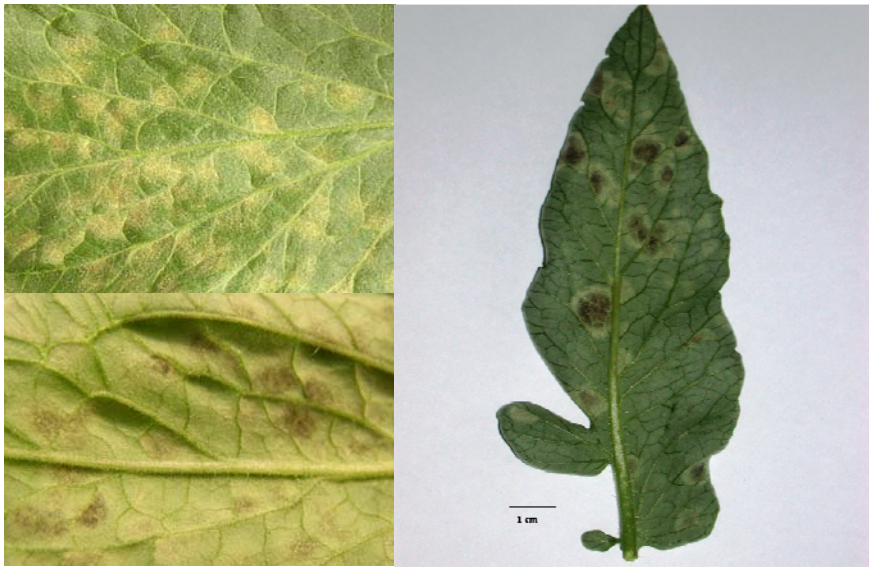
Epidemiological Investigations of Black Leaf Mold (*Pseudocercospora fuligena* (Roldan) Deighton) on Tomato (*Solanum lycopersicum* L.) under Protected Cultivation



Der Naturwissenschaftlichen Fakultät
der Gottfried Wilhelm Leibniz Universität Hannover
zur Erlangung des Grades eines

Doktors der Gartenbauwissenschaften

- Dr. rer. hort. -

genehmigte Dissertation

von

Zelalem Mersha Ayele (MSc.)

geboren am 20.10.1972 in Melkawerer, Äthiopien

2008

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Angefertigt am
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ABSTRACT

Macroscopic observations of symptoms and signs of the disease as well as isolation, culturing, inoculation (Koch's postulate) and microscopic characterizations (morphological and molecular) of fungal structures from axenic cultures proved *Pseudocercospora fuligena* to be the causative agent of black leaf mold (BLM) in Thailand. Macroscopically, the appearance of indistinct effuse patches on tomato leaves, amphigenous fructification and prolific fuliginous appearance of sporulating lesions, predominantly on the abaxial side and as infection progresses on the adaxial side too, were some of the peculiar features of the disease. Microscopically, the basic fungal structures like conidial dimension and segments (11-128 µm in length x 3.5–9 µm in width, with up to 12 septations) as well as fascicles of conidiophores of *P. fuligena* were observed. Despite its slowness, *P. fuligena* grew on all the ten artificial growth media tested but improved growth and sporulation occurred on tomato oatmeal agar and carrot leaf decoction agar, both supplemented with $CaCO_3$. Stomatal penetration and egress of *P. fuligena* were testified after light and scanning electron microscopy observations of cleared as well as intact leaves with the staining using aqueous acid fuchsin solution. Further conidiogenesis studies on cleared and stained leaves indicated prevalence of primary and secondary infection hyphae and exponential progression of blocked stomatal apertures.

With this background information of the pathogen, further studies on determinants of the "infection chain" in terms of favorability of temperature (*T*), wetness duration (*WD*) and leaf age (*A*) were carried out under ambient (AE) and controlled environment (CE) experiments. *P. fuligena* under artificial inoculation in CE experiments was highly favored by a temperature of 28°C. Considering the high mean daily *T* that prevailed under AE experiments in greenhouses at central Thailand (duration of < 11 h at $T \leq 28$°C and of about 17 hours at $T \leq 30$°C), *P. fuligena* could be assumed to cause the disease even at a *T* that surpasses the limit of 28°C. *WD* of at least 1 day after inoculation (DAI) was found to be a necessity for *P. fuligena* infection to take place. In all the tested parameters, young and fully expanded leaves of 3 and 5 weeks age as well as the youngest leaves (1 week old) were more susceptible than leaves of 7 weeks age. Eventually, all these determinant factors, *i.e. T*, *WD* and *A*, influenced the incubation (*IP*) and latent (*LP*) periods. For instance, under CE both the respective *IP* and *LP* were 2.2 to 5 days and 3 to 9.6 days shorter at 28°C than at 24°C. Whereas a respective *IP* and *LP* of 11 to 13 and 12.5 to 18 days were recorded at 28°C from CE experiments, it was even shorter in one of the AE experiments lasting only 9.2 to 14.0 (*IP*) and 10.4 to 16.0 (*LP*) days. Under natural infection, however, *IP* extended from an average of 11 to 25 days.

To test the effect of BLM vis-à-vis host growth, four sequential plantings were carried out in 2005. Weekly assessments of BLM incidence (*DI*) and severity (*DS*) fitted well to logistic functions. The sigmoidal shapes suggested that multiple cycles of infection occurred on leaves of tomato season-long. At times of heavy disease epidemics, a clear effect of reduced host growth, particularly in terms of healthy leaf area (*HLA*), was discernible from a comparison of treatments with (F) and without (NF) fungicide spray. A standardized disease severity (*DS**) proportion of 0.3 from the two peak-epidemics plantings resulted in a 68% loss of *HLA* when treatments *F* and *NF* were compared. Comparison of healthy leaf area index of healthy plants ($HLAI_{HP}$) with that of diseased plants in the F ($HLAI_F$) and NF ($HLAI_{NF}$) treatments showed a respective *HLAI* loss of 11 and 50%. Two applications of the fungicide mancozeb at 4 and 6 weeks after transplanting (WAT) reduced BLM epidemics by 60 to 90%.

Prevalence of such distinction in level of BLM epidemics amongst the plantings of 2005 led us to widen the area of investigation of the seasonal epidemics in relation to weather and yield. From the 24 fortnightly and monthly plantings, three BLM peak-epidemic periods were identified, *i.e.* August and September 2005 (1) and 2006 (2) and December to January 2005/06 (3). While maximum *DS* at the last assessment date of non-fungicide sprayed plants of these periods were 0.81, 0.50 and 0.56, the integral variable *DS** was 0.30, 0.14 and 0.20 for the 1st, 2nd and 3rd peak-epidemic periods, respectively. Similarly, favorability indices of temperature (FI_T) were 0.90, 0.61 and 0.66 and of relative humidity (FI_{RH}) were 0.51, 0.35 and 0.44 for the peak-epidemic periods 1 to 3, respectively. Actual comparisons of F and NF treatments of these three peak-epidemic periods resulted in an average of 31.1% marketable yield loss. Marketable yield (*MY*) was negatively but poorly ($r^2 = 0.021$) correlated with that of *DS**. Integrating maximum plant height (*MPH*) in the model $MY = (a + b \cdot MPH) \cdot (1 - c \cdot DS^*)$, however, improved the fit ($R^2 = 0.35$) with high significance all the parameters. The estimated coefficient of parameter *c* in the model suggested that a 1% *DS** reduced marketable yield by ≈ 1.2%. Interestingly, the actual *MY* losses from the comparison of the F and NF treatments of the 1st and 3rd peak-epidemics were close to the prediction of this model.

Besides BLM, early blight (*Alternaria solani*) occurred during the cool season plantings in October and November but was only dominant for the first 3 to 6 WAT and taken over by BLM thereafter. Taking into consideration the shared and overlapping niche of both diseases and that early blight has minor to negligible importance in the BioNet™ greenhouse, only the impact of BLM was analyzed in the seasonal dynamic study.

In an effort to pinpoint the source of epidemics and design strategies of BLM management, the vertical distribution of BLM was studied across the canopy of the tomato cultivar FMTT260 in the BioNet™ greenhouse. After 16 weeks of a natural epidemic, *DS* of the lower

canopy layer (0-50 cm) was 42% and significantly higher compared to 26% of the middle (51-150 cm) and 5% of the upper (> 150 cm) layer. During the growing period of another natural epidemic, a similar distribution of BLM was observed, but higher *DS* and bigger lesions were detected on leaf positions 5 to 10 (in ascending order from bottom up) than on the first four leaves borne in the nursery. Further non-destructive samplings revealed the earliest BLM symptoms on leaf positions 5 to 8. When cohorts of 5 leaves were formed starting from the bottom, BLM incidence of leaves of the 1st cohort was only 79% while the next two cohorts reached 100%. In artificially inoculated plants, however, BLM was clearly more prevalent in the middle layer of the plant canopy as compared to the lower and the top part. Plants inoculated at 4 weeks after transplanting and monitored 10 days later, showed a *DS* of 43, 67 and 21% on the 1st, 2nd and 3rd cohorts, respectively. Thus, given equal chance of *P. fuligena* inoculum to infect all leaves of a tomato plant at one time, more BLM developed on fully expanded younger leaves than older ones. The high BLM severity of the lower canopy in natural epidemics is not related to the age of tomato leaves, but attributed to the proximity to substrate evaporation coupled with the down-hanging nature of FMTT260 leaves that created a confounding microclimate which led to higher relative humidity within the range of about 50-70 cm.

As greenhouse improvements were mainly focused on reducing high temperature and excluding insects and vectors of viral diseases, the horizon of this research was extended to elucidate the impact of four cooling methods on epidemics of BLM and EB. These cooling methods included the fan and pad cooling system (FAD), natural ventilation (NV) using two mesh sizes (50 and 78, BioNet™ and Econet-T™, respectively) and NV plus shading a nearly infrared pigment on the roof of a 78-mesh greenhouse (Econir). Severities of both diseases (DS_{TOT}), *i.e.* black leaf mold (DS_{BLM}) and early blight (DS_{EB}), were positively related to that of increased cooling and *RH*. There was 50 to 94% and 54 to 61% more disease in the FAD greenhouse during the hot-wet and cool-dry seasons, respectively, as compared to the other three. Next to FAD were the Econir and BioNet™ greenhouses in which average seasonal temperature was 1 to 2ºC higher and relative humidity (*RH*) 10 to 20% less than in FAD. Lowest disease was observed in the Econet-T greenhouse where temperature was highest during noontime. Cooling of the greenhouse in FAD was accompanied by high *RH*, which unabatedly created a fertile ground for epidemics of both diseases. DS_{EB} was dominant in the FAD greenhouse, particularly in season 2 (cool-dry), constituting 68.9% of the $AUDPC_{TOT}$.

