated with the formation of Appressoria Like swollen, globular Structures (ALS) at the hyphal apices (Fig. 1g). In the colonized Varuna leaf tissue, hyphal growth within the intercellular spaces of the mesophyll cells was observed at 96 hpi (Fig. 1m, n), which was accompanied by cell death and tissue collapse (Fig. 1m′). Besides fungal proliferation within the leaf tissue, extensive mycelial growth on the leaf surface of Gre-0 and Varuna was also observed (Fig. 1j). By 6 dpi, re-emergence of fungal hyphae could be seen through natural opening like stomata and leaf margins in Varuna (Fig. 1o). Intercellular fungal growth and re-emergence of the fungus in Gre-0 could not be observed even beyond 7 dpi.

Disease progression during *A. brassicae* infection is accompanied by ROS, cell death and callose production

To further characterize and compare the defense response of the two compatible hosts to *A. brassicae* infection, penetration associated: (i) induction of H_2O_2 with 3,3′-diaminobenzidine (DAB) staining, (ii) host cell death with

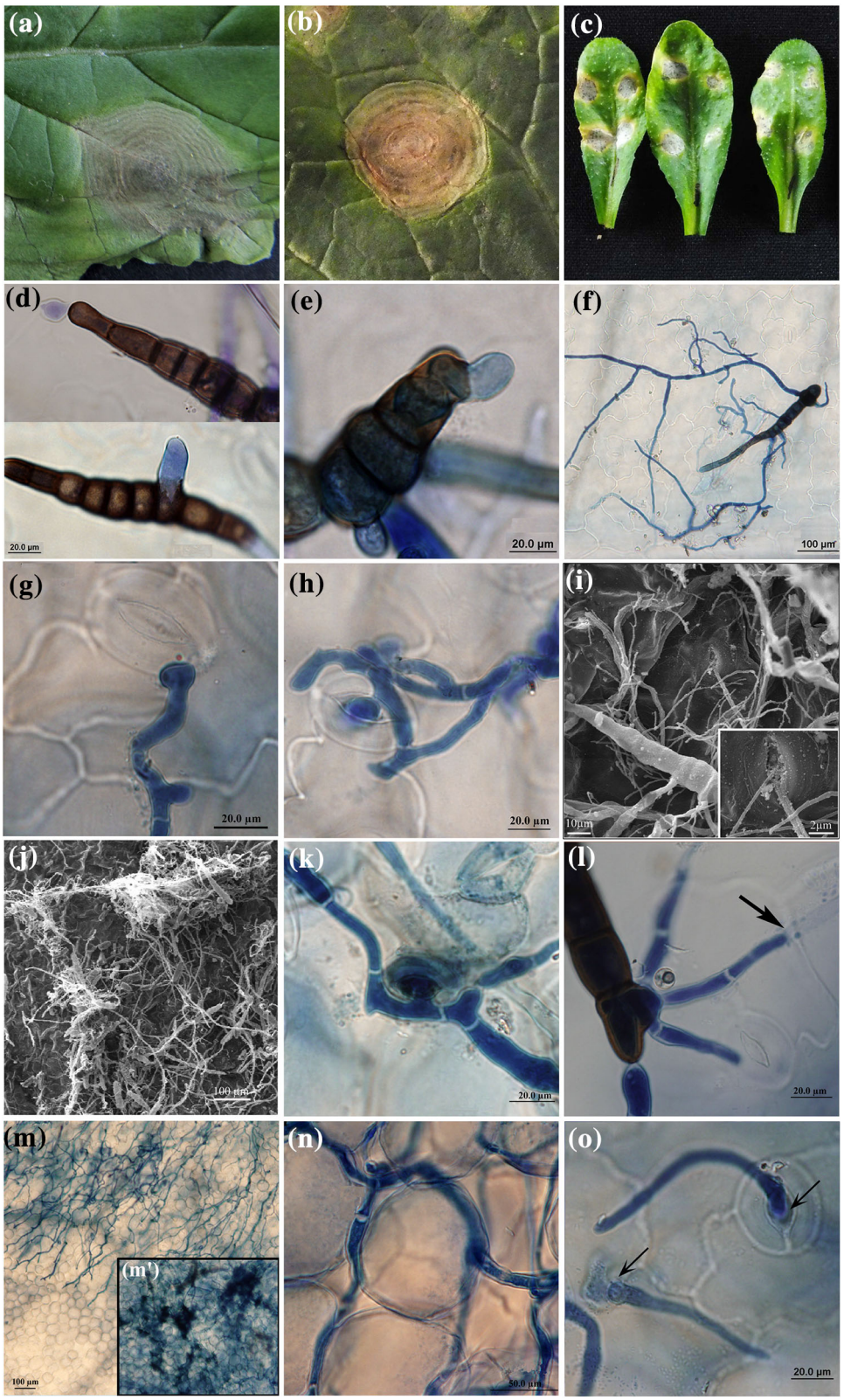

Fig. 1 Progression of *Alternaria brassicae* infection on *B. juncea* cv. Varuna and *A. thaliana* accession Gre-0. Comparative analysis of macroscopic infection phenotypes 7 dpi on Varuna leaf upon artificial inoculation (**a**), under field conditions (**b**), and upon artificial inoculation on Gre-0 leaf surface (**c**). Initiation of spore germination at 6 hpi from terminal and intercalary cells of conidiophores on Varuna (**d**) and Gre-0 (**e**). Conidia with multiple branched hyphae were observed at 24 hpi on the surface of Varuna (**f**). Hyphae forming ALS near the stomatal opening on Varuna leaf surface (**g**). Penetration via stomata on Varuna leaf surface (**h**, **i**), Gre-0 (**k**) at 48 hpi. SEM image showing hyphal growth on the Varuna leaf surface at 72 hpi (**j**). Direct fungal penetration through epidermal cell junctions on Gre-0 leaf surface at 48 hpi (**l**). Fungal invasion into the healthy mesophyll cells at 96 hpi (**m**) and darkly stained dead cells at the site of inoculation at 96 hpi (**m′**). Ramified hyphal growth between the two mesophyll cells at 96 hpi (**n**). Re-emergence of young fungal hyphae through the stomata of Varuna 6 dpi (**o**)

lactophenol trypan blue stain, and (iii) deposition of callose using aniline blue were monitored.

Reactive oxygen species (ROS) and callose deposition in the cell wall plays a major role in plant-pathogen interaction. ROS formation was assessed at the site of

