academicJournals

Vol. 7(33), pp. 4284-4290, 16 August, 2013
DOI: 10.5897/AJMR2013.5585
ISSN 1996-0808 ©2013 Academic Journals
http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Microscopic study of *Alternaria brassicae* infection processes in *Brassica juncea* cultivars by drop plus agarose method

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Accepted 25 June, 2013

***Alternaria* blight caused by *Alternaria brassicae* (Berk) Sacc. is one of the most serious diseases of Indian mustard [*Brassica juncea* (L.) Czern and Coss.] grown as edible oilseed crop in India. Poorly characterized tolerance mechanisms against this pathogen further confirm the strategies that can be undertaken to design durable resistance or effective disease control measures. The host-pathogen interaction was studied and showed that the initial infection processes involving conidium germination and mode of penetration were similar in both cultivars, although considerably late in 'PAB 9511' evidently due to tolerance. However, apparent differences between the two cultivars were noticeable by 1 dpi. At the plant surface, impeded fungal growth, less proliferation of spores and active suppression of the dense hyphal moulds are the factors involved in the expression of tolerance against *A. brassicae* in cultivar PAB 9511. Our findings have notably advanced our understanding of the mechanism of tolerance in this patho-system.**

Key words: Artificial inoculation, *Brassica juncea*, *Alternaria brassicae*, pathogenicity, plant-pathogen interaction.

INTRODUCTION

Indian mustard [*Brassica juncea* (L.) Czern and Coss.] is one of the most important oilseed crops, alone contributes about 80% of the total rapeseed-mustard cultivated (6.69 million ha) with the all time high production of 8.17 million tons in India (Chauhan et al., 2012). It fulfills nearly 27% of vegetable oil requirements of the country (Sharma et al., 2002). *Alternaria* blight disease caused by a necrotrophic fungus (Tewari, 1991) lowers yield 47% in the entire mustard growing area (Meena et al., 2010a). Various methods like cultural control and chemical control is often used, but none effectively control this disease on their own. Since *Alternaria* blight is increasingly destructive in oil production, ways of controlling the disease need to be developed. Studies of host pathogen interactions at the cellular level will contribute to the development of more effective disease control measures. Therefore, an attempt has been taken to determine the factors which are involved in the expression of tolerance in tolerant PAB9511 and the factors governing the susceptibility of the cultivar Varuna by the light microscopic studies with the drop plus agarose artificial inoculation method (Giri et al., 2013) in the artificial conditions. Here we describe in detail the previously unreported infection processes of *Alternaria brassicae* on two contrasting cultivars of *B.*

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juncea ‘Varuna’ and ‘PAB 9511’.

MATERIALS AND METHODS

Host cultivars

Two *Brassica juncea* cultivars that is. ‘PAB9511’ and ‘Varuna’, were used in this study. Seeds were grown in plastic inserts (7.5 cm X 5 cm; 2 seeds per insert) containing a compost mixture consisting of vermicompost, peat moss and soil under the greenhouse conditions at 22/18 (± 1)°C (day/night) with light intensity of 150 µE m^{-2} s^{-1} for 12-h light/dark cycles.

Alternaria brassicae

Alternaria brassicae (Berk.) Sacc. was isolated from a diseased leaf of *Brassica juncea* cultivar ‘Varuna’ at Crop Research Centre (CRC) Pantnagar India. Pure single spore culture of *A. brassicae* was developed with the help of stereo microscope (Nikon make) and maintained on potato dextrose agar (PDA) slants at 4°C.

Inoculum preparation

A. brassicae was subcultured from the 7-day old culture on V-8 agar medium (10% V-8 juice, 0.02% $CaCO_3$ and 2% agar) incubated at 22°C. A conidial suspension was prepared by scraping the mycelia and spores from the surface of the actively growing fungal culture into autoclaved distilled water and filtered using four layered cheese cloth to remove most of the mycelia. The filtered spore suspension was centrifuged at 2000 X g for 5 min and resuspended in deionized water. This centrifugation was repeated one more time in order to ensure a clear spore suspension free of metabolites. After the final wash, supernatant was discarded and spores were resuspended in water containing 0.05% Tween-20 as an adhesive. The concentration of spore suspension was adjusted to $5x10^4$ spores ml^{-1} using a haemocytometer (Sharma et al., 2007).