Key words: Black leaf mold, *Pseudocercospora fuligena*, epidemiology, tomato, protected cultivation

ZUSAMMENFASSUNG

Makroskopische Beobachtungen von Krankheitssymptomen, Isolierung, Kultivierung und Inokulation von Tomate in Anwendung der Kochschen Postulate sowie die mikroskopische Charakterisierung (morphologisch und molekular) aus Reinkulturen bewiesen, dass *Pseudocercospora fuligena* der Erreger des Schwarzen Blattschimmels (BLM – black leaf mold) in Thailand ist. Auf makroskopischer Ebene waren das Auftreten undeutlicher ausgebreiteter Flecken auf beiden Seiten der Tomatenblätter sowie die Produktion von schwarzen Sporen in großen Mengen überwiegend auf der Blattunter-, mit fortschreitender Infektion auch auf der Oberseite auffällige Merkmale. Auch mikroskopisch deckten sich die im Rahmen dieser Arbeit beobachteten grundlegenden pilzlichen Strukturen, wie Größe und Segmentierung der Konidien (11-128 µm Länge x 3,5-9 µm Breite mit bis zu 12 Septen) sowie die Bündel der Konidienträger, mit früheren Beschreibungen von *P. fuligena*. Trotz seines langsamen Wachstums auf künstlichen Nährmedien wuchs *P. fuligena* auf allen zehn getesteten Medien, jedoch wurden ein starkes Wachstum und eine vermehrte Sporulation nur auf Tomaten-Hafermehl-Agar und Karottenblattsud–Agar beobachtet, wobei beiden Medien $CaCO_3$ zugesetzt wurde. Das Eindringen und Austreten von *P. fuligena* durch die Stomata wurden mittels Licht- und Laserscanmikroskopie an aufgehellten und intakten Blättern durch eine einfache Färbung mittels wässriger Fuchsinsäure-Lösung nachgewiesen. Weitere Untersuchungen der Konidienbildung an aufgehellten und gefärbten Blättern zeigten ein häufiges Auftreten primärer und sekundärer Infektionshyphen sowie einen exponentiellen Anstieg blockierter Stomata-Öffnungen.

Mit diesen Hintergrundinformationen über das Pathogen wurden weitere Studien zu Einflussfaktoren der Infektkette sowohl unter gegebenen Bedingungen (AE – ambient environment) als auch kontrollierten Bedingungen (CE – controlled environment) durchgeführt. Dies geschah hauptsächlich im Hinblick auf Wetter-Parameter wie Temperatur (*T*) und Benetzungsdauer (*WD* – wetness duration) sowie Blattalter (*A* – age). *P. fuligena* wurde bei künstlicher Inokulation unter CE bei 28°C stark begünstigt. Bedenkt man die hohe durchschnittliche Tagestemperatur in den Gewächshäusern in Zentralthailand (durchschnittlich 11 h pro Tag ≤ 28°C und ca. 17 h pro Tag ≤ 30°C), kann davon ausgegangen werden, dass *P. fuligena* auch bei Temperaturen > 28°C noch BLM verursacht. Eine *WD* von mindestens 1 Tag nach Inokulation ist für eine Infektion mit *P. fuligena* notwendig. Bei allen getesteten Parametern waren junge, voll ausgebreitete Blätter mit einem Alter von 3 bis 5 Wochen sowie junge Blätter mit einem Alter von 1 Woche anfälliger als

Blätter, die bereits ein Alter von 7 Wochen hatten. All diese determinierenden Faktoren, d.h. *T*, *WD* und *A*, beeinflussten die Inkubations- (*IP*) und Latenzperioden (*LP*). Beispielsweise waren bei 28°C unter CE die jeweiligen *IP* und *LP* 2,2 bis 5 Tage und 3 bis 9,6 Tage kürzer als bei 24°C. Während in den CE-Experimenten bei 28°C *IP* bzw. *LP* von 11 bis 13 Tagen bzw. von 12,5 bis 18 Tagen dauerten, waren die beiden Perioden in einem der AE-Experimente nur 9,2 bis 14,0 bzw. 10,4 bis 16,0 Tage lang. Bei einer natürlichen Infektion verlängerte sich *IP* allerdings auf 11 bis 25 Tage.

Um die Auswirkungen von BLM auf das Wirtswachstum zu überprüfen, wurden 2005 vier sequenzielle Pflanzungen durchgeführt. Der Krankheitsbefall, der wöchentlich als Befallshäufigkeit (*DI*) und Befallstärke (*DS*) geschätzt wurde, konnte gut mit logistischen Funktionen beschrieben werden. Die sigmoiden Kurvenverläufe der Epidemien deuteten darauf hin, dass im Laufe der Saison multiple Infektionszyklen auf den Blättern von Tomaten auftraten. Bei einem Vergleich von Behandlungen mit (F) und ohne (NF) Fungizid zeigte sich, dass starker Befall das Wirtswachstums deutlich reduziert, insbesondere die gesunde Blattfläche (*HLA* – healthy leaf area). Die standardisierte Befallsstärke (*DS**) von 0,3 (als Proportion) in den zwei stärksten ungestörten Epidemien ergab einen Verlust der *HLA* von 68% bei einem Vergleich der Behandlungen F und NF. Vergleicht man den gesunden Blattflächeindex (*HLAI* – healthy leaf area index) von gesunden Pflanzen ($HLAI_{HP}$) mit dem von befallenen Pflanzen in den F- ($HLAI_F$) bzw. NF-Varianten ($HLAI_{NF}$), ergab sich ein *HLAI*-Verlust von 11 bzw. 50%. Zwei Applikationen des Fungizids Mancozeb 4 und 6 Wochen nach dem Umpflanzen verminderten den BLM-Befall um 60 bis 90%.

Das Auftreten solcher Unterschiede in der Epidemiestärke zwischen den Pflanzungen 2005 veranlasste uns zu weiteren Untersuchungen der saisonalen Entwicklung von BLM in Bezug zu Wetter und Ertrag. Während der 24 14-täglichen bzw. monatlichen Pflanzungen (Juni 2005 bis März 2007) wurden drei Perioden mit besonders hohen BLM-Epidemien festgestellt: die August- und September-Pflanzungen in 2005 (1) und 2006 (2) sowie die Dezember- und Januar-Pflanzungen in 2005/06 (3). Die maximalen *DS* bei der letzten Bonitur der nicht-fungizidbehandelten Pflanzen dieser drei Perioden waren 0,81, 0,50 und 0,56. Die Integralvariable *DS** (das ist die mittlere Befallsstärke während der Dauer der Beobachtung) erreichte 0,30, 0,14 und 0,20 (als Proportion) in der ersten, zweiten und dritten Spitzenbefallszeit. Die "Favorability"-Indizes von Temperatur (FI_T) bzw. relativer Feuchtigkeit (FI_{RH}) für die drei Perioden mit hohen Epidemien waren 0,90, 0,61 und 0,66 (FI_T) bzw. 0,51, 0,35 und 0,44 (FI_{RH}). Bei einem Vergleich des vermarktbaren Ertrags der F- und NF-Varianten in den drei Perioden ergab sich ein durchschnittlicher Verlust von 31.1%.

Der vermarktbare Ertrag (*MY*) war nur sehr schwach (r^2 = 0,021) mit *DS** korreliert. Wenn aber die maximale Pflanzenhöhe (*MPH*) in dem Modell $MY = (a + b \cdot MPH) \cdot (1 - c \cdot DS^*)$ mitberücksichtigt wurde, erhöhte sich das Bestimmtheitsmaß auf R^2 = 0,35 mit hoher Signifikanz aller Parameter. Der Wert des Koeffizienten *c* zeigte, dass 1% *DS** den vermarktbaren Ertrag um1,2% reduzierte. Der mit dem Modell vorausgesagte Verlust bei der 1. und 3. Periode mit hohem Befall stimmte mit dem Verlust überein, der sich aus der Ertragsdifferenz zwischen den F- und NF-Varianten ergab.

Neben BLM trat die Dürrfleckenkrankheit (Early blight = EB), hervorgerufen durch *Alternaria solani*, in den Pflanzungen während der kalten Saison von Oktober bis November auf, war aber nur in den ersten 3 bis 6 WAT dominant und wurde später von BLM übertroffen. Da beide Krankheiten sich überlappende Nischen teilten und unter Berücksichtigung der geringen Bedeutung der Dürrfleckenkrankheit im BioNet™-Gewächshaus, in dem die Untersuchung durchgeführt wurde, wurde nur der Einfluss von BLM in der Studie der saisonalen Dynamik analysiert.

Um die Ausgangquelle der Epidemien zu lokalisieren und eine Strategie zum Management von BLM zu entwerfen, wurde die vertikale Verteilung von BLM im Bestand der Tomatensorte FMTT260 in einem BioNet™-Gewächshaus untersucht. Nach einer 16-wöchigen unbeeinflussten Epidemie war die Befallsstärke (*DS*) im unteren Bestandesbereich (0 – 50 cm) mit 42% signifikant höher als 26% im mittleren (51 -150 cm) bzw. 5% im oberen (> 150 cm) Bereich. Bei einer anderen natürlichen Epidemie wurde eine ähnliche Verteilung der Krankheit beobachtet, wobei aber auf den Blattpositionen 5 bis 10 (in aufsteigender Reihenfolge vom Substrat) *DS* höher und die Läsionen größer waren als auf den ersten vier Blättern, die bereits in der Anzucht gebildet worden waren. In weiteren Stichproben wurden die ersten Symptome an den Blattpositionen 5 bis 8 festgestellt. Bildet man Kohorten von jeweils 5 Blattpositionen von unten beginnend, war die Befallshäufigkeit der Blätter bei der ersten Kohorte nur 79%, während bei den nächsten beiden 100% erreicht wurde. An Pflanzen, die künstlich inokuliert wurden, trat BLM stärker im mittleren Bestandesbereich auf als oben und unten. Bei Pflanzen, die 4 Wochen nach dem Auspflanzen komplett inokuliert und 10 Tage später bonitiert wurden, betrugen die Befallsstärken der drei ersten Kohorten 43, 67 und 21%. Daher entstand mehr Befall an vollständig entfalteten jungen als an alten Blättern, obwohl für alle Blätter der Tomatenpflanze die Infektionswahrscheinlichkeit gleich war. Der hohe BLM-Befall des unteren Bestandesbereichs bei ungestorten Epidemien steht nicht in Beziehung zu dem Alter der Blätter, sondern ist auf die Nähe zu der Evaporation des Substrats

und die herabhängenden Tomatenblätter zurückzuführen, wodurch ein Mikroklima mit hoher relativer Feuchtigkeit in der Bestandeshöhe von 50 bis 70 cm entsteht.

Da Gewächshausverbesserungen auf eine Verminderung der hohen Temperaturen und auf das Fernhalten von Insekten und Krankheitsvektoren zielen, wurden auch Untersuchungen zur Auswirkung von Kühlmethoden auf Epidemie von BLM und EB durchgeführt. Die getesteten Kühlmethoden waren die Mattenkühlung (fan and pad = FAD), die natürliche Ventilation (NV) unter Verwendung von zwei Maschengrößen (50 und 78, BioNet^{TM} bzw. Econet T^{TM}) und NV in Kombination mit einem nahezu infraroten Pigment auf dem Dach eines 78-maschigen Gewächshauses (Econir). Die Gesamtbefallsstärke beider Krankheiten (DS_{TOT}), d.h. des Schwarzen Blattschimmels (DS_{BLM}) wie auch der Dürrfleckenkrankheit (DS_{EB}), war negativ mit der Temperatur, aber positiv mit *RH* korreliert. In dem FAD-Gewächshaus wurde 50 – 94 % (heiß – feuchte Saison) und 54 – 61 % (kühl – trockene Saison) mehr Befall im Vergleich zu den anderen drei Gewächshäusern festgestellt. Etwas niedriger als in FAD war der Befall in den Econir- und BioNet-Gewächshäusern, in denen die durchschnittliche saisonale Temperatur 1 bis 2 °C höher und die relative Feuchtigkeit 10 bis 20% niedriger war als in FAD. Der geringste Befall wurde im Econet-Gewächshaus beobachtet, in dem die Temperatur in der Mittagszeit am höchsten war. Die Kühlung des FAD-Gewächshauses führte zu hoher *RH*, die permanent günstige Bedingungen für die Epidemien beider Krankheiten lieferte. In Saison 2 (kühl - trocken) war EB im FAD-Gewächshaus besonders dominant und hatte bei der letzten Bonitur einen Anteil von 68.9% des Gesamtbefalls.

