Histological Characterization of Host-Pathogen Interactions in Two *Brassica rapa* Accessions Exhibiting Resistance to *Albugo candida*

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ABSTRACT

Oilseed mustard, *Brassica juncea*, is highly susceptible to white rust disease caused by *Albugo candida*. The disease causes extensive yield loss in the crop and therefore it is crucial to identify and introgress loci conferring resistance to the disease in the commercial varieties. Most accessions belonging to the Indian gene-pool are highly susceptible to the disease; however, resistance sources have been previously identified in the east European *B. juncea* germplasm and also in the diploid progenitor species, *B. rapa*. We had recently identified two novel sources of resistance to *A. candida* in the *B. rapa* germplasm, YSPB-24 and Candle. In the present study, both accessions were characterized for their host-pathogen interactions. The two accessions are highly resistant to the pathogen and no pustules were observed on the cotyledonary tissue upon infection, unlike susceptible forms. In YSPB-24, unlike Candle, distinct necrosis was visible at the site of infection as early as 5 days post inoculation (5 DPI). Light microscopic observations of trypan blue stained infected cotyledonary tissue revealed that the pathogen was able to germinate and penetrate the host tissue in both YSPB-24 and Candle, similar to susceptible accessions during the initial stages of infection. However, subsequent hyphal growth and penetration was restricted, with no significant growth seen beyond 3 DPI. In contrast, in the susceptible lines, hyphal growth was profuse, and increased with disease progression, culminating in pustule formation at 11 DPI. Hypersensitive response, as detected using DAB staining, was found only in the resistant accessions, YSPB-24 and Candle. Being completely resistant to *A. candida*, the two accessions identified in the present study would be useful resources for breeding durable resistance in *B. juncea*.

Key words: ***Albugo candida*, *Brassica rapa*, resistance, white rust**

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Introduction

White rust, caused by *Albugo candida* (Pers. ex Lev.) Kuntze, is a major disease of Brassica species, including oilseed crops, *Brassica juncea* (Indian mustard) and *B. rapa*. In India, approximately 80% of the total area under rapeseed-mustard cultivation is occupied by *B. juncea*. The disease has been reported to cause massive yield losses (20-60%), with maximum damage occurring at the staghead formation stage (Saharan et al., 1984; Kolte, 2002; Sachan et al., 2004; Kumar & Kalha, 2005; Kaur et al., 2008).

Oomycte *A. candida* is an obligate biotroph and requires host tissue for its survival and reproduction. The first symptoms of the disease are white blisters (hence the name) that appear on the surface of the leaves, stems and pods. Systemic spread of the disease results in distortion, hypertrophy and formation of stagheads (hypertrophy of the inflorescence). Based on the host specificity, at least 17 different *A. candida* races have been identified (Minchinton et al., 2005). Of these, race 2 predominantly infects *B. juncea* (AABB genome), however, it has been found to infect several genotypes of *B. rapa* (AA genome) as well (Petrie, 1988). The primary source of infection is through the thick walled oospores which can resist dry conditions and high temperature and thus survive for long in the plant debris (Verma and Petrie, 1980). Infection is initiated by the germination of the zoospores that is facilitated by the moisture present on the plant surface. The germ tubes enter through the stomata and colonize the mesophyll cells through formation of primary and secondary hyphae from which multiple haustoria develop that penetrate individual cells (Verma et al., 1975; Liu et al., 1989). In susceptible hosts, hyphae extend intercellularly, penetrating deep into the mesophyll layer and produce multiple haustoria (Liu et al., 1989; Liu and Rimmer, 1990). In completely resistant hosts, after initial hyphae initiation, the growth was found

restricted and host cell death was observed at the site of penetration (Liu et al., 1989).

Most accessions of the Indian gene pool of *B. juncea* and *B.rapa* are highly susceptible to the disease. However, a few resistant accessions have been identified in the east European germplasm of *B. juncea,* and in *B. rapa*. In majority of the studies, a single major locus conferring resistance to *A. candida* has been identified in Brassica species including *B. juncea* (Tiwari et al., 1988; Prabhu et al., 1998; Panjabi-Massand et al., 2010; Bhayana et al., 2020), *B. rapa* (Kole et al., 1996; 2002), and *B. napus* (Ferreira et al., 1995). Resistance to *A. candida* isolate AcB1 has been mapped to linkage groups A04 and A05 in the east European accessions Heera and Donskaja respectively (Panjabi-Massand et al., 2010) and to A06 in the Chinese vegetable type mustard, Tumida (Bhayana et al., 2020). Owing to the limited sources of resistance available, finding additional such resources is crucial for breeding endeavors.