Sample preparation for light microscopy

Drop plus agarose artificial inoculation method (Giri et al., 2013) was used to study the process of spore germination on glass slide as well as on cotyledons of both cultivars. Inoculated cotyledons and glass slides were sampled at 15 min, 30 min, 1 h, 3 h, 6 h, 12 h post inoculation (hpi), and then daily for the 4^{th} d post-inoculation (dpi). The sampled cotyledons where drop of inoculum was placed along with agarose was decolourized in an acetic acid: ethanol: water (2:2:1) solution at 25°C for overnight. Cotyledons were then washed with two changes of DI water and stained with 1% cotton blue in lactophenol (Garg et al., 2010). Whole wet mounts of cotyledons on microscope glass slides were examined and photographed using a fluorescent microscope Nikon Eclipse 80i. The length of aerial hypha were measured by observing 10 spores at random at inoculated sites at 1, 3 , 6, 12 hpi and daily for 2 dpi. At 3 dpi it was not possible to measure the hyphal length due to extensive mycelial growth.

Statistical analysis

The hyphal elongation data were analyzed separately by ANOVA using SAS (9.3) version. It is also used to calculate the significant difference ($P< 0.001$) between the two *B. juncea* cultivars and glass slide.

RESULTS

Conidial germination and fungal development on glass slides and host

Conidia of *A. brassicae* were muriform, beaked, bottle shaped, and measuring 92.3-102.5 µm long and 10.5-20.5 µm wide (Figure 3a). Conidia had 6-10 or even more transverse septa and a few or none longitudinal septa. The longitudinal septa, when found, were only in the middle cells. The conidium was developed from a bud formed by the apical cell of conidiophore. The main body of the conidium was oblong with its formal end protruding and the terminal cell tapered into a beak. The beak length varied from 41-51.25 µm long. Conidial germination began within 1 hpi on both the cultivars as well as on glass slides. Germination was observed as either a small swelling at the end of the spore or a very short germ tube (Figures 1d, 2d, 3c). At 1 hpi, there were no significant differences observed in average hyphal length between the two cultivars and between PAB9511 and glass slides (Table 1). More than 90% of the conidia have been germinated by 3 hpi onto the surface of cultivars as well as on glass slides. At initial hour of infection (6 and 12 hpi) multiple germ tubes have been appeared in the conidia irrespective of the surface (Figures 1f, 2f, 2g, 2h, 3e and 3g). At 6 and 12 hpi germ tubes that emerged from conidia continued growth on the surface of cotyledons and on glass slide (Figures 1f, 1g, 2f, 2g, 3e, 3f). Hyphal length was significantly less ($P<0.001$) on glass slides compared with either ‘PAB 9511’ or ‘Varuna’ at all the time points of observation. The growth of fungal mycelium is almost two fold at all the time intervals taken as the infection proceeds (Table 1). In ‘Varuna’ penetration of fungal hyphae into intercellular spaces was observed at 12 hpi (Figure 1h) whereas, no such penetration was evident in ‘PAB 9511’. At 1 dpi apart from the increase in the hyphal length many changes have been observed. Slightly swollen hyphal apices were evident, mainly on ‘Varuna’ (12.3 µm) and on glass slides (10.25 µm) at 1 dpi (Figures 1i and 3h). Hyphal branching was seen on both the cultivars and on the glass slides (Figures 1j, 2i, 3i). In ‘Varuna’ the hyphal apices showed dichotomous branching that gave rise to simple appresorium like structure (Fig 1k), however dichotomous branching of the terminal hyphae was not evident in the ‘PAB 9511’,despite a slight increase in diameter of the hyphal cells being apparent in this cultivar (Figure 2l). Appresorium like structure and infection thread (Figure 3h) are the two hyphal modifications observed in *Alternaria brassicae.* Penetration of appresorium like structure was evident only through stomata in ‘Varuna’ (Figure 1j and 1n), whereas penetration of infection thread into the intercellular spaces observed in both the cultivars (Figures 1o, 2j and 2k). In addition to hyphal modifications swollen hyphal apices also approaches towards stomata only in ‘Varuna’ (Figure 1l and 1m). Both type of entry, entry through the natural openings (stomatal