Table 1 Germination and infection processes of *Alternaria brassicae* isolate J_3 on Varuna and Gre-0

Plant	No. of spores germinated/total no. of spores		Total penetration sites[d]		Mode of penetration[e]	
	6 hpi[a]	24 hpi[c]	24 hpi	48 hpi	Stomata	Cell junction
Gre-0	437/562 (77.8)[b]	2883/2892 (99.6)	341	884	1131 (92.3)	94 (8.3)
Varuna	260/318 (81.7)	2235/2253 (99.2)	577	1443	1875 (92.8)	145 (7.2)

For Gre-0: A total of 40 infection sites were scored from three independent experiments

For Varuna: A total of 36 infection sites were scored from two independent experiments

[a]Total spores represent spore count from three leaves each collected from two experiments

[b]Numbers within parentheses indicate the percentage of spore germination

[c]Total spores represent the spore count from all the leaves collected for host responses (H_2O_2, cell death and callose deposition). The experiments were repeated three times with similar results

[d]Total penetration site represent the sum of all the penetration sites observed in experiments evaluating various host responses upon infection

[e]Numbers within parentheses indicate the percentage of penetration sites through stomata and cell junction

Table 2 Quantitative analysis of H_2O_2 accumulation, cell death and callose deposition at the penetration sites upon *A. brassicae* infection

Host	Hpi	$H_2O_2^a$	Cell death[b]	Callose[c]
Gre-0	24	27.5–32.3	0–9.5	6.2–8.8
	48	29.5–38.8	6.8–10.1	17.3–20.7
Varuna	24	26.5–26.6	18.2–19.3	16.6–18.3
	48	56.4–57.8	50.4–52.1	46.6–48.3

For Gre-0: Results compiled from three independent experiments per time point

For Varuna: Results compiled from two independent experiments per time point

[a, b, c]Range of % penetration sites associated with H_2O_2 production, cell death and callose deposition respectively

infection in a temporal fashion. At 24 hpi, only 30.5% of penetration sites had an associated DAB staining in *Arabidopsis* (Table 2). The staining was observed not only in the stomatal guard cells at the site of penetration but also in the adjoining epidermal cells. Additionally, H_2O_2 production could be detected in a discrete cluster of mesophyll cells beneath the invading hyphae (Fig. 2a, b) which spread to larger patches of mesophyll and epidermal cells by 48, 72 and 96 hpi (Fig. 3a). ROS was also detected in the growing tip of the fungal hyphae (non-penetrating) and in penetrating ALS (24 hpi) (Fig. 2h–j).