Schlagworte: Schwarzen Blattschimmel, *Pseudocercospora fuligena*, Epidemiologie, Tomate, geschützter Anbau

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ABBREVIATIONS

***A*:**	Leaf age
AE:	Ambient environment
AIT:	Asian Institute of Technology
ANOVA:	Analysis of variance
***AUDPC*:**	Area under the disease progress curve
***AUHGC*:**	Area under the host growth curve
AVRDC:	Asian Vegetable Research and Development Centre
BLM:	Black Leaf Mold
BMA:	Biomalt agar
CE:	Controlled environment
CLDA:	Carrot leaf decoction agar
CRD:	Completely randomized design
DAI:	Days after inoculation
DAS:	Days after sowing
DAT:	Days after transplanting
DCT:	Direct conidial transfer
***DI*:**	Disease incidence on a plant basis
$\boldsymbol{DI_{SP}}$**:**	BLM incidence of sporulating units
$\boldsymbol{DI_{SY}}$**:**	BLM incidence of symptomatic units
$\boldsymbol{DI_{BLM}}$**:**	Disease incidence of black leaf mold
$\boldsymbol{DI_{EB}}$**:**	Disease incidence of early blight
***DS*:**	Disease severity on a plant basis
$\boldsymbol{DS_{BLM}}$**:**	Disease severity of black leaf mold
$\boldsymbol{DS_{EB}}$**:**	Disease severity of early blight
$\boldsymbol{DS_{TOT}}$**:**	Disease severity of black leaf mold and early blight
EB:	Early blight
***FI*:**	Favorability index
$\boldsymbol{FI_{RH:}}$	Favorability index of relative humidity
$\boldsymbol{FI_{RHD:}}$	Favorability index of day relative humidity
$\boldsymbol{FI_{RHN:}}$	Favorability index of night relative humidity
$\boldsymbol{FI_{T}}$**:**	Favorability index of temperature
$\boldsymbol{FI_{TD}}$**:**	Favorability index of day temperature

FI_{TN}: Favorability index of night temperature

HAI: Hours after inoculation

HLA: Healthy leaf area

HLAI: Healthy leaf area index

$HLAI_F$: Healthy leaf area index with fungicide treatments

$HLAI_{NF}$: Healthy leaf area index with out fungicide treatments

$HLAI_{HP}$: Healthy leaf area index of healthy plants

I_H: Inoculated with high inoculum concentration

I_L: Inoculated with low inoculum concentration

IP: Incubation period

ITS: Internal transcribed spacer

LA_C: Leaf area of the compound leaf

LA_T: Leaf area of the terminal leaflet

LL_C: Leaf length of the compound leaf

LL_T: Leaf length of the terminal leaflet

LP: Latent period

LT: Lesion transfer

PCR: Polymerase chain reaction

PH: Plant height

RH: Relative humidity

RH_{DM}: Daily mean relative humidity

SCT: Suspension conidial transfer

SEM: Scanning electron microscopy

T: Temperature

T_{DM}: Daily mean temperature

TLA: Total leaf area

TLC: Total lesion count

TLN: Total leaf number

T_{max}: Maximum temperature

T_{min}: Minimum temperature

TOA: Tomato oatmeal agar

WAT: Weeks after transplanting

WD: Wetness duration

GENERAL INTRODUCTION

The ability of agriculture to support the growing population has been a concern for generations and continues to be high on the global policy agenda (Rosegrant and Cline, 2003). The threat from plant diseases alone accounts for at least 10% of crop losses globally and is thus partly responsible for the suffering of 800 million people who lack adequate food (Strange and Scott, 2005). Implementation of global policies towards achieving self-sufficiency and sustainable food production has made the choice of intensification amongst the best options. One of these options is protected cultivation, which along with other strategies entails boosting productivity within a give piece of small land area. More importantly, in many Asian countries and other parts of the globe, there is a dramatic tendency of shift towards protected cultivation owing to dynamic fluxes of harsh environments and pest problems causing a significant reduction of productivity, dwindling cropland due to natural calamities, rapidly expanding human settlements and abandonment of fertile lands because of mismanagements. Besides, production in protected systems is one more development in the trend towards better quality, more diversified and increased value added produce for consumer selection of vegetables like tomato (Jones, 2008) and other agricultural products.

Protected cultivation systems which are implemented through use of cladding materials to create a secluded environment for the growth of a crop, however, are also accompanied by many pests that are favored by the long-standing warmth and relative humidity. Averting pest problems in such systems are mainly entertained through exclusion, a strategy which helped a lot for insect pests and viral vectors but not for fungal pathogens and other microbes owing to their microscopic nature and hence multitude of options for their entrance into these protected systems (Paulitz and Bélanger, 2001). Consequently, fungal pathogens that are highly favored by confined warmth and humidity are gaining more and more economic importance and thereby forced the farming community to dwell much on use of fungicides to maintain productivity. This trend of heavy fungicide reliance is reflected mainly in Asian countries for production of vegetables and fruits in terms of global fungicide sales (Kuck and Gisi, 2007). In Thailand, for instance, there has been an increasing tendency in the use of pesticides over the past decades which resulted in associated environment and health problems (Ecobichon, 2001; Thapinta and Hudak, 2000). Despite concerted efforts of comprehensive extension works in use of biological and other integrated strategies to combat pests and mitigate hazardous impacts of fungicides (Lakchai and Chiradej, personal communication), there is a need for epidemiological studies to augment these efforts and come up with basic information

on disease forecasting and setting thresholds for effective and sustainable disease management.

This dissertation covers a wide array of epidemiological investigations that were primarily undertaken on the fungal pathogen *Pseudocercospora fuligena* (Roldan) Deighton (syn. *Cercospora fuligena* Roldan) and epidemics of the black leaf mold (BLM) disease it causes on tomato (*Solanum lycopersicum* L.). Besides, in one of the component studies, epidemics of BLM and early blight (EB) caused by the necrotrophic fungus *Alternaria solani* (Ellis & Martin) Jones & Grout, were compared amongst four greenhouses with different cooling methods.

The research work was carried out at the Institute of Plant Diseases and Plant Protection of Leibniz Universität Hannover (LUH), Germany, and at the greenhouse construction area of the "Protected Cultivation Project" located in the campus of Asian Institute of Technology (AIT), Thailand, from October 2004 until December 2007. The host plant, tomato (*S. lycopersicum*), a model crop chosen for an integrated multidisciplinary research in the project, is a highly cherished and most widely grown solanaceaous vegetable next to potato globally (Rubatzky and Yamaguchi, 1997). The diversity of pathogens on tomato also emphasizes its growing importance as a favorable model for studying plant-pathogen interactions (Meissner *et al.*, 1997; Arie *et al.*, 2007). Tomato belongs to the family solanaceae and genus Solanum that is grown for its edible fruit (Jones, 2008). *S. lycopersicum* is believed to have originated in the coastal strip of western South America from the equator to about 30° latitude south (Papadopoulos, 1991). The tomato plant cannot tolerate frost, and high temperatures above 35°C which reduce fruit set and inhibit development of normal fruit color. The optimal temperature range for normal plant growth and fruit set is between 18.5 and 26.5°C with day and night temperature ranges between 21 to 29.5°C and 18.5 to 21°C, respectively (Jones, 2008). Whereas acreages of land devoted to tomato is getting bigger owing to the increasing demand of tomato for its lycopene and other important components with respect to human health, its productivity is declining due to biotic and abiotic stresses. In Thailand, for instance, despite the steady increment of tomato production, productivity is declining (FAO, 2007). Although some preliminary experiments of artificial inoculations at LUH were conducted on the cultivars King Kong and Lizzy, all the main experiments were performed using the fresh market tropical tomato (FMTT260) an F1-hybrid from AVRDC (Shanhua, Taiwan).

BLM is widespread in the tropics and subtropics with its origin assumed to be in Asia since most reports are from this continent (Hartman and Wang, 1993; Wang *et al.*, 1996). Its distribution is mapped by CAB International (1982). Economic damage on tomato is reported

from the Philippines (Roldan, 1938), Japan (Yamada, 1951), India (Mohanty and Mohanty, 1955), Taiwan (Hartman and Wang, 1992) and other Asian countries (Hsieh and Goh, 1990; Hartman *et al.*, 1991; Wang *et al.*, 1996). In Thailand, except an earlier report of Chandrasrikul (1962), there is lack of well-documented study about the disease until a recent survey (Kandziora, personal communication) that highlighted its importance as a major fungal foliar disease of tomato under protected cultivation. This dissertation covers results from a follow-up research in the past three years (2004-2007) which addressed this aspect from the seasonal dynamic study of BLM epidemics and its effect on yield.

The causative agent of BLM was first reported as *Cercospora canescans* Ellis & G. Martin by Solheim and Stevens (1931). It was then appropriately described and categorized as *Cercospora fuligena* first in the Philippines (Roldan, 1938) and its distinguishing feature of fuliginous sporulation was further elaborated in the book of Chupp (1954). It assumed its current nomenclature *Pseudocercospora fuligena* in 1976 (Deighton, 1976). Researches on this fungus were mainly undertaken at the world's vegetable centre (AVRDC) with the latest scientifically refereed publication being from Wang *et al.* (1996). The horizon of the disease is extending with a recent report from Roraima, Brazil (Halfeld-Viera, 2006). *P. fuligena* is highly favored by high humidity, moderate to high temperatures and low night temperatures which result in extended periods of leaf wetness (Chupp, 1954; Blazquez and Alfieri, 1974; Sherf and MacNab, 1986; Blazquez, 1991; Wang *et al.*, 1996).

Initial symptoms of the disease appear as small, pale yellow lesions with no definite margin on either the upper or lower leaf surface. These lesions have white fungal growth that turns gray to black as the fungus sporulates first at the lower side and later on black sooty fungal growth will occur on both leaf surfaces. Although comprehensive phylogenetic and condiogenesis studies about the genus *Pseudocercospora* are extensively underway (Beilharz, 1994; Goodwin *et al.*, 2001; Babu *et al.*, 2002; Beilharz *et al.*, 2004; Crous *et al.*, 2004; Grice *et al.*, 2006), the area of epidemiological studies of *P. fuligena* on tomato remained scanty for more than a decade. Research studies in this dissertation will attempt to bridge this gap by reviewing past efforts and incorporating new findings which could be of paramount importance towards a sustained management this foliar disease in the future.