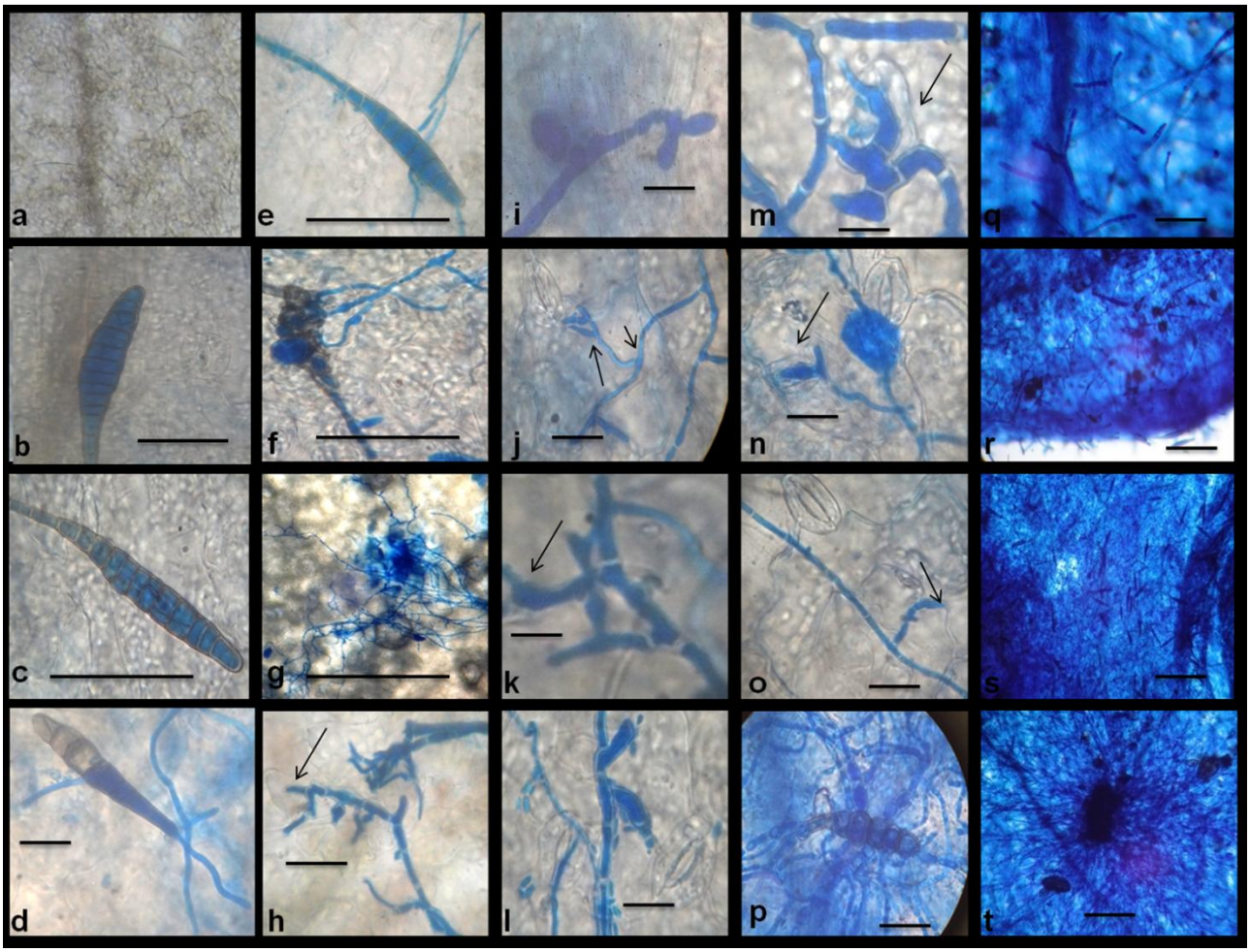

Figure 1. Light microscopy showing infection behavior of *Alternaria brassicae* (Pantnagar isolate) onto cotyledon of *B. juncea* cultivar 'Varuna'. A) Control with no conidia. B) Conidium after 15 minutes post inoculation (mpi). C) Conidium after 30 mpi. D) Germ tube emerged from conidium after 1 hour post inoculation (hpi). E) Elongation of germ tube at 3 hpi F) Formation of several germ tubes and their elongation at 6 hpi. G) Fungal hyphae continued growth on the surface of cotyledonary tissue at 12 hpi. H) Penetration of fungal hyphae (arrow) into intercellular spaces at 12 hpi. I) Swollen hyphal apices at 1 day post inoculation (dpi). J) Emergence of lateral hyphal branches (arrow) from aerial hyphae and appresorium like structure approaches towards stomata (big arrow) at 1 dpi. K) Repeated dichotomous branching (arrow) of the terminal hyphae leading to formation of appresoria like structure at 1 dpi. L) Swollen hyphal apices approaching towards stomata at 1 dpi. M) Penetration through stomata (arrow) by swollen hyphal apices at 1 dpi. N) Terminal hyphae leading to formation of appresoria like structure and its penetration (arrow) through stomata at 1dpi. O) Differentiation of hyphae into infection thread and its penetration (arrow) through intercellular spaces at 1 dpi. P) Significant increase in the length of the fungal