Traditionally, the formation of ROS in response to pathogen invasion is associated with hypersensitive response (HR) and programmed cell death. The kinetics of cell death and its extent was assessed during the course of *A. brassicae* pathogenesis in both the hosts by Trypan blue staining. Only a small fraction of penetration sites were associated with cell death (5.6% in Gre-0) early during the host invasion which increased at 48 hpi (Table 2). Interestingly, during the initial phase of fungal ingress, the cell death was mostly confined to a few mesophyll cells present below the penetrating stomata while the guard cells or the adjoining epidermal cells did not show any signs of cell death despite the accumulation of ROS (Fig. 2a′, b′). These trends of H_2O_2 accumulation and associated cell death were also observed in the natural host Varuna. In Varuna, with the passage of time (96 hpi), the zone of dead cells extended over a larger area coincident with the ramified growth of the fungal hyphae (Figs. 2c, 3b). Microscopically it was observed that the leading edge of the invading hyphae grew into the intercellular space between the mesophyll cells without causing any visible cell death (Figs. 1n, 2f) although extensive cell death was seen in the already colonized zone (Fig. 2d, e). As expected for a necrotroph, the extent of cell death increased with the progression of the fungal growth and by 7 dpi, cell death had progressed in the veins of the infected brassica leaves as well (Fig. 2g).

Additionally, in the infected Gre-0 and Varuna leaves, callose was predominantly deposited in the epidermal cells surrounding the site of fungal penetration at 24 hpi, which extended to larger patches of cells as the fungal growth extended (Fig. 3c; Table 2).

Defense related gene expression in response to *A. brassicae* in Gre-0

Previous studies in the susceptible host *B. juncea* have shown that the transcript level of the defense related gene PR1 is highly induced in response to *A. brassicae* and *A. brassicicola* infection (Mazumder et al. 2013; Nayanakantha et al. 2016). To compare if the compatible *Arabidopsis* accession also elicited similar defense responses, the transcription level of three representative