In the present study, we characterized two novel sources of resistance to *A. candida* AcB1 isolate (our unpublished results) for their host-pathogen interactions using histological studies. Results revealed that both the *B. rapa* accessions, YSPB-24 and Candle, exhibited complete resistance to *A. candida* isolate AcB1. The pathogen growth was restricted within three days of infection and a hypersensitive (HR) response was generated in both accessions.

Materials and Methods

Plant material

Three accessions of *B. rapa*: Candle, YSPB-24 (yellow sarson), Pant-toria and one *B. juncea* variety, Varuna, were used in the present study. Of these, YSPB-24 and Candle are resistant, while Pant-toria and Varuna are susceptible to the pathogen, *A. candida*. Seed material for all these accessions were obtained from Centre for Genetic Manipulation of Crop Plants, University of Delhi, South Campus, New Delhi.

White rust assay

Brassica seeds were sown in 7 cm pots containing a 1:1 mixture of soilrite and soil in plant growth room maintained at 22°C with a 16-hour light/ 8-hour dark photoperiod. Following germination, only five to six seedlings were retained in each pot. Each round of inoculation comprised of multiple such pots arranged in trays. In each tray, two pots containing seedlings of Varuna were also placed as a positive control (Fig. 1). In the highly susceptible variety, Varuna, intercellular mycelia are observed within 2 DPI, which then grow profusely and extensive white pustules are observed on the abaxial cotyledonary surface by 11 DPI (Panjabi-Massand et al., 2010).

For the assay, a previously described *A. candida* race 2 isolate, AcB1 (Panjabi-Massand et al., 2010), was used. The isolate was maintained on cotyledons of the highly susceptible Varuna seedlings. Zoosporangia were collected from freshly infected Varuna cotyledons and suspended in sterile double distilled water. The spore suspension was then incubated at 4°C for 3 hr to release the zoospores. Inoculum was prepared by adjusting the spore count to 10^5 spores/ml. For the assay, 10 µl of this suspension was drop inoculated on each of the two cotyledons of seven day old Brassica seedlings (Fig. 1). The inoculated seedlings were kept in the dark for 48 hrs in an infection room maintained at 18°C with 98-100% relative humidity. After incubation, the trays were shifted to growth room with 16-hour light/ 8-hour dark photoperiod and 80% relative humidity.

Histological studies

A. candida infected cotyledons of YSPB-24, Pant-toria, Candle and the positive control, Varuna were harvested at various time intervals post inoculation and stained with trypan blue (Koch and Slusarenko, 1990). For this, the cotyledons were boiled for 2 minutes in ethanol: lactophenol (1:1) solution having trypan blue (250 µg/ml) and then cleared in chloral hydrate solution (2.5 g/ml distilled water). The cleared cotyledons were mounted in 50% glycerol and *A. candida* growth progression was observed using Zeiss Scope A1.microscope.

Hypersensitive response due to *A. candida* infection was observed by staining with 3, 3-diaminobenzidine (DAB, Sigma D5637). DAB detects hydrogen peroxide (H_2O_2), which is generated during HR in the plants. In the presence of peroxidases, when DAB comes in contact with H_2O_2 it polymerizes to yield a reddish brown polymer (Thordal-Christensen et al., 1997). For DAB staining, infected and control (water inoculated) seedlings were collected 7 DPI in 15 ml Falcon tubes containing 2 ml of 1 mg/ml DAB solution (pH 7.0, adjusted with sodium phosphate buffer). The tubes were kept for 12 hrs at 18°C, subsequent to which the cotyledons were boiled for 10-15 min in ethanol: acetic

Figure 1: *Brassica* plant growth and infections with *Albugo candida* in controlled growth room conditions. The pictures represent: (A) trays harbouring *Brassica* seedlings grown in growth room for white rust infections, (B) inoculation of *Brassica* cotyledons with *A. candida* spore suspension, (C) seedlings maintained in dark conditions post-inoculation, (D) pots uncovered after 48 hrs post inoculation and maintained at 16hr/8hr photoperiod. High humidity was maintained by using humidifiers, highlighted with blue arrow in (C).

acid: glycerol (3:1:1) solution to remove chlorophyll (Thordal-Christensen et al., 1997). The cotyledons were mounted in 50% glycerol and observed using bright field microscopy (Zeiss Scope.A1 microscope).