As part of agro-ecosystems management, plant disease epidemiology is receiving a new dimension and purpose with a change in the philosophy and strategy of pest control in the new ideas of integrated pest management. This could principally be achieved through integrated multidisciplinary efforts that would eventually help in manipulating the three epidemiological

strategies, *i.e.* eliminate or reduce the initial inoculum or delay its appearance, slow the rate of disease increase and shorten the time of exposure to the pathogen (Berger, 1977; Zadoks and Schein, 1979; Kranz, 2003; Nutter, 2007). Within the framework of this dissertation, an attempt was made to cover the epidemiological aspects of BLM from the perspectives of its causative agent to the effect it causes on its host. Moreover, as part of disease management, its seasonal dynamics as a function of weather vis-à-vis yield and fungicide application was studied. Together with the comparative epidemics in four different greenhouses, outputs from this study would enable designing future integrated strategies towards managing the disease. To put it in a nutshell, this dissertation is composed of three main components each of which entail two specific chapters within themselves.

The first component of this study was devoted to aspects related to the pathogen, *P. fuligena*. In one of chapters, *in vitro* and *in vivo* studies of its biology and infection mechanisms were presented. Identification and characterization of the pathogen were done in collaboration with the Centraalbureau voor Schimmelcultures. Infection mechanisms like modes of penetration and egress were studied microscopically at LUH, AIT and Thailand Science Park. The second chapter aimed at determining monocyclic components of BLM from non-destructive and detached leaf experiments under controlled and ambient environments. Disease progresses of symptomatic and sporulating experimental units were compared with the prevailing favorability factors like temperature and wetness durations. Besides, leaf age at a time of inoculation was tested in all the experiments. Monocyclic components measured in terms of incubation and latent periods of BLM across these factors were compared.

The next component focused on investigation and quantification of the effect of the disease on the host and its distribution across the canopy. This study, as a prelude to the economic importance of BLM under protected cultivation in Thailand, was performed on selected four plantings. These plantings were mainly targeted to acquire information from the hot-wet (Kleinhenz *et al.*, 2006) or rainy season (Thapinta and Hudak, 2000). Healthy leaf area index of the fresh market tropical tomato cultivar FMTT260 was compared amongst treatments of the plantings and that of fungicide applications. Such an approach to verify the effects of a given pathosystem was reported from earlier research (Waggoner and Berger, 1987; Seem, 1988; de Jesus Junior *et al.*, 2003; Mersha and Hau, 2008). The other chapter, as part and parcel of the study on the host, researched spatio-temporal dynamics of BLM across plant canopy of FMTT260. With this research, it was possible to pinpoint disease zonation across

the canopy and more importantly to justify reasons that caused such patterns like concentration of BLM on the lower canopy of the plant.

The last component was mainly related to seasonal dynamics and comparative epidemics studies that would ultimately help in prediction and designing a sound disease management strategy for BLM. One of the chapters accordingly focused on studying the seasonal dynamics of BLM epidemics from 24 monthly and bi-monthly plantings. The final output from this research showed the relationship between the three components, *i.e.* disease-weather-yield across the plantings. Favorability indices of temperature and relative humidity were then regressed against BLM severity. Marketable yield too was compared with host parameters like plant height and leaf number, separately and in a multiple regression. Further regressions were done to relate severity and mean plant height with that of marketable yield. The sixth chapter addressed the comparative epidemics of the foliar diseases BLM and early blight across four greenhouses with different cooling methods.

Chapter 1: Biology and infection mechanisms of *Pseudocercospora fuligena* (Roldan) Deighton causing black leaf mold disease on tomato (*Solanum lycopersicum* L.)

ABSTRACT

In this study, aspects related to identification, *in vitro* and *in vivo* characterization of conidial morphology and infection mechanisms of the hyphomycetous fung*us P. fuligena* were investigated. Macroscopic observations of symptoms and signs of the disease as well as isolation, culturing and characterization (morphological and molecular) of its causative agent from axenic cultures and the follow-up inoculations on tomato (according to Koch's postulate) proved *Pseudocercospora fuligena* to be the causative agent of black leaf mold (BLM) in Thailand. Macroscopically, appearance of indistinct effuse patches on both sides of tomato leaves, amphigenous fructification and prolific production of fuliginous lesions predominantly on abaxial side and as infection progresses also on the adaxial side were some of the peculiar features of the disease observed in this research. Microscopically, too, basic fungal structures like conidial dimension and segments (11–128 µm in length x 3.5–9 µm in width, with up to 12 septations) as well as fascicles of conidiophores observed in this study fit to earlier descriptions of *P. fuligena*.

Direct transfer of conidia from profusely sporulating lesions was found to be the easiest and quickest method of isolation that was less prone to contamination for *in vitro* experimentation of *P. fuligena*. Despite its slowness, *P. fuligena* grew well on ten artificial culture media tested but improved growth and sporulation occurred on tomato oatmeal agar and carrot leaf decoction agar, both supplemented with $CaCO_3$.

Stomatal penetration and egress of *P. fuligena* were testified after light and scanning electron microscopy of cleared as well as intact leaves. Further pathogenesis studies on artificially inoculated, cleared and acid fuchsin stained leaves indicated prevalence of primary and secondary infection hyphae. Besides, *in situ* progression of blocked stomatal apertures which averaged 154, 401 and 2043 μm^2 area at 7, 12 and 17 days after inoculation, respectively was described well with an exponential growth function.

Key words: *Pseudocercospora fuligena*, macroscopic and microscopic features, *in vitro*, *in vivo*, penetration, egress

INTRODUCTION

The hyphomycetous fungus *Pseudocercospora fuligena* (Roldan) Deighton (syn. *Cercospora fuligena* Roldan) is known to cause black leaf mold (BLM) disease, also synonymously called "*Cercospora* leaf mold" in earlier literature. The disease and the pathogen were first described from the Philippines by Roldan (1938) and further elaborated by Chupp (1954) who considered all species of *Cercospora* on tomato be *C. fuligena*. The pathogen was further characterized and assumed its current taxonomic nomenclature after Deighton (1976). *P. fuligena* belongs to the group dothideales and the teleomorphs known belong to *Mycosphaerella* within ascomycotina (Goodwin *et al.*, 2001; Crous *et al.*, 2004; Agrios, 2005). There are, however, several thousand anamorph species that lack known teleomorphs (Crous and Braun, 2003).

BLM is widespread in the tropics and subtropics with its origin assumed to be in Asia since most reports are from this continent (Hartman and Wang, 1993; Wang *et al.*, 1996). Its distribution is mapped by CAB International (1982). Economic damage caused on tomato (*Solanum lycopersicum* L.) is reported from India (Mohanty and Mohanty, 1955), Japan (Yamada, 1951), Philippines (Roldan, 1938), Taiwan (Hartman and Wang, 1992) and other Asian countries (Hsieh and Goh, 1990; Hartman *et al.*, 1991; Wang *et al.*, 1996). In Thailand, apart from an earlier report of Chandrasrikul (1962), well documented and published studies about the pathogen and the disease it causes were very few until a recent survey (Kandziora, personal communication) implicated its importance as a major fungal foliar disease of tomato under protected cultivation.

A follow-up research in the past three years (2004-2007) has confirmed this situation whereby average yield losses of up to 31.3% were recorded under ambient greenhouse condition of high BLM epidemics during the hot wet seasons of 2005 and 2006 (Mersha and Hau, unpublished). Halfeld-Vieira *et al.* (2006) similarly reported severe damage of some tomato varieties (cv. Santa Clara) under protected cultivation in Ruraima, Brazil. In Taiwan, Hartman *et al.* (1991) and Hartman and Wang (1992) recorded 54 to 86% BLM severity on susceptible varieties without fungicide application which resulted in a yield of loss of up to 32%.

BLM symptoms begin with the development of irregularly shaped chlorotic spots with sporulation initially evident mainly on abaxial side of the tomato leaves. During advanced stages, the lesions enlarge and coalesce with abundant dark sporulation on both surfaces. Later at high disease level, leaves roll upward, die prematurely and generally remain hanging on the plant with a soot-covered appearance (Wang *et al.*, 1995, 1996; Anonymous, 2004b).

The dry-spored and air-dispersed conidia of *P. fuligena* can survive from one crop to the next without an intermediate host, and thus crop debris can serve as an important source of primary inoculum. *P. fuligena* overwinters in old diseased plant parts (Chupp and Sherf, 1960; Sherf and MacNab, 1986) for a time duration of up to 7 months on unburied crop debris in the field and up to 18 months in dry conditions (Wang *et al.*, 1996). Besides, other plants of solanaceous family, for instance *Solanum nigrum* (Blazquez, 1991; Hartman *et al.*, 1991) were found to be alternate hosts for the pathogen. Wang *et al.* (1996) reported that conidia still germinate after 18 months stored at low temperatures (4 to 20°C), but not at 28°C or higher.
Although its natural host range appears to be narrow, the fungus infects other *Lycopersicon* spp. (Hartman and Wang, 1993) and *Solanum nigrum* but no infection was seen on *Nicotiana benthamiana* (Hartman *et al.*, 1991). Wang *et al.* (1995) investigated the host range of *P. fuligena* by inoculating 137 accessions representing 26 species and five genera of solanaceous plants. The disease developed on 106 accessions, specifically on black nightshade, some eggplant-related species, several *Capsicum* species, and on all *Lycopersicon* spp., although the range of disease severity varied greatly among the wild tomato accessions.
Despite the growing tendency of tomato production under protected cultivation (Jones, 2008) and the high favorability of such confined environments that led to a significant yield loss due to BLM during the warm and humid seasons, works related to the disease are scanty if not non-existent in the past years after the latest publication of Wang *et al.* (1996) a decade ago. Exceptions are only few yearly reports and fact sheets (Anonymous, 2003, 2004a, 2004b) of the world vegetable centre (formerly known as Asian vegetable research and development centre, AVRDC). In Thailand, apart from the above-mentioned report of Chandrasrikul (1962) regarding prevalence of the disease in the country, there is no single study on the biology and infections mechanisms of the causative agent, *i.e. P. fuligena*.

Hence, this research was initiated with the objectives of studying the identification and characterization of the causative agent of BLM under protected cultivation in Thailand and acquiring an insight into its biology and infection mechanisms. Its ultimate goal is to update previous information about the pathogen and the disease it causes, to present outcomes of studies on conidial morphology and *in vitro* cultural studies, to describe mechanisms of penetration, egress and *in situ* progression of the disease. With an overall additional information and review of past works on *P. fuligena*, this research would contribute its share in elucidating basic understanding of the biology and infection mechanisms of the pathogen.

MATERIALS AND METHODS

Symptomatic characterization, isolation, culturing and identification of BLM

Macroscopic appearances of signs and symptoms of black leaf mold disease from both the adaxial and abaxial leaf surfaces of tomato (*S. lycopersicum*) cv. FMMT260 were captured with a sharply focused digital camera. For microscopic observations of the fungal structures, the sooty part of a profusely sporulating lesion was slightly touched with a camelhair brush and the conidia (and conidial fascicles including conidiophores) were then fallen onto a drop of 0.5% acid-fuchsin on a clean glass slide. Samples were then immediately covered with cover slip and mounted on a microscope (100x and 400x magnifications) fitted with a camera. Within a time of 30 seconds to 1 minute, well-focused pictures of the fungal structures were shot from different spots of the slide. The acid-fuchsin stain was prepared after intense stirring of the aqueous mixture using vortex magnetic stirrer for a minute and filtering through double-layered cheesecloth to avoid appearance of any crystal clods during mounting and microscopic observation. Accordingly, conidial dimensions were measured and the number of segments per conidia was counted.