hyphae and lateral branch length at 2 dpi. Q) & R) Extensive growth of both primary hyphae and of lateral branches at 3dpi. S) Excessive proliferation of the spores at 3 dpi. T) Certain inoculation sites frequently appeared as dense hyphal 'moulds' as a result of the extensive mycelia growth at 4dpi. Scale bar: (B, C, E, P, Q, R, S, T) = 100µM; (H, I, J, K, L, M, N) = 20 µM ; (D & O) = 50 µM ; (F & G) = 500 µM

penetration) and direct entry (epidermal penetration) have been observed in both the cultivars, but stomatal entry is not evident in 'PAB 9511' cultivar at 1 dpi. At 2 dpi lateral branches also increased in length on 'Varuna' cultivar (Figure 1p) and on glass slide (Figure 3i), whereas impeded fungal growth was observed in 'PAB 9511'. Stomatal penetration of appresorium like structure was clearly evident in 'PAB 9511' (Figures 2m and 2n) and appresorium like development was also seen in glass slide (Figure 3j). At 3 dpi extensive growth of both primary and secondary hyphae have been observed in 'Varuna' cultivar (Figure 1q and 1r) and on glass slide (Figure 3k), however no such growth was evident in 'PAB 9511'. The proliferation of spores has been observed in Varuna (Figure 1s) and on glass slides (Figure 3l) whereas no proliferation was observed in PAB 9511.

At 3 dpi onwards measurement of length of aerial hyphae was not possible on the 'Varuna' cultivar because