Results

Host pathogen interaction

Both YSPB-24 and Candle were found to be completely resistant to the *A. candida* isolate, AcB1, with no pustules seen in any of the cotyledons tested. However, the disease response was a little different. Post inoculation, the YSBP-24 cotyledons developed a white necrotic patch at the site of the inoculation (adaxial surface) that was visible by 5 DPI and developed into a distinct patch by 11 DPI (Fig. 2B). However, no visible changes were observed in the Candle seedlings post inoculation (Fig. 2A). The susceptible accession, Pant-toria, developed multiple pustules on the both surfaces of cotyledon (Fig. 2C), unlike Varuna wherein pustules were observed predominantly on the lower surface of the cotyledons at 11 DP1 (Fig. 2D).

To study the progression of the disease in the three *B. rapa* accessions, YSPB-24, Pant-toria and Candle, inoculated cotyledons of each accession were harvested at different time points; 1, 3, 5, 7 and 11 DPI and stained with trypan blue. For a comparative analysis, cotyledons of the highly susceptible variety of *B. juncea*, Varuna (positive control), was also included in each infection experiment.

Microscopic observations of trypan blue stained infected cotyledonary tissue at 1 DPI (24 hr) revealed spore germination, formation of germ-tube and appressorium in all the accessions, irrespective of being resistant or susceptible to *A. candida*. Consistent with the previous reports (Liu et al., 1989; Bansal et al., 2005), the germinating *A. candida* spores were found to enter the leaf tissue through the stomata (Fig. 3A). In most cases, distinct appressoria were observed, but in some cases germ tubes were found to directly enter through the stomata. To further investigate, if variability existed in the spore germination time across the accessions, infected samples were collected at different time intervals within 24 hours of infection. In all accessions, spores of the pathogen were found to germinate within ~3.5 hours post inoculation (Fig. 3A 1-3). Hence there was no visible difference in the spore germination ability of the pathogen across the different accessions.

At 3 DPI, intercellular growth of the fungal hyphae was observed in all accessions with haustoria formation, however the extent of hyphal growth was distinctly different between the resistant and susceptible accessions. In both the resistant *B. rapa* accessions Candle and YSPB-24, the hyphal growth was much restricted (Fig. 3B1; C1), unlike the susceptible accessions Pant-toria and Varuna wherein the mycelium growth was higher (Fig. 3D1; E1). At 5 and 7 DPI, no enhancement in the hyphal growth was observed in both the resistant