Further tests on conidial germination were carried out after conidia of *P. fuligena* were brushed off from infected leaves using a camelhair brush and dropped on to potato dextrose agar (PDA), tomato oatmeal agar (TOA) and water. Germination of conidia in the respective medium was inspected after 24 hours under Olympus CX 40 light microscope fitted with a camera and scaled using Motic Images Plus (Version 2.0). The germination test was further performed on detached leaves that were put in small tightly closed plastic containers at dry, moist and wet conditions.

An experiment to identify an easy and fast method of isolation of *P. fuligena* was conducted using the recommended media, TOA (Hartman *et al.*, 1991), both at Leibniz Universität Hannover (LUH), Germany and at Asian Institute of Technology (AIT), Thailand. The three methods of isolation tested were direct conidial transfer (DCT), conidial suspension transfer (CST) and transfer of lesion from advancing margin (LT). Whereas, a 6-mm diameter lesion with profuse sporulation was taken and aseptically slided at four lines running across the diameter of 9-cm diameter sterilized Petri dish for DCT, a conidial suspension of 2×10^4 mL^{-1} was uniformly spread over the surface of the media for CST. For the last method, four lesions with complete black sporulating appearance were aseptically put at four places within the Petri dish. The experiment was carried out in a completely randomized design with three treatments

(DCT, CST and LT) replicated twenty times. Evaluation of growth and contamination was carried out on each Petri dish 72 hours after isolation following the scaling in Table 1.

Table 1. Scaling (0 to 3) of colony growth of *P. fuligena* and extent of contamination while using the three methods of isolation (DCT - direct conidial transfer, CST - conidial suspension transfer and LT - transfer of lesion from advancing margin).

Scale	Growth	Contamination
0	Poor (no growth)	Pure (non-contaminated)
1	Low growth (< 25% visible)	Less contaminated (e.g. at corner or confined)
2	Intermediate (26 up to 60% visible)	Highly contaminated (but culturable, confined)
3	High (> 60% visible)	Spoiled (discarded, not usable)

An experiment comprising 10 different artificial growth media, with or without sealing, was run on 9-cm diameter sterilized plastic Petri dishes. Alphabetically ordered, the media tested included biomalt agar (BMA), carrot leaf decoction agar (CLDA), carrot leaf oatmeal agar (CLOA), malt extract agar (MEA), oatmeal agar (OMA), potato carrot agar (PCA), potato dextrose agar (PDA), tomato oatmeal agar (TOA), vegetable agar (V_8) and water agar (WA). Refer the appendix at the end of this chapter for composition and preparation of each culture medium.

Following the above-mentioned DCT method, profusely sporulating lesions were brought from the greenhouse, pieces were cut aseptically using a 4-mm diameter cork borer and three pieces were aseptically and gently pressed onto each media (point inoculation). Each medium was replicated five times with three cut pieces put in each Petri dish forming a total of 15 replications. A week later (7 days after inoculation), each medium was evaluated for growth (radial expansion in mm) as well as extent of contamination following the same scaling in Table 1.

Further experiments on identification of *P. fuligena* were carried out at LUH and AIT. At LUH, DNA extraction and inoculation of three axenic cultures isolated from heavily BLM infected leaves that were brought from AIT in 2003 (Kandziora, personal communication) and 2004 were performed using Chelex® 100 method (Wichura, 2007). The complete internal transcribed spacer (ITS) region was amplified using the universal ITS1 and ITS4 primers

(White *et al.*, 1990; Goodwin *et al.*, 2001). The PCR product was then sent for sequencing to the company MWG Biotech AG (Germany). To identify the extent of homology to closely related species, a BLAST (Altschul *et al.*, 1997) search was performed. The DNA sequences were further aligned with the profile mode of Clustal X 1.81 (Thompson *et al.*, 1997).

In 2005, samples of BLM diseased tomato leaves with profuse sporulation were collected, immersed in 70% ethanol for one minute and then gently rinsed in two sets of distilled water, each lasting for a minute. Leaf samples were then blotted dry on sterile filter papers, laid flat in between A4 sized papers, pressed tightly and sent as herbarium to the Centraalbureau voor Schimmelcultures (The Netherlands) for further confirmation of the causative organism of the disease.

Penetration and infection mechanisms of *P. fuligena*

Stomatal composition of young tomato terminal leaflets (from the leaf at the 6th internode of 2 weeks old plants) was determined using a simple light microscopy. Leaflets were laid flat on a glass slide (on either abaxial or adaxial side) and the number of stomatal openings within the focused area was counted.

Leaflets on other plants of the same age were marked and inoculated with a conidial suspension of 2 x 10^4 mL^{-1} on both sides. Those inoculated plants were covered with black plastic bags and kept for 16 hours under high humidity (95 to 99%) in the greenhouse. 48 to 96 hours after inoculation (HAI), plants were carried to the laboratory and the inoculated (and fully expanded) leaves were stained with drops of 0.05% aqueous acid fuchsin solution. These inoculated leaflets were laid flat and fuchsin solution droplets were poured until the whole leaflet area was covered with the droplets. After half an hour, 6 mm of stained leaflet section was randomly cut with a cork borer and observed under a light microscope with high light intensity (augmented with external light illuminator).

From the same inoculated leaflets, samples were prepared for scanning electron microscopy (Hitachi 3400N-SEM, Japan) following the modified methods of Babu *et al.* (2002) and Blodgett and Stewart (2002). However, due to unavailability of a critical point drier (EMS – 850) using CO_2 as transition fluid, most of the attempts for observation of the penetration process using SEM were not successful. Nevertheless, observation of egress using SEM was possible following the same procedure with the samples only air-dried. Further infection, sporulation and progression of substomatal stroma were followed using simple techniques of light microscopy after whole leaf clearing of heavily infected leaf sections. For the light

microscopy, two techniques of leaf clearing, *i.e.* those of Bruzzese and Hasan (1983) and de Luna *et al.* (2002) were compared.

Data collection and analyses

Data from quantification of the radial colony growth were analyzed using ANOVA (SAS, 2003) and further separation of means was done using Tukey's test at $p < 0.05$. For the analyses of the frequency counts and scale comparisons of the three isolation methods tested, the exact Wilcoxon test (Mehta and Patel, 2001) was used. All regression analyses were made using SigmaPlot (2006).

RESULTS

Macroscopic and microscopic observations of symptoms and signs of BLM

After natural and artificial inoculations, the initial macroscopic symptoms of black leaf mold caused by *P. fuligena* appeared as small, pale brown yellow effuse patches on both the upper (Fig. 1a) and lower (Fig. 1b and c) sides of tomato leaves. In a day or two, depending on favorability, lesions on the lower surface turned gray then black (fuliginous) as the fungus sporulated (Fig. 1b and c). Sporulation and the fuliginous appearance were thus predominant but not exclusively hypophylous since the adaxial side also showed these symptoms at times of heavy epidemics. Amphigenous fruiting is mentioned as one feature distinguishing BLM from leaf mold caused by *Cladosporium fulvum* (Anonymous, 2004b; Deighton, 1976). Later, during heavy infection, lesions coalesced leading to a complete drying of the leaf. Unless mechanically disturbed, for instance by shaking or swift shocks of the hanging strings (tomato stakes), there was no visible effect of defoliation as the infected leaves always remained hanging on the stem.

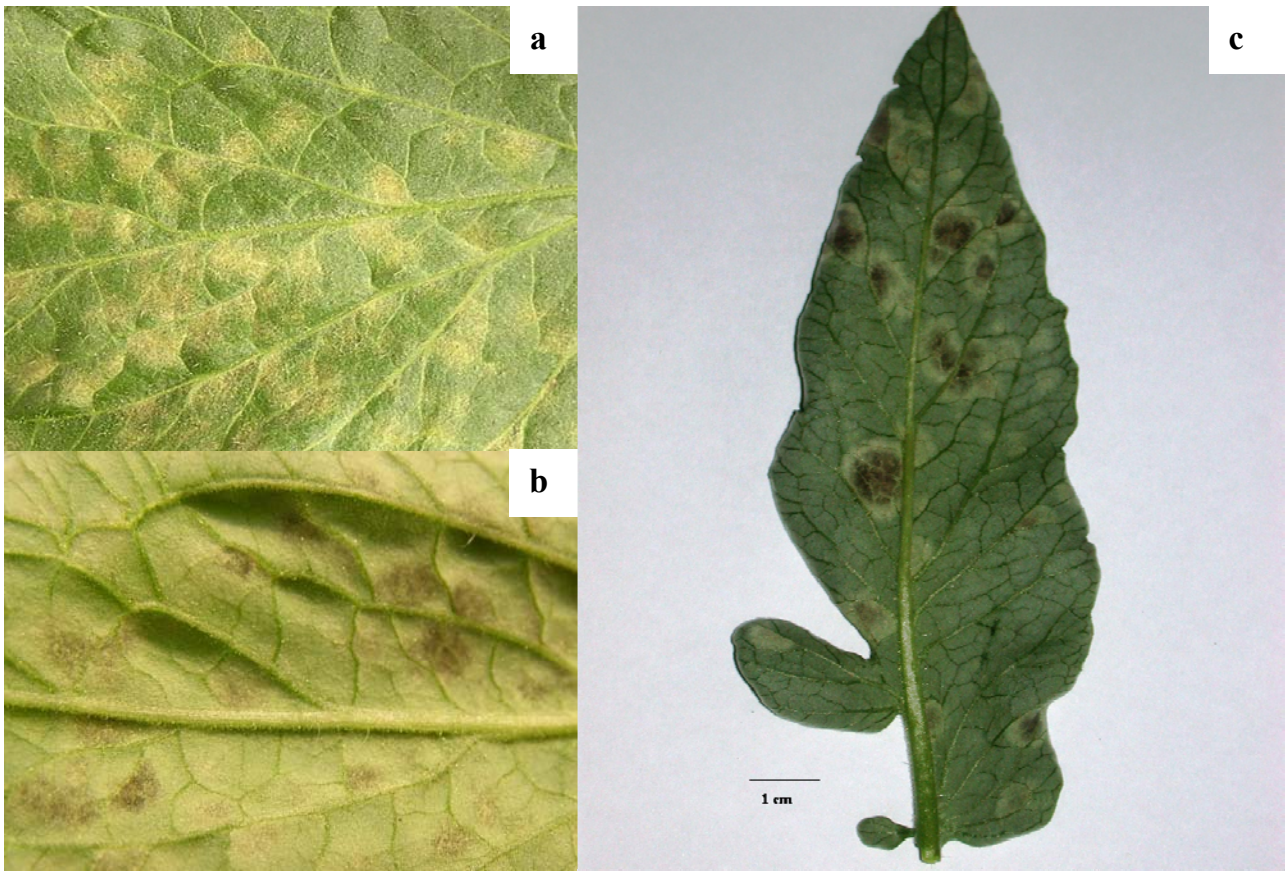

Fig. 1. Symptoms of black leaf mold caused by *P. fuligena* on the adaxial (a) and abaxial (b and c) leaf surface of *S. lycopersicum* cv. FMTT260.