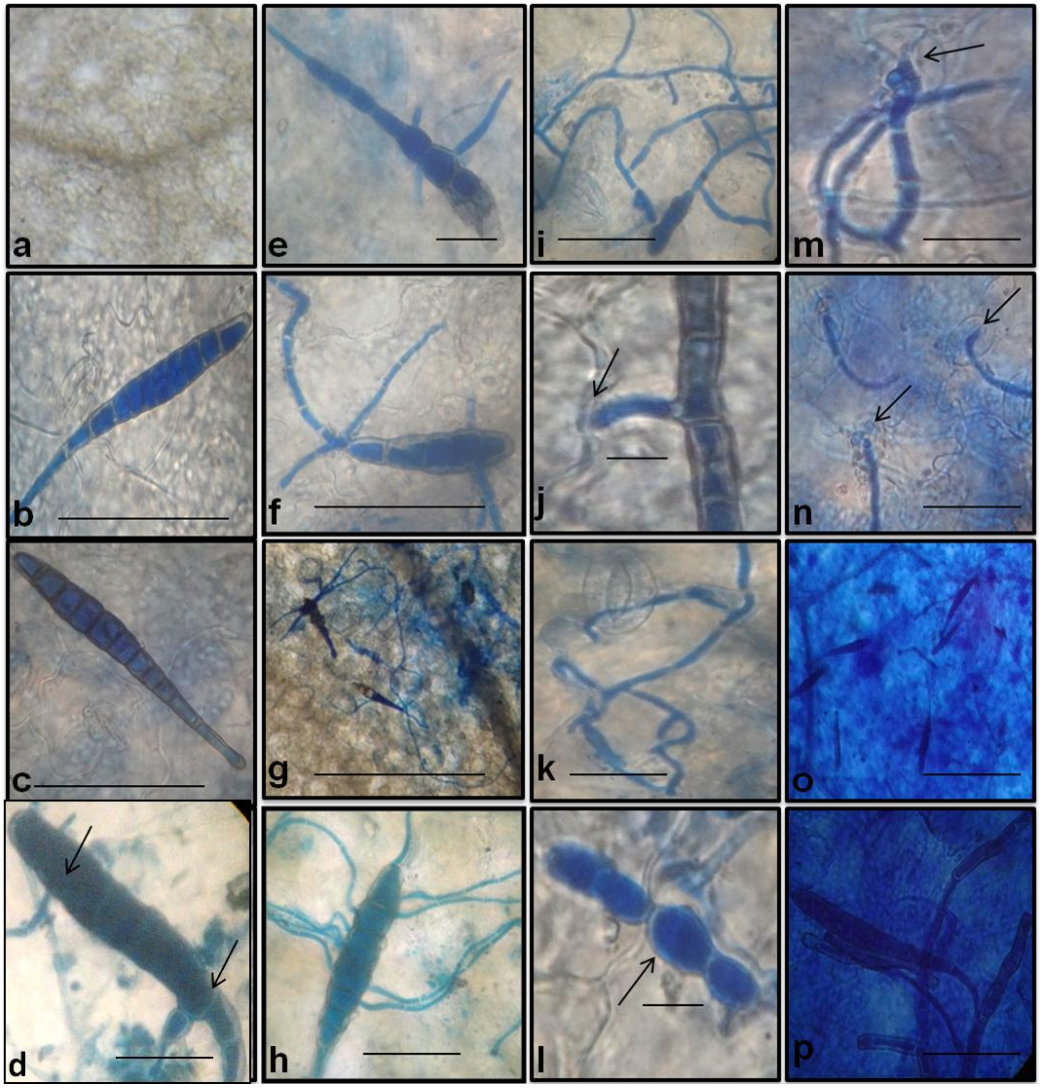

Figure 2. Light microscopy showing infection behavior of *Alternaria brassicae* (Pantnagar isolate) onto cotyledon of *B. juncea* 'PAB9511' cultivar. A) Control with no conidia. B) Conidia after 15 (mpi). C) Conidia after 30 mpi. D) Germ tube emerged (arrow) from conidia after 1 (hpi). E) and F) Elongation of germ tube at 3 hpi and 6 hpi. G) Fungal hyphae continued growth on the surface of cotyledonary tissue at 12 hpi. H) Formation of several germ tubes and their elongation at 12 hpi. I) Small lateral branches emerged from the hyphae at 1 dpi. J) & K) Differentiation of hyphae into infection thread and its penetration (arrow) through intercellular spaces at 1dpi. L) No dichotomous branching, but slight increase in diameter (arrow) of the hyphal cells at 1 dpi. M) Terminal hyphae leading to the formation of appresoria like structure and its penetration (arrow) through stomata at 2 dpi. N) Multiple penetrations (arrow) through stomata by fungal appresoria like structure at 2 dpi. O) No proliferation of the spores at 3 dpi. P) No dense hyphal 'moulds' were seen at 4dpi. Scale bar: (B, C, E, N, O) = 100µM; (D, K, L, M) = 20µM; (J & Q) =50 µM; (F, G, H & I) = 500 µM

of extensive mycelial growth. At 4 dpi growth of hyphae on the surface of 'Varuna' (Figure 1t) and on glass slide (Figure 3 m) at certain inoculation sites frequently appeared as dense hyphal 'mounds' as a result of the extensive mycelial growth but this kind of hyphal 'mounds' was not evident in 'PAB 9511'. The histology of early stages of infection was found to be similar in both the cultivar but a little late in 'PAB 9511' cultivar.