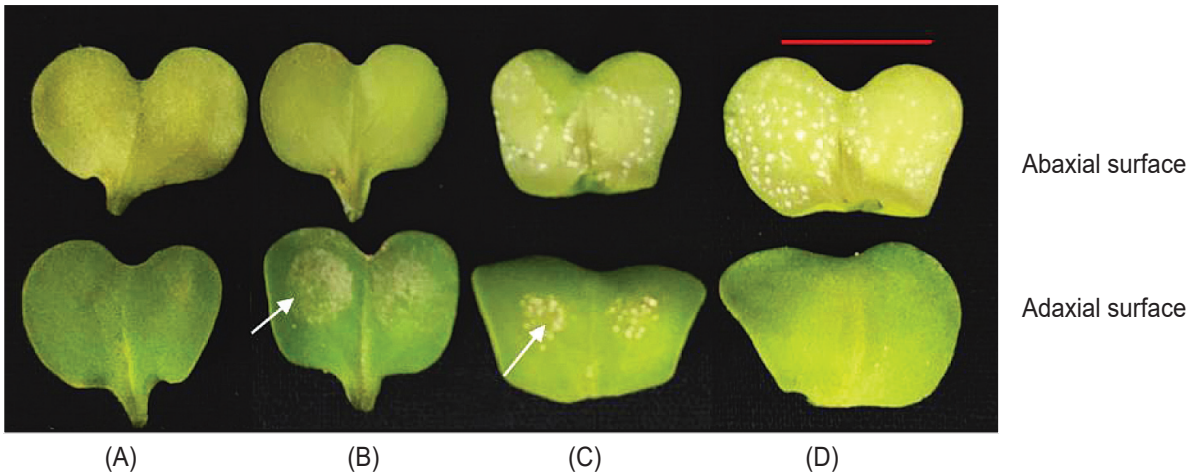

Figure 2: Disease response of *Brassica rapa* and *B. juncea* post infection with *A. candida*. The disease reactions observed on the abaxial and adaxial cotyledonary surface at 11 DPI on each of the different *Brassica* accessions: (A) Candle, (B) YSPB-24, (C) Pant-toria and (D) Varuna is shown. Both Candle and YSPB-24 are resistant to the pathogen. Unlike Candle where no visible changes are seen on the cotyledonary surface (A), YSPB-24 exhibits a clear hypersensitive response at the site of the inoculation (B); adaxial surface, marked with an arrow). Both Pant-toria (C) and Varuna (D) are highly susceptible and exhibit pustules on the cotyledons, more predominantly in the adaxial side. Bar=1cm.