Microscopic observation of conidial structure showed subhyaline to pale olivaceous, obclavate to cylindrical, attenuated tip, long obconic to long obconically truncated base, with slight constrictions at the septa, straight to mildly curved and unthickened scars. Conidia, stained with 0.5% aqueous fuchsin solution and mounted on microscope a minute later, showed a better contrast (Fig. 2a). The conidial dimension ranged between 3.5-9 × 11-128 µm in width and length, respectively. More than 80% of conidia had a conidial length between 21 and 60 µm (Fig. 2d) and the average conidial length recorded was 45.9 µm (n = 192). The number of segments per conidium reached up to a maximum of 12 and showed a high linear correlation (r = 0.9) with that of conidial length (Fig. 2e). Germinated conidia were stained similarly and individual conidia were observed 12 hours after keeping the slides in a moist chamber (Fig. 2b). Fasciculate conidiophores with sporulating conidia were viewed 5 minutes after dyeing (Fig. 2c). Conidiophores were typically loosely fasciculate, pale olivacious to pale brown, uniform in color, straight to sinuous, tip rounded or truncated, sometimes once geniculate and not branched.

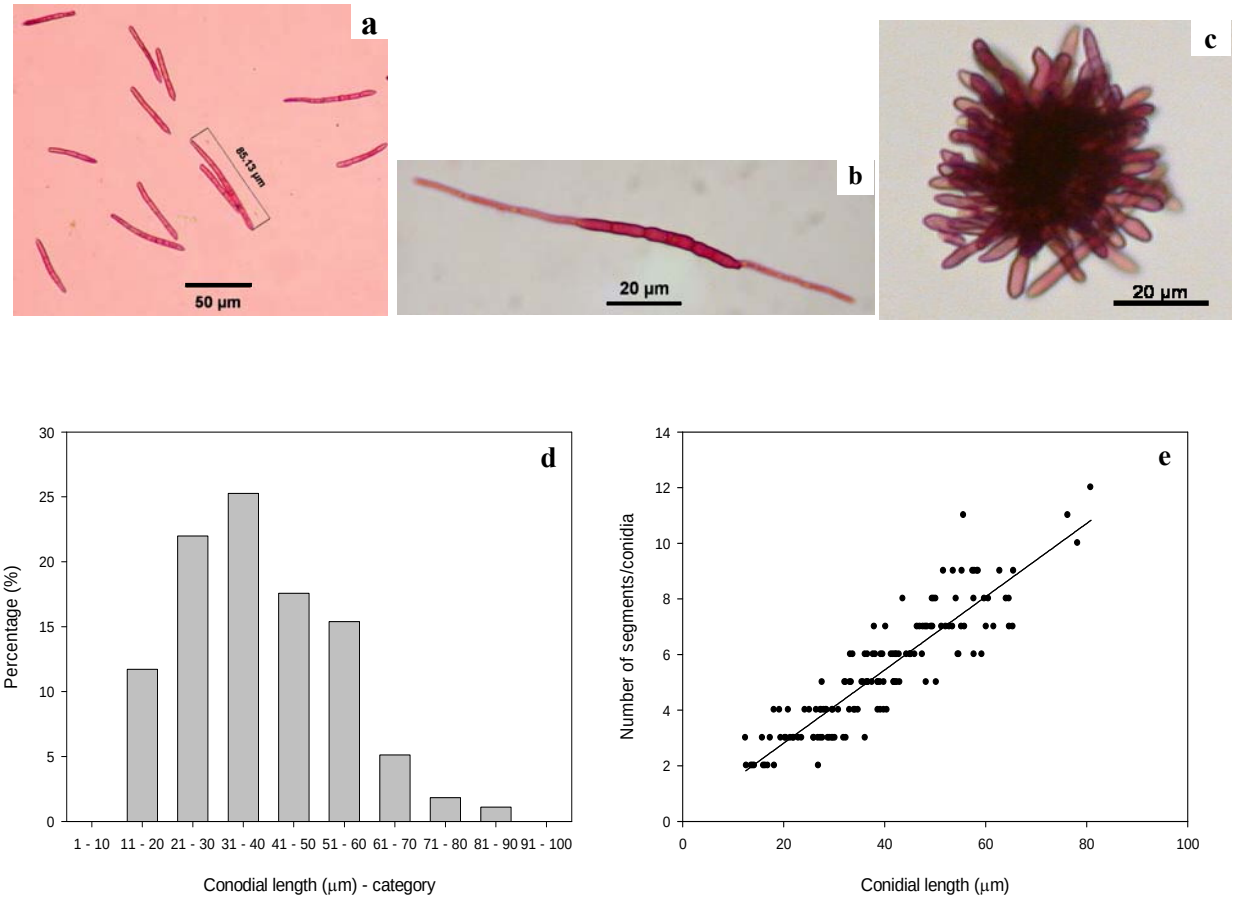

Fig. 2. Fuchsin stained conidia (a), germinating conidia (b) and fasciculate conidiophores (c) of *P. fuligena* and frequency distribution of conidial length (d) and number of segments of each conidium (e) of *P. fuligena*.

Isolation and identification of *P. fuligena*

Amongst the three methods of isolation tested, direct conidial transfer (DCT) proved to be the easiest and most efficient one. According to the ordinal scaling used (0-3), isolation using a DCT produced less contamination and more growth, as compared to either conidial suspension (CST) or lesion (LT) transfer (Table 2). Statistically, the paired probability comparisons between DCT and CST (0.0001), DCT and LT (0.0088) and that of CST and LT (0.0088) according to Wilcoxon's exact test indicated a significant difference between the three methods in terms of contamination (Table 2). A similar result was recorded for the growth whereby growth of *P. fuligena* in DCT proved to significantly higher than in both CST ($p = 0.0003$) and LT ($p = 0.0002$). Since *P. fuligena* grew slowly on artificial media, inoculum was widely spread on the growth media using zigzag or cross streaking. This method was found to be practical and by far more prolific growth of the fungus within a short time was achieved as compared to point inoculations (Figure not presented). For these reasons, later experiments involving *in vitro* isolation and inoculation of *P. fuligena* were done using DCT.

Table 2. Frequency count of 9-cm Petri dishes that were compared for growth and contamination of *P. fuligena* according to a rating scale of 0 to 3 after using the three isolation methods (DCT = direct conidial transfer, CST = conidial suspension transfer and LT = lesion transfer).

Method	Contamination scale*					Growth scale*				
	0	1	2	3	Significance**	0	1	2	3	Significance**
DCT	10	7	3	0	a	0	3	5	12	a
CST	0	7	8	5	b	5	5	9	1	b
LT	1	14	5	0	c	0	12	8	0	b

* Key for the scales are presented in Table 1

** According to Wilcoxon's exact test

To identify the causative agent according to Koch's postulate, suspensions of 5 x 10^5 fungal propagules from TOA plated pure cultures of two isolates were inoculated on three varieties, namely King Kong 2, Lizzy and FMTT260. Out of them only the one which later on was

proved as *Pseudocercospora sp.* (after DNA extraction using molecular techniques) showed the symptoms. The output from the molecular characterization matched that of the earlier pure culture identification by the fungal biodiversity centre (Centraalbureau voor Schimmelcultures – CBS) in The Netherlands. The sequencing comparison of the purified sample as shown by Clustal X (1.81) and further tested for the homology alignments using BLAST from NCBI showed 100% similarity (429/433 bp) to that of *Pseudocercospora sp.* Further confirmation that proceeded from AIT after dried herbarium were sent to the CBS reconfirmed the causal pathogen of the leaf mold on tomato in this research to be *P. fuligena* (Pedro W. Crous, personal communication).

Culturing and sporulation of *P. fuligena* in artificial growth medium

Further experiments on optimization of growth conditions for *P. fuligena* under laboratory conditions showed better performance of the pathogen in recommended media like TOA (Hartman *et al.*, 1991) and CLDA (Kilpatrick and Johnson, 1956) supplemented with $CaCO_3$. Point inoculation using DCT in a non-sealed Perti dish showed a radial growth of the 6-mm cut pieces to 13.6 mm in TOA and 13.1 mm in CLDA 7 days after inoculation (DAI) which was significantly different from the growth in the other media (Table 3). In relative terms, however, *P. fuligena* grew well in all but less in OMA, PCA, V_8 and WA. Better growth was also seen in non-sealed (NS) Petri dishes as compared to those wrapped and sealed (S) with parafilm. In four of the growth media, *i.e.* BMA, CLDA, MEA and TOA, the difference between S and NS Petri dishes was statistically significant (Table 3).

Germination was nearly the same in TOA, PDA and water with 96, 95 and 98% conidia germinated after 24 hours in three of the medium respectively. Similar extensive germ tube growth of the conidia was observed on filter paper soaked in TOA. Further experiments in closed plastic containers proved that *P. fuligena* germinated at high humidity without presence of free water. Of the conidia that were brushed off to the leaflets in the plastic containers under dry, moist and wet conditions, 0, 80 and 95% germinated after 48 hours.

Attempts to induce successful sporulation in artificial growth media using different techniques was not successful as expected and hence all experiments that were based at LUH were done by quantifying the propagules (mycelia-conidia-conidiophore).

Table 3. Growth (diameter expansion in mm) of *P. fuligena* in ten different artificial media with (S) and without (NS) sealing Petri dishes as was observed 7 days after inoculation.

Sealing		Media types									
		BMA	CLDA	CLOA	MEA	OMA	PCA	PDA	TOA	V_8	WA
NS	Mean	11.7bc	13.1a	11.1bc	10.4c	6.4d	8.6d	10.9bc	13.6a	7.8d	9.2d
	SE	0.42	0.35	0.32	0.26	0.22	0.33	0.50	0.30	0.27	0.40
S	Mean	10.3c	11.9bc	11.0bc	7.9d	6.6d	8.7d	10.2c	12.2b	7.2d	8.3d
	SE	0.30	0.41	0.34	0.27	0.17	0.30	0.24	0.41	0.33	0.33

NB: Details of preparation and description of each growth medium are provided at the appendix section of this chapter.

Penetration and egress of *P. fuligena*

Stomata of tomato leaves including the subsidiary and guard cells were easily visible on both leaf surfaces of the intact (Fig. 4a) and cleared (Fig. 4b) leaves of FMTT260 from light microscopy and using 500x magnifications from scanning electron microscopy (Fig. 4c). Though tomato plants under natural light situation are known to be amphistomatous, the abaxial surface was found to be more stomatophorous (with 148.4 ± 8.3 stomata/mm^2 leaf surface) and hairy as compared to the adaxial surface (with 80.2 ± 7.6 stomata/mm^2 leaf surface) which often had a thicker cuticle.

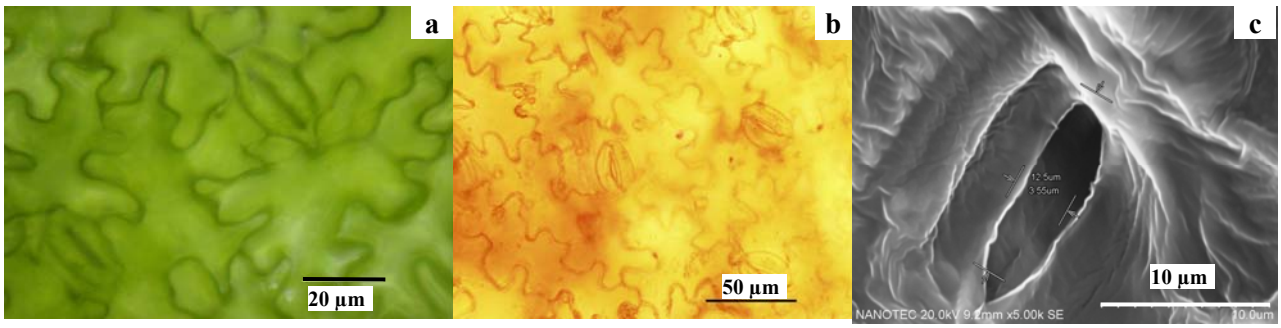

Fig. 4. Subsidiary cells, guard cells and stomata of a tomato leaf as seen through light microscopy of intact (a) and cleared (b) leaves as well as observation from SEM (c).