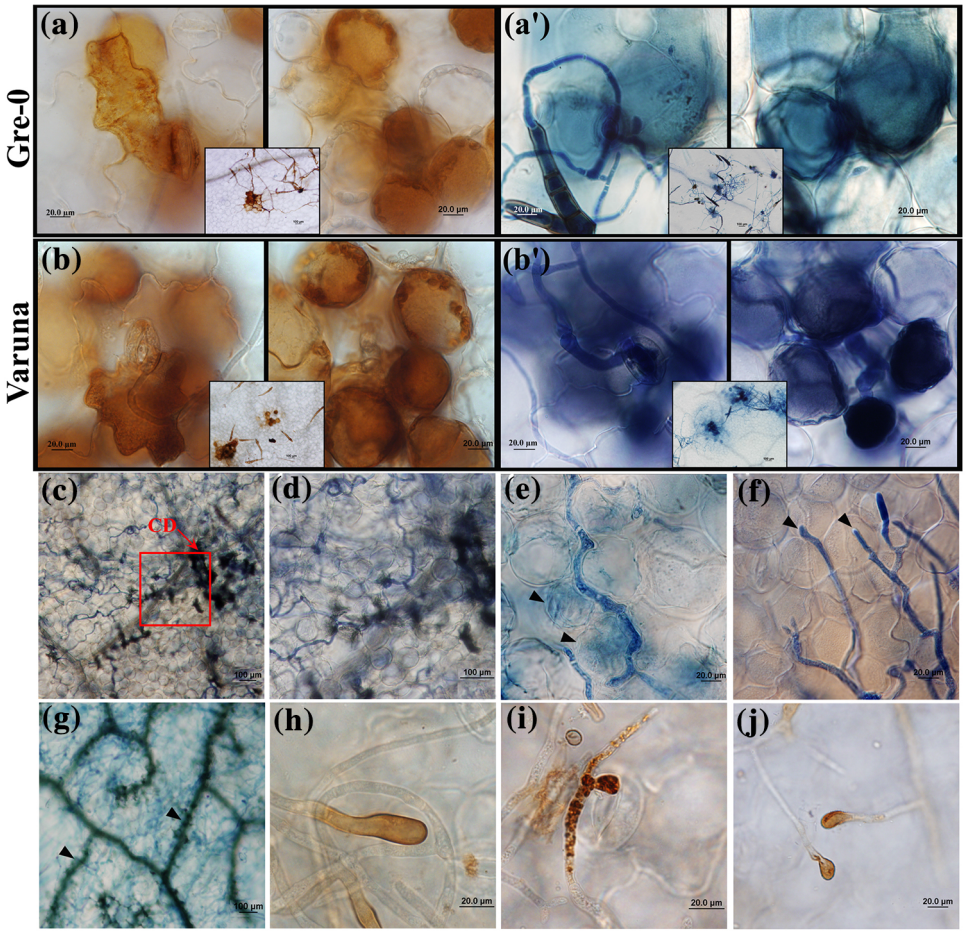

Fig. 2 Cellular responses to the *Alternaria brassicae* infection on Gre-0 and Varuna. Representative images of infected Gre-0 and Varuna leaves stained with 3,3'-diaminobenzidine (DAB) to detect hydrogen peroxide (H_2O_2) accumulation (reddish brown) and trypan blue to detect cell death (blue). At 24 hpi, H_2O_2 accumulates in epidermal and mesophyll cells at the site of fungal penetration, whereas cell death occurs only in mesophyll both in Gre-0 (**a**, **a**′) and Varuna (**b, b**′). The insets in **a**, **b**, **a**′ and **b**′ shows localization of DAB and cell death respectively around the penetrated sites in Gre-0 and Varuna at a lower magnification. Infected Varuna leaves showing cell death (CD) following invasion by the fungal hyphae into the healthy mesophyll cells-96 hpi (**c**, **d**). Slight coloration of the mesophyll cells (arrowheads) indicates the initiation of cell death around the fungus at 96 hpi (**e**). Leading edge of the invading hyphae grows into the intercellular space between the mesophyll cells without causing any visible cell death (arrowheads)-96 hpi (**f**). The extensive spread of cell death observed in Varuna leaf veins (arrowheads) at 7 dpi (**g**). H_2O_2 accumulation was observed at fungal hyphal tip and ALS on Varuna (**h**, **i**) and Gre-0 (**j**)

markers for JA/Ethylene pathway-PDF1.2, and Salicylic Acid pathway- PR1, and PR5 were evaluated for infection (Fig. 4). Similar to *B. juncea*, the marker for the SA pathway: PR1 showed a maximum induction, while the JA pathway marker (PDF1.2) showed a comparatively moderate induction.

Discussions

Compared to biotrophic phytopathogens, our understanding of the mechanisms of pathogenesis and host responses towards necrotrophic pathogens is still in infancy. This study is the first to define host–pathogen interaction involving *A. brassicae* and *A. thaliana*. Here, for the first time, we show that *Arabidopsis* accession Gre-0 (Greenville, USA) supports substantial *A. brassicae* growth. Furthermore, *A. brassicae* infection processes and the induced host responses are analogous between the model host Gre-0 and its natural host Varuna.

Unlike biotrophs, necrotrophs are believed to facilitate their entry into the host cells by degrading the host cell walls, thus relinquishing the need for developing specialized infection structures like appressoria (Kübicek et al. 2014). Although, appressoria like structures have been earlier reported for other *Alternaria* spp. e.g. *A. brassicicola*, *A. raphanin*, *A. alternata,* these fungi habitually penetrate directly through cell junctions (McRoberts and Lennard 1996). The appearance of unmelanised ALS associated with host penetration events in our study was