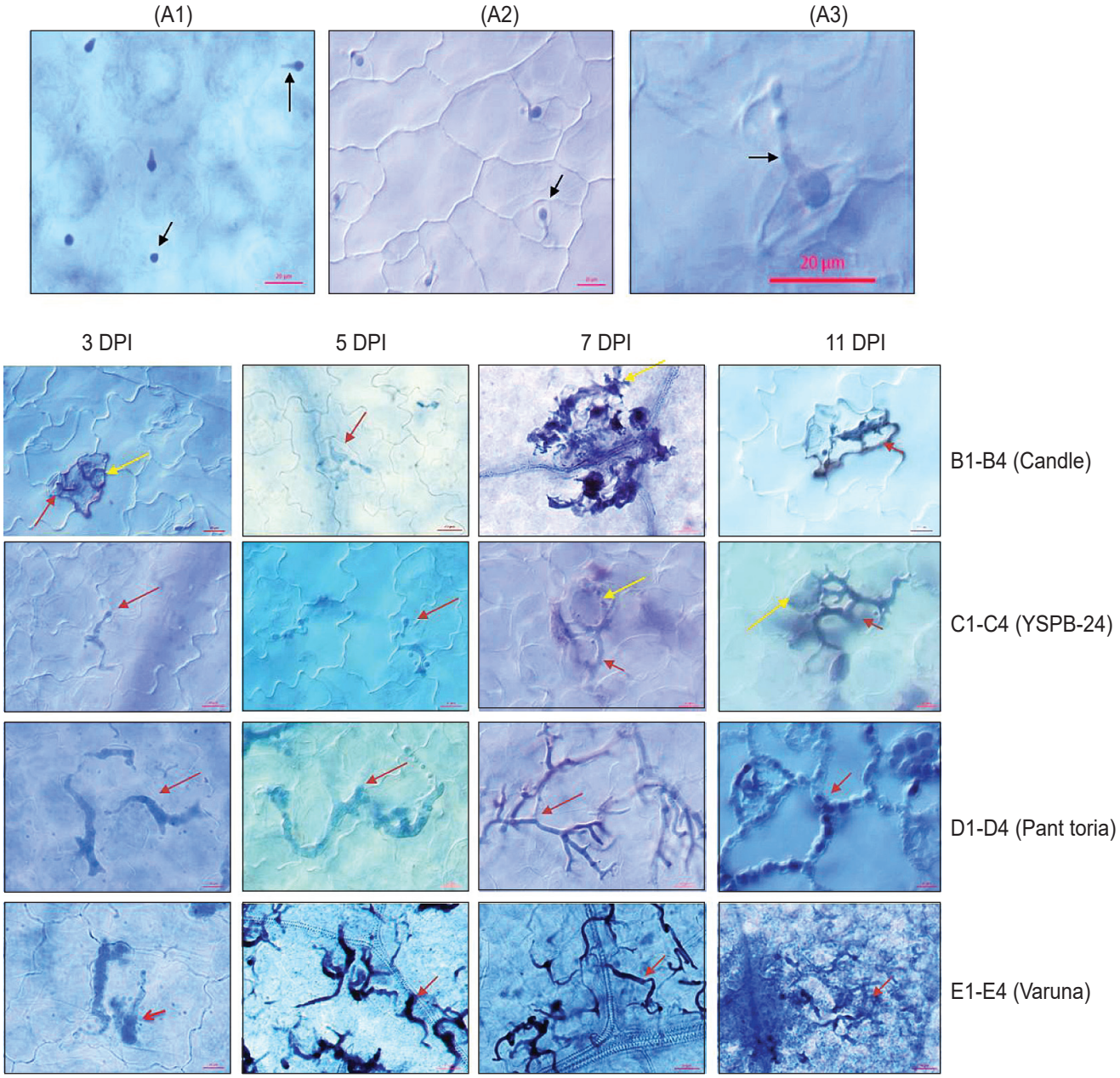

Figure 3: Histology of host-pathogen interaction of *Albugo candida* race 2 on *Brassica rapa* and *B. juncea*. In all accessions zoospore germination was observed at ~3.5 hrs (a1) and by 24 hrs (1 DPI), the growing germ tube was seen entering through the stomata (A2). A single magnified germinated zoospore entering the stomata is shown (A3). Disease progression on *B. rapa* accessions: (B) Candle, (C) YSPB-24, (D) Pant-toria and *B. juncea* cultivar (E) Varuna at 3, 5, 7 and 11 DPI has been shown. The resistant accessions, Candle and YSPB-24 had little fungal mycelia visible at 3 and 5 DPI (B1-B2; C1-C2), which was found to marginally increase at 7 and 11 DPI (B3-B4; C3-C4). However, in case of the susceptible accessions; Pant-toria and Varuna, the mycelial growth was significantly higher at 3 DPI (D1, E1), which increased profusely at 5 and 7 DPI (D2-D3; E2-E3) and at 11 DPI, pustules were observed in both accessions (D4, E4). At all stages, Varuna cotyledons had comparatively higher extent of fungal growth as compared to Pant-toria. Bar=20µm

accessions (Fig. 3B2-3; C2-3). However, in Pant-toria and Varuna, the hyphae had grown profusely and had penetrated more deeply into the mesophyll tissue (Figure 3D2-3; E2-3). At 11 DPI, in the resistant accessions, hyphal growth was restricted and similar to that observed at 7 DPI (Fig. 3B4; C4). In the susceptible accessions, hyphae had spread extensively, and pustule formation was observed (Fig. 3D4, E4). Between the two susceptible accessions, at all the stages of infection analysed, the extent of hyphal growth and also the