DISCUSSION

Drop plus agarose artificial inoculation method is an efficient, rapid and inexpensive method. It has the advantage of being accurate and precise. By employing this method it would be easier to handle the inoculated plants. This method could also be used at the cotyledonary stage, where it will take rather less time to assess the

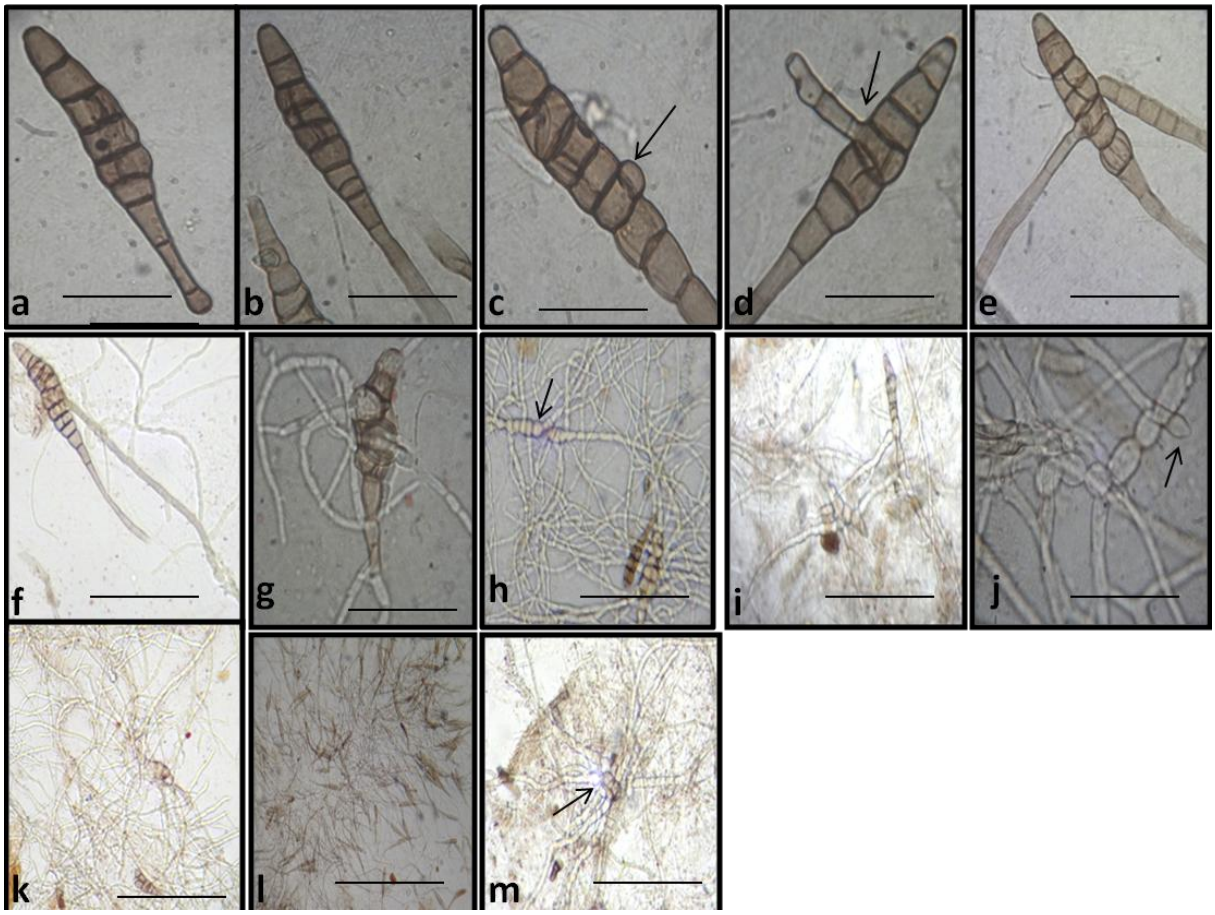

Figure 3. Growth of *Alternaria brassicae* (Pantnagar islolate) on the surface of glass slide on water. A) and B) Conidium at 15 and 30 mpi. C) Germ tube emerged from conidium after 1 hpi. D) E) and F) Fungal hyphae continued growth on the surface at 3, 6, 12 hpi. G) Branching of the germ tubes at 12 hpi. H) Swollen hyphal apices and extensive network of infection thread at 1 dpi I) Emergence of lateral branches at 1 dpi J) Appresorium like development at 1 dpi K) Significant increase in the length of the fungal hyphae and lateral branch length at 2 dpi. L) Proliferation of spores at 3 dpi. M) Dense mould observed at 4 dpi. Scale bar: (A, B, E, G) = 100µM; C= 20 µM; D= 50 µM; (F, H, I, J, K, L, M) = 500 µM.