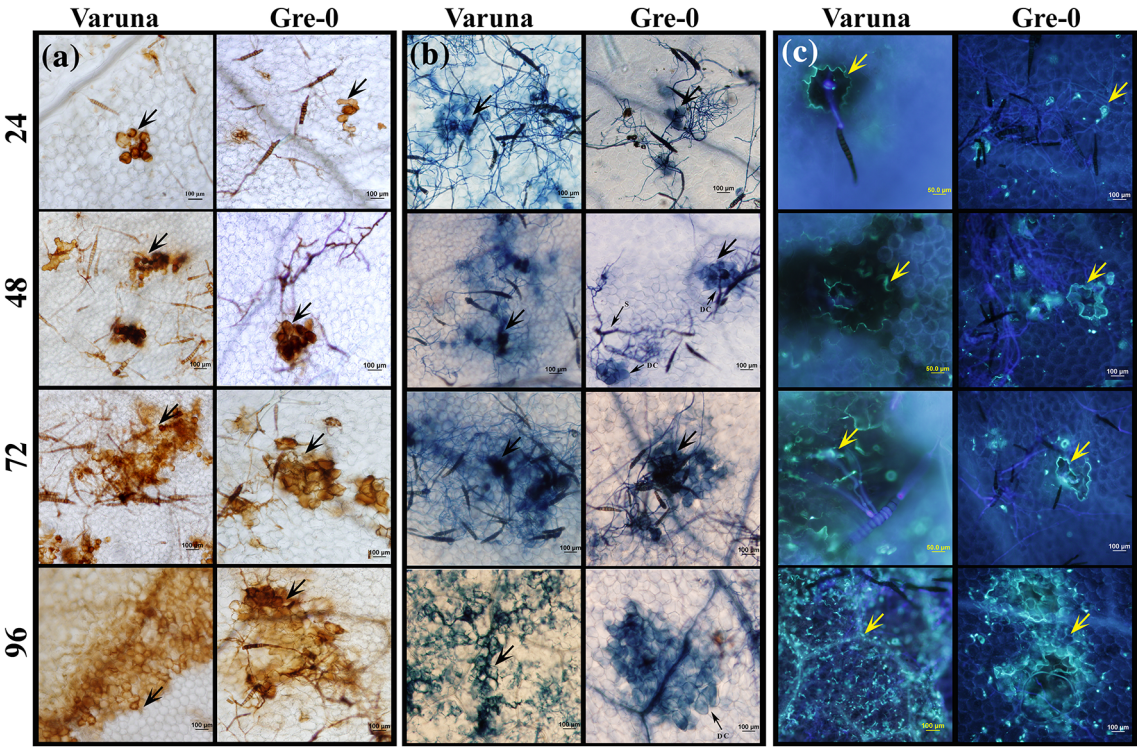

Fig. 3 Hydrogen peroxide accumulation, cell death and callose deposition in Gre-0 and Varuna in response to *A. brassicae* infection. Representative pictures of inoculated leaf samples collected at 24, 48, 72 and 96 hpi were assayed for H_2O_2 accumulation (arrow) (**a**); cell death (arrow) (**b**) and callose deposition (blue fluorescence, yellow arrow) (**c**). The production of H_2O_2, cell death and callose deposition are restricted to a few cells during the early phases. Their spread tightly correlates with spread of the disease symptoms

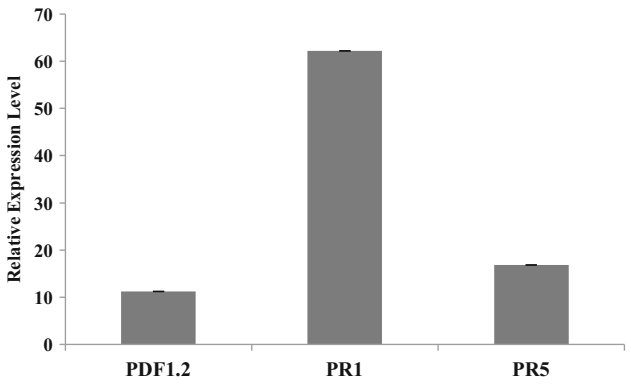

Fig. 4 Expression pattern of defense related genes: PDF1.2, PR1, and PR5, at 4 days post infection (dpi) in the compatible *Arabidopsis* accession Gre-0. The mean values of two independent biological replicates are shown

unforeseen since *A. brassicae* predominantly (> 90%) entered the host via stomata. Formation of similar unmelanised compound appressoria for host penetration has been recently reported for several other necrotrophs viz. *S. sclerotiorum*, and *B. cinerea* (Dai et al. 2006; van Kan 2005).