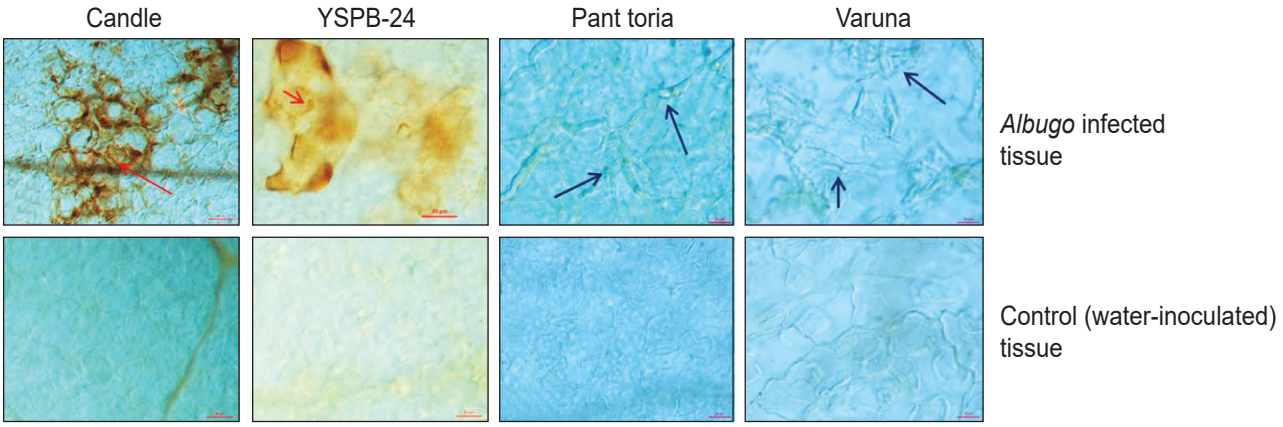

Figure 4: DAB stained tissues of control and *A. candida* infected *Brassica* accessions collected at 7 DPI. *Albugo* infected cotyledonary tissue of the resistant accessions, Candle and YSBP-24, exhibited a hypersensitive response, visible as brown coloration (A1, B1; highlighted by arrows). Both the susceptible accessions, Pant-toria and Varuna, had no such hypersensitive response (C1, D1). Arrows highlight the hyphal growth visible in Pant-toria and Varuna cotyledons. Bar=20µm

pustules formation (at 11 DPI) was more profuse in Varuna as compared to Pant-toria.

To investigate whether *Albugo* infection generated a hypersensitive response (HR) in any of the four Brassica accessions, cotyledons at 7 DPI were subjected to DAB staining.

Of the four accessions, HR was found in both the resistant accessions, Candle and YSPB-24, visualized as brown coloration of the DAB stained tissue infected with *A. candida* (Fig. 4). In the control samples (water inoculated) of both accessions, no such coloration was observed. As mentioned previously, HR response was also clearly visible on the surface of infected YSPB-24 cotyledons in the form of a necrotic spot. In the case of susceptible accessions Pant-toria and Varuna, no HR was observed (Fig. 4).

Discussion

In the present study, two newly identified sources of resistance to *A. candida* in the *B. rapa* germplasm were characterized for host-pathogen interactions. Both accessions, YSPB-24 and Candle, were found to possess complete resistance to the pathogen, with no pustule formation seen on the infected cotyledons. Upon infection, seedlings of YSPB-24, unlike Candle, developed a clear necrotic patch (indicating HR) on the adaxial surface at the site of the inoculation that was clearly visible as early as 5 DPI.

Histological studies of infected cotyledonary tissue revealed that initial disease progression in both the resistant accessions were similar to that observed in the susceptible Pant-toria (*B. rapa*) and Varuna (*B. juncea*). In all accessions, zoospore germination, elongation of germ tubes and invasion of the plant tissue was observed within 1 DPI. Zoospores germinated within ~3.5 hours of inoculation in all the accessions. By 3 DPI, both resistant hosts exhibited restricted hyphal growth, and no increase in hyphal mass was observed in the subsequent stages analysed. In the susceptible host, Pant-toria, at each of the stages analysed (1, 3, 5, 7 and 11 DPI), the pathogen was found to grow and propagate extensively with deeply penetrated mycelia, and pustules appeared on the cotyledonary surface 11 DPI. This was more or less similar the growth of the pathogen on Varuna seedlings, as has been previously described. (Panjabi-Massand et al., 2010).

Previous studies based on the histology of host-pathogen interactions between *A. candida* and accessions of *B. rapa*, *B. juncea* and *B. napus* have reported similar observations (Verma et al., 1975, Liu et al., 1989, Bansal et al., 2005, Panjabi-Massand et al., 2010). In one of the earliest reports (Verma et al., 1975), initial disease progression was found to be similar in both susceptible (*B. juncea* and *B. rapa*) and resistant (*B. napus*) hosts with differences observed only after the formation of the first haustorium. Hyphal growth ceased within 2-3 days in the resistant host. Liu et al. (1989) reported that zoospore germination occurred 2-3 hr post inoculation (with *A. candida* race 7) in both the susceptible and resistant hosts in *B. napus*. In this study also, disease progression varied between the susceptible and resistant hosts in terms of the stage of development of the first haustoria.

With the limited sources of resistance available in

the *B. juncea* germplasm against this highly prevalent and destructive pathogen, any new resistant accession identified is important as it would benefit breeding endeavours towards building durable resistance against the pathogen. Both the *B. rapa* accessions characterized in the study impart complete resistance to the pathogen, and therefore will be useful sources for introgression of the resistant trait in commercial varieties.

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