symptoms of different cultivars compared with upto 1-2 months when other methods are utilized. As this method increases the inoculation time of the inoculum at the infection court, it would be beneficial to understand the infection behavior of *A. brassicae* onto the cotyledonary surface of two cultivars of *Brassica juncea.* The morphology and septation of conidia of *A. brassicae* resembles the genus *Alternaria.* At the initial hours of infection the absence of significant differences in conidial germination among glass slide and on cultivars of *Brassica*, suggests that signal for germination of conidia are triggered by the availability of adequate moisture irrespective of the nature of the cultivars. These results further suggest that both the cultivars are equally likely to be infected at this early stage. Enhancement of hyphal activity on 'Varuna' as compared with 'PAB9511' and with glass slide, suggests that 'Varuna' prompted hyphal activity on its surface. It could be due to the host factors present in 'Varuna' cultivar which is required for the fungal growth; this is in accordance as many times *B. juncea* Varuna leaf extract could be used for culturing *A. brassicae* isolates (Meena et al., 2012). In contrast significantly impeded hyphal growth on the surface of 'PAB 9511' at 2 dpi suggests that this cultivar might be producing certain antifungal/fungistatic compounds, as suggested by Kowalska and Niks (1999) for a resistant flax (*Linum usitatissimum*) genotype against *Melampsora lini* and by (Blakeman and Sztejnberg, 1973) in beetroot (*Beta vulgaris*) against *Botrytis cinerea*. It is noteworthy that interplay between *A. brassicae* and the cotyledons of 'PAB 9511' or 'Varuna' were evident by 1 dpi. *A. brassicae* enter the cotyledons of *B. juncea* both by direct (epidermal) and stomatal penetration. Both the penetrations were also evident in *A. ricini* on castor leaf, *A. porri* on onion leaf (McKenzie et al., 1993; Aveling et al., 1994; Suheri and Price, 2000; Babu et al., 2009). But penetration was only through stomata in *A. eichhorniae* on water hyacinth leaves (Shabana et al., 1997) and in *A.*

Table 1. Infection process of *Alternaria brassicae* Pantnagar isolate on two *Brassica juncea* cultivars 'Varuna' and 'PAB9511' and on glass slide surface

	Average hyphal length						
Hours/days/minutes Post-inoculation	**PAB9511 cultivar (µm)**	**Varuna cultivar (µm)**	**Glass slide (µm)**	**ANOVA of PAB9511, Varuna and glass slide***	**ANOVA of PAB9511 vs. Varuna****	**ANOVA of PAB9511 vs. glass slide*****	**ANOVA of Varuna vs. glass slide********
15 mpi	-	-	-				
30 mpi	-	-	-				
1hpi	18.57	28.85	10.53	P<0.001 (27, 8.48, 43.91)	n.s.	n.s.	P<0.001
3hpi	51.41	74.08	31.92	P<0.001 (27, 1.48, 2.83)	P<0.001	P<0.001	P<0.001
6hpi	112.1	155.4	66.5	P<0.001 (27, 1.64, 1.50)	P<0.001	P<0.001	P<0.001
12hpi	334.6	412.2	203.6	P<0.001 (27, 2.65, 0.83)	P<0.001	P<0.001	P<0.001
1dpi	509.3	718.1	562.4	P<0.001 (27, 4.14, 0.6)	P<0.001	P<0.001	P<0.001
2dpi	645.7	820.7	718.2	P<0.001 (27, 12.09, 1.66)	P<0.001	P<0.001	P<0.001