Stomatal penetration of *P. fuligena* was visible through the simple staining technique using 0.05% aqueous acid fuchsin solution. On leaves that were inoculated in the greenhouse,

observation of the primary infection hyphae was possible 48 hours after inoculation (Fig. 5a). This observation was also possible on detached leaves whereby conidia were dropped onto the abaxial side of leaves that were then left in highly humid plastic cans. The stomata penetration in case of detached leaves was observed randomly in a range of 48-96 hours after inoculation (Fig. 5b). While direct penetration of primary germ tube was seen through simple dyeing technique of the leaves, further spread of the pathogen by secondary infection hyphae were seen both in intact (Fig. 5c) and cleared leaves (Fig. 5d).

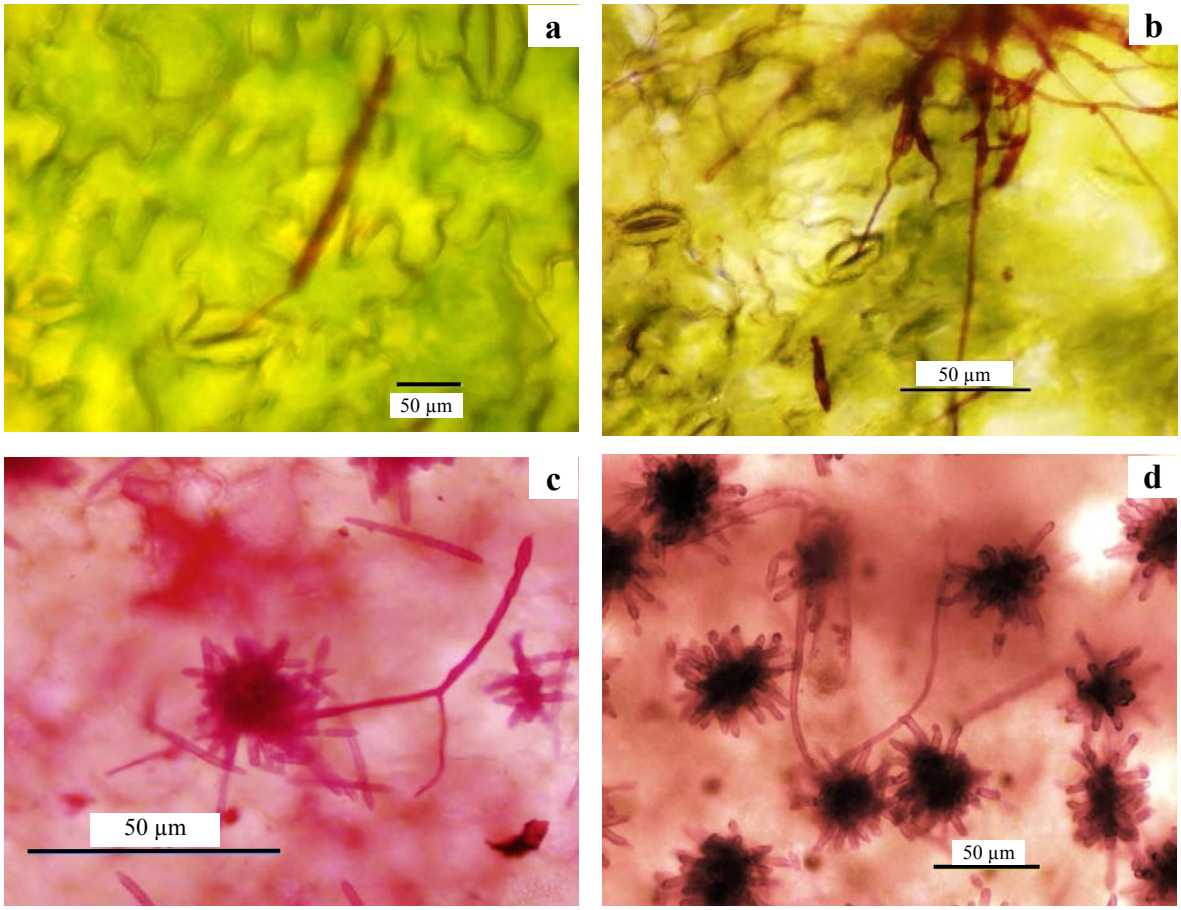

Fig. 5. Light microscopy of stained (0.05% acid fuchsin) germinating conidia producing primary infection germ tube (hypha) and penetrating through stomata of intact leaves (a and b) as well as further dispersal and penetrations through secondary hyphae as seen through cleared leaves (c and d).

Using both methods of leaf clearing and dyeing, it was possible to comprehend the mode of egress and thus progress of *in situ* infection from observed blockage of stomatal aperture. The earliest symptomatic observations started about 8 DAI under optimal conditions and thus observations in cleared leaves started a day earlier at 7 DAI (Fig. 6a) and then at 12 DAI (Fig. 6b) and finally at 17 DAI (Fig. 6c). The area (μm^2), diameter (µm) and perimeter (µm) of

these localized spherical infection sites were statistically significant among the three observation times, *i.e.* 7, 12 and 17 DAI (Fig. 6d). Besides, the progress of *in situ* infection during the first 17 days fitted best to two parametric exponential growth function with $R^2 > 0.81$ (Fig. 6e).

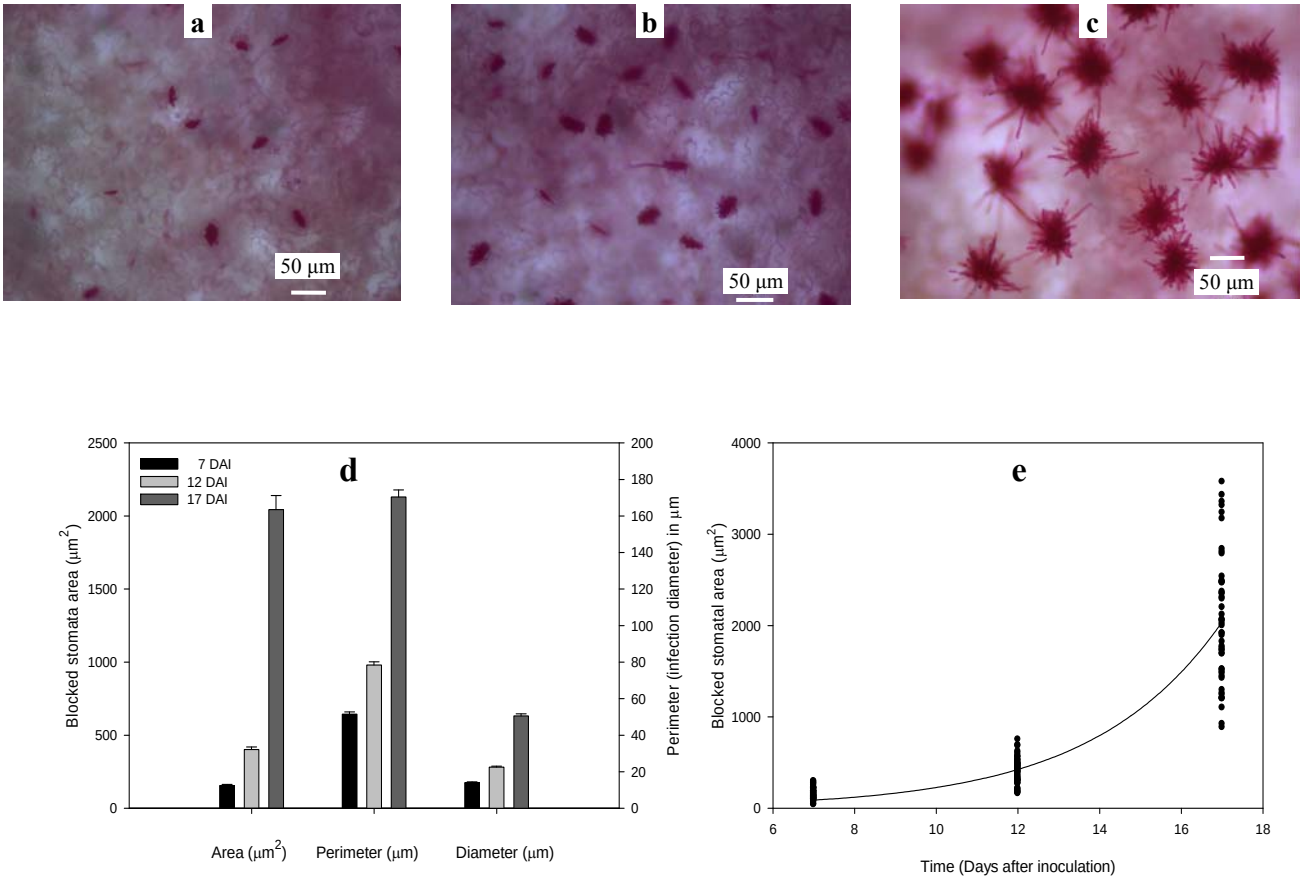

Fig. 6. Light microscopy of *P. fuligena* pathogenesis and fascicle formation through stomata 7 (a), 12 (b) and 17 (c) days after inoculation as well as dimension measurements of area, perimeter and diameter of each fascicle (d) with an exponential function fitted to area of blocked stomata across the three observation times (e). NB: average temperature and relative humidity at the time of inoculation were 29 (± 1°C) and 95%, respectively.

Microscopic observation of inoculated leaves at 21 DAI showed the extent of enlargement of each fasciculate conidioma and the conidia which would soon be released and serve for dispersing the pathogen (Fig. 7). Diameter of the area covered with conidiophores was approximately in a range of 98 µm to 263 µm as could be seen from the two selected observations of the light microscopy (Fig. 7a and b).

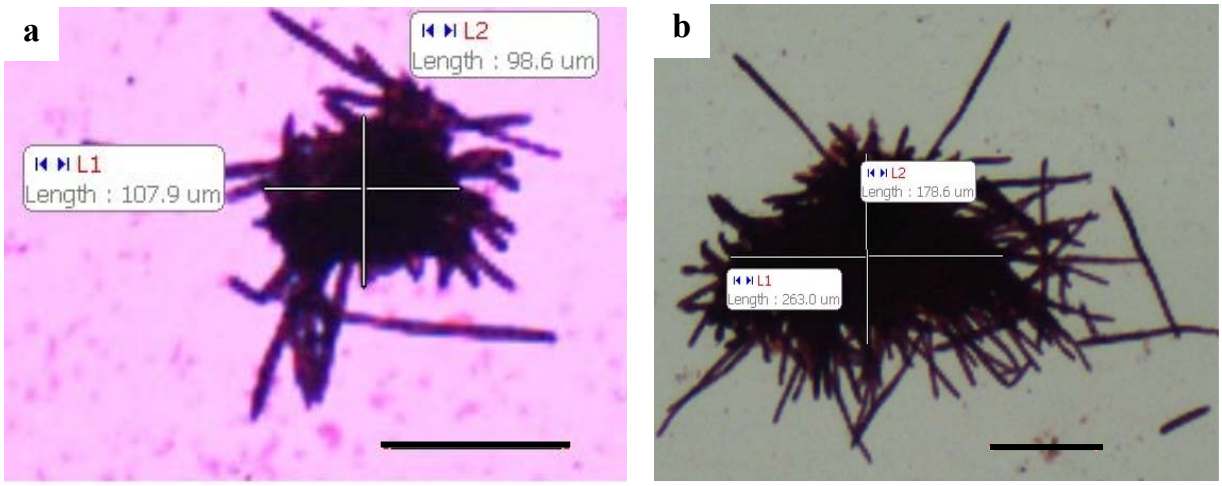

Fig. 7. Samples of fasciculate conidiophores and conidia of *P. fuligena* observed under light microscopy 21 days after inoculation and the respective dimension measurements of sampled infection sites. NB: Scale bars represent 100 µm.