The host responses to infection, like accumulation of ROS, cell death, and callose deposition, are reciprocally correlated with disease resistance in many biotrophic and hemi-biotrophic pathosystem (Koch and Slusarenko 1990; O'Connell et al. 2004; Soylu 2004). On the contrary, the successful necrotrophic infections are associated with extensive cell death and ROS production during the establishment and disease progression phase (Laluk and Mengiste 2010; Williams et al. 2011). Production of ROS and the concomitant cell death during the disease progression has been reported earlier in mustard and *Arabidopsis* (Col-0) upon infection with *A. brassicicola*-a closely related *Alternaria* sp. (Meur et al. 2015; Su'udi et al. 2011). In our study, restricted H_2O_2 accumulation and cell death during the early phase of infection and a more robust H_2O_2 generation accompanied by spreading cell death in the post establishment phase was observed in both the compatible hosts and it positively correlated with the disease spread. Our observations suggest that for a successful compatible interaction, a fine balance must be reached between the suppression of ROS and cell death at the initial stage of infection followed by extensive triggering of the same, post establishment. Besides the host induced ROS, *A. brassicae* also produces ROS at the hyphal tips during the compatible interaction (Fig. 2i, j). A close association of ROS generation and fungal aggressiveness has been explicitly

recorded for various other necrotrophic fungi and bacteria. ROS-deficient mutants of *S. sclerotiorum* (*Ssnox1*), *B. cinerea* (*bcnoxA* and *bcnoxB*) and *A. alternata* (*AaNoxA*) have compromised virulence and have defective development (Kim et al. 2011; Segmuller et al. 2008; Takemoto et al. 2007).

It is generally believed that toxins secreted by necrotrophs induce host cell death ahead of extensive colonization which usually appears as a chlorotic halo surrounding the advancing necrotic zone (Glazebrook 2005). *A. brassicae* is known to secrete Destruxin B both in vitro and *in planta* that has phytotoxic properties (Parada et al. 2007). Surprisingly, in *B. juncea* at the advancing infection front, intercellular fungal hyphal growth without any associated cell death was observed. No chlorotic zone surrounding the necrotic lesion was seen in these infected samples. Additionally, earlier observation that *A. brassicae* suppress plant ROS and cell death during penetration, indicates that there might be a short biotrophic-like phase where the fungus suppresses plant defense responses before triggering massive cell death. Similar observations have been reported for the broad spectrum necrotroph *S. sclerotiorum* infecting tomato (Kabbage et al. 2013, 2015). However, from our results, it is not clear if the cell death observed is necrosis or programmed cell death. Furthermore, activation of Salicylic acid mediated pathway in the *Arabidopsis* accession (susceptible) is in agreement with a similar observation reported in *B. juncea* (Mazumder et al. 2013; Nayanakantha et al. 2016).

Callose deposition at the penetration site to stall the hyphal entry is a hallmark of plant response to infection especially against biotrophic fungi. Although in the present study, callose deposition does not appear to contribute to resistance as generalized callose deposits were observed at the sites of successful penetration and establishment in both the compatible hosts. Rather, callose deposition appears to be a default plant response to infection. Nevertheless, we cannot rule out the role of callose in basal resistance against *A. brassicae* as the callose synthase mutant *gsl5*/*pmr4* showed enhanced susceptibility to the fungus (data not shown).

In this study, the comparative analysis of the processes of pathogenesis, infection and the host defense responses in the natural host *B. juncea* and a model host *Arabidopsis* Gre-0 reveal significant parallels between the two pathosystems. The extent of ROS, cell death and callose accumulation show a spatial and temporal correlation with the developing symptom, thus hinting at their putative role in susceptibility. Here we provide the foundation for future studies to investigate the plant defense mechanism and associated regulatory pathways that impart resistance against Alternaria leaf blight using the *Arabidopsis* model.

Acknowledgements We thank Nottingham Arabidopsis Stock Center (NASC) for distribution of *Arabidopsis* accession. We acknowledge Central Instrument Facility-University of Delhi, South Campus and Centre for Genetically Modifies Crop Plants, University of Delhi for sharing plant growth space. This work was financially supported by the grants from Science and engineering Research Board (SB/FT/LS-327/2012) and Department Of Biotechnology (BT/PR13379/GBD/27/263/2009) and research fellowship to Sayanti Mandal and Sivasubramanian Rajarammohan from University Grants Commission (UGC), Government of India.

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