Analysis of variance (ANOVA) for hyphal length at 1, 3, 6, 12 hpi and at 1 and 2 dpi as an average of 10 random spores. Where there were significant differences, the numbers in parenthesis represent the error degree of freedom, root mean square error (Root MSE) and the coefficient of variation, respectively ; * Overall analysis of variance between 'PAB 9511', 'Varuna' and glass slide data in relation to hyphal length (1, 3,6,12 hpi and at 1 and 2 dpi) ; **Analysis of variance between 'PAB 9511' with 'Varuna' data in relation to hyphal length (1, 3,6,12 hpi and at 1 and 2 dpi) ; *** Analysis of variance between 'PAB 9511' with glass slide data in relation to hyphal length (1, 3,6,12 hpi and at 1 and 2 dpi) ; **** Analysis of variance between 'Varuna' with and glass slide data in relation to hyphal length (1, 3, 6, 12 hpi and at 1 and 2 dpi).

alternata on grape fruits (Swart et al., 1995), whereas penetration was always direct through the epidermal cell layers in *A. linicola* on water agar and linseed leaves (Vloutoglou et al., 1996) and in *A. alternate* on mulberry leaves (Gupta et al., 1997). The formation of several germ tubes and appressoria from a conidium is a common feature of this genus, as reported in *A. porri* (Aveling et al., 1994; Everts and Lacy, 1996), *A. linicola* (Vloutoglou et al., 1996), *A. alternata* (Gupta et al., 1997) and *A. cassia* (Mims et al., 1997).

Here terminal hyphae produces appressoria like structure which penetrates only the stomata not the epidermal cells (Figure 1n) this is in contrast to *A. ricini* which produced appressoria only for direct penetration through epidermal cells, whereas appressoria were not observed on the leaf surface where the germ tubes entered through stomata. However, on onion leaves, germ tubes from the conidia of *A. porri* produced appressoria on epidermal cells as well as on stomata (Aveling et al., 1994). The present results confirmed the earlier findings of Tsuneda and Skoropad (1978) who also studied the behavior of *A. brassicae* on intact and on excised leaves of rapeseed. Furthermore, the frequency of stomatal penetration was more on 'Varuna' cultivar at 1dpi as compared to the 'PAB 9511' cultivar where it was frequently observed at 2 dpi. At the later stages of infection process in Varuna cultivar, the fungus appeared to have ramified through the cotyledonary tissue between the upper and lower epidermis (Figure 1q and 1r) as the spots developed and conidia emerged through both surfaces of host cotyledonary tissue. This is in accordance with the study carried out by (Babu et al., 2007) who observed the release of conidia through stomata as well as through ruptured epidermis apparently without any preference for the emergence route in *Cercospora ricinella* on castor leaf. Conidiophores emerged by piercing or rupturing epidermal cells over stomatal complex when there was no emergence through that stomatal opening. A similar observation was found with *C. ricinella* (Babu et al., 2007) and *A. ricini* (Babu et al., 2009) on castor leaf indicating that the emergence of conidia through stomata was at random, rather than a preferred route, and that the development of conidiophores from the internal mycelium lacked any special orientation towards stomata. This feature of *A. brassicae* on mustard leaf and *A. ricini* and *C. ricinella* on castor leaf is in contrast to the well-oriented spore release observed by *C. moricola* (Gupta et al., 1995) and *Pseudocercospora mori* (Babu et al., 2002) on mulberry leaf. In *C. moricola* the conidia were discharged in clusters through stomata and by rupture of epidermal layers at the bases of trichomes. In *P. mori* the discharge of conidia was solely through stomata as the stromata inside the leaf tissue were always oriented below stomata for the release of conidia.

Presence of nutrients is essential for hyphal development, penetration and for subsequent establishment of a successful invasion. From the present study, it seems that impeded hyphal growth, no dichotomous branching, late penetration of hyphae on the PAB 9511 cultivar are the most important factors involved in the infection processes of *A. brassicae* that are responsible for the tolerance by which this cultivar actively suppresses the colonization and sporulation of the pathogen. Whereas more hyphal growth, dichotomous branching, early penetration, excessive proliferation governs the susceptible nature of Varuna cultivar. The observation of slower infec-

infection process in ‘PAB 9511’ clearly resembled those of previous reports where a similar sequence of events in infection was observed rather slower in resistant genotype in comparison to susceptible genotype (Yang et al., 1992; Tewari 1986).

Acknowledgement

Authors are grateful to DBT for providing the financial support in the form of R & D support under the project Programme mode support in Agricultural Biotechnology.

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