Despite the lack of cross sectional studies to follow events of conidiogenesis, the formation of conidioma and egress was clearly seen in SEM (Fig. 8). The emergence of early conidiogenous cells through the stoma guard cells (Fig. 8a) and the formation of the fasciculate conidiophores (Fig. 8b) were evident. Later progression of the sporulation with released conidia was clearly visible too (Fig. 8c).

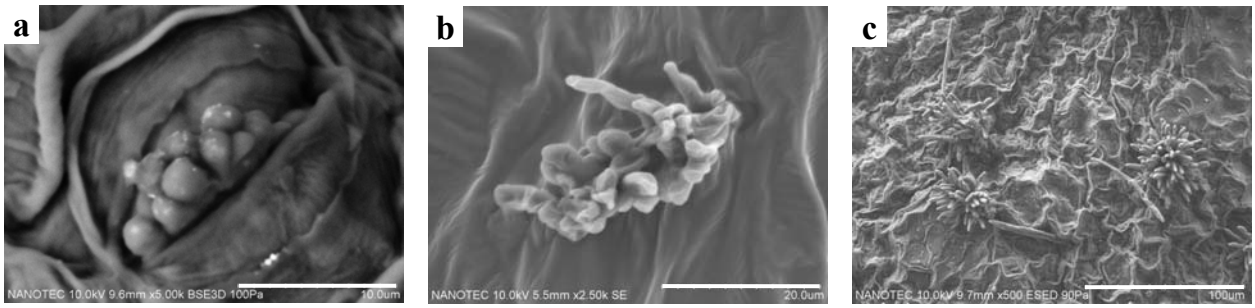

Fig. 8. Fascicles of conidiophores emerging through stoma (a and b) and prolific sporulation of conidiophores and conidia (c) of *P. fuligena* as seen through scanning electron microscopy (SEM). NB: Scale bars represent 10, 20 and 100 µm in a, b and c, respectively.

DISCUSSION

Macroscopic observations of symptoms and signs of black leaf mold (BLM) from natural and artificial inoculations corroborated many of the earlier research outputs. Effuse patches of the lesion appearance and amphigenous fructification were consistently detected from all the observations. Initial lesion appearances of BLM (*P. fuligena*) and leaf mold caused by *Fulvia fulva* (Cooke) Cif. (syn. *Cladosporium fulvum* Cooke) are very similar in that leaf lesions of both turn pale-green to yellow on the upper leaf surface and both fungi sporulate profusely on the lower leaf surfaces (Anonymous, 2004b; Sherf and MacNab, 1986). Symptoms of leaf mold (*F. fulva*) appear olive-green to grayish purple in color whereas BLM (*P. fuligena*) is initially white in color and turns gray to black as infection progresses (Sherf and MacNab, 1986; Hartman *et al.*, 1991). Moreover, the amphigenous fructification is mentioned (Anonymous, 2004b; Deighton, 1976) as one distinguishing feature of BLM from that of leaf mold caused by *Fulvia fulva*.

Chupp (1954) mentioned the indistinct discolorations of the lesions of *Cercospora fuligena* as peculiar feature amongst other *Cercospora* species. The phenomena of early fuliginous sporulating lesions of *P. fuligena*, prominently on the abaxial side and then on the adaxial side as infection progresses, could also be taken as a unique feature to distinguish the symptoms of BLM from other leaf molds. This feature led, one among other reasons, earlier taxonomists to categorize the fungus to *Cercospora fuligena* (Chupp, 1954). Hartman *et al.* (1991) indicated the distinct morphology of conidia which can help to readily distinguish them microscopically from that of *Cladosporium fulvum*. The latter, for instance, is known to produce the asexual conidia on clustered or single, tall dark, upright, variously branched conidiophores (Joosten and de Wit, 1999; Ulloa and Hanlin, 2000; Thomma *et al.*, 2005) near the apex unlike the fasiculate types of *P. fuligena*. Hartman *et al.* (1991) further stated the need for additional research on geographical distribution and importance of these two diseases in areas of tropical and subtropical countries because of similarity of the field symptoms. Even other *Pseudocercospora* species, for instance *P. mori* on mulberry, are reported to show similar disease symptoms in India (Babu *et al.*, 2002) and Australia (Grice *et al.*, 2006).

Wang *et al.* (1996) gave details of conidial morphology and color changes of BLM from an observation of plants inoculated with *P. fuligena*. At times of heavy infection, lesions coalesced and leaves curled upward and died prematurely with many remaining on the plant (Wang *et al.*, 1995). The same phenomenon was observed in this research, which on the other

hand, was in contrary to earlier reports of Hartman *et al.*, (1991) in which premature defoliation due to BLM was mentioned. It is not uncommon, however, with some cercosporoid fungi like the one on peanut (*Arachis hypogaea*) to induce or accelerate senescence and abscission of leaves (Ketring and Melouk, 1982).

Considering the slow growth of *P. fuligena*, the selected method of isolation, *i.e.* direct transfer of conidia from the profusely sporulating lesions, was found to be efficient and less prone to contamination than the other two methods tested in this research. This went in agreement with observations of Beilharz (1994) for other species of *Pseudocercospora* on native Australian plants. Since establishment of disease by artificial inoculation is essential for studies of various aspects of plant pathology, including epidemiology, disease resistance, host-parasite interactions and disease control, *in vitro* studies that were carried out both from pure cultures at LUH and from freshly infected and sporulating leaves at AIT have been informative of the nature of growth of the pathogen in artificial media. The point inoculations of 6-mm diameter pure culture pieces in this study showed a diameter expansion which has nearly doubled after 7 days in dark chamber at 28°C in tomato oatmeal agar (TOA). The slow growth of other cercosporoid fungi is also mentioned in earlier reports of Beilharz (1994).

Regarding the preference of growth media, *P. fuligena* grew slowly but indiscriminately on all the ten growth media tested in this research. Better growth performance was, however, recorded on TOA, the medium vastly used at AVRDC based researches, and CLDA, a medium recommended for sporulation of *Cercospora* species (Kilpatrick and Johnson, 1956) both supplemented with $CaCO_3$. Earlier works of Hartman *et al.* (1991) have reported similar results and recommended the use of TOA as an appropriate medium for the growth of *P. fuligena*. Furthermore, enhanced growth and hence harvesting more inoculum of *P. fuligena* was achieved through zigzag and radial spreading instead of point inoculations. These experiments yielded about 10^4 to 10^5 propagules per Petri dish per 100 mL deionized water at 14 DAI.

The count from a pure culture at LUH resulted in 5.0 x 10^4 and 6.0 x 10^4 propagules/mL under dark (28 ± 0.6ºC) and 1.0 x 10^5 and 1.2 x 10^5 propagules/mL in black light (25 ± 1.5ºC) incubations from sealed and non-sealed Petri plates, respectively. Despite indicators of better conidial production in cultures incubated under black light in non-sealed but once wounded cultures, sporulation of *P. fuligena* in this research was generally poor. Propagules (mycelium-conidiophore-conidia) from these cultures resulted in only nearly 20% conidia as compared to the whole propagule composition.

Dhingra and Sinclair (1995) and Campbell *et al.* (2003) summarized basic growth factors and mechanisms to induce sporulation of fungal pathogens. One was the spectrum of light, as growth was enhanced by light of wavelengths longer than 500 ηm, while sporulation was enhanced by light of wavelengths shorter than 500 ηm. Moreover, whereas alternating temperatures with a diurnal photoperiod and darkness and mycelial wounding favored sporulation, sealing of Petri plates was assumed to have an inhibitory effect on sporulation. Campbell *et al.* (2003), for instance, recorded 800% increase of conidial numbers of *Pyrenophora semeniperda* with wounding cultures grown at 25°C in 12 h alternating with cool-white light. With respect to *P. fuligena*, Yamada (1951) cultured the fungus and reported that the pathogen grew poorly on artificial media but grew well on Fermi agar and sporulated well on tomato plant decoction agar. Similarly, Hartman *et al.* (1991) harvested conidia from tomato leaf extract-oatmeal agar but not from that potato dextrose agar. Beilharz (1994) succeeded in inducing sporulation of *Pseudocercospora* spp. on *Solanum* after incubating at 24°C for three days in the dark, then under intermittent (12/12 hr) fluorescent and near UV lights at room temperatures for 11 days. With a conidial count of only 20% as compared to total count of propagules from wounded cultures in black light, we propose a continued research in the future incorporating all aspects of inducing sporulation, particularly the use of optimal temperature and interchanging light spectrum. Because of lack of prolific sporulation, quantification of inoculum density for all experiments at LUH was summarized as propagule (mycelium-conidiophore-conidia) counts.

Basic microscopic fungal structures and descriptions of *P. fuligena* from this research fitted within the range of morphological variations accepted in literature. For instance, dimensions of conidia (length x width) ranged between 25-70 µm x 3.6-5 µm (Blazquez and Alfieri, 1974), 20-90 x 2.5-4 µm with 2-9 septations (Hsieh and Goh, 1990), 9-137 µm x 3.5-6.1 µm with 2-27 septations (Hartman *et al.*, 1991), 15-120 µm x 3.5-5 µm (Blazquez, 1991) and 29-110 µm x 2.5-5.0 µm (Halfeld-Vieira *et al.*, 2006). Our observations (11-128 µm x 3.5-9 µm with up to 12 septations) were close to all, particularly to the report from Taiwan as described by Hsieh and Goh (1990). Conidium length in cercosporoid fungi may vary as much as ten-fold in a given sample (Chupp, 1954) and conidiophores too are highly variable in length (Solheim and Stevens, 1931). A major cause of measurement variations within a given species could be attributed to the prevailing conditions during mounting. One amongst others is relative humidity since it causes excessive elongation of conidia and conidiophores as underscored by Welles (1925) and Webster and Weber (2007). Some of the differences in

conidia and conidiophore dimensions could also be attributed to the time duration between sample preparation and microscopic observation. Because of high moisture affinity of *P. fuligena* conidia, it would be appropriate to measure the conidial dimension immediately after mounting on the slide. Besides, differences in conidium length and septation in several species of *Cercospora* could also be attributed to the type of growth medium as reported from Ekpo and Esuruoso (1978).

Further attempts to study *in vivo* and *in situ* fungal structures like observations of conidioma and conidiophores using leaf clearing and dyeing techniques were successful. Among the two whole leaf clearing methods used, the method of de Luna *et al.* (2002) was preferred considering its simplicity and omission of intermediary transfers and thus less time required to complete the procedure. In this method, cut pieces were put in vials containing 70 parts Ethanol (absolute 99.7%) and 30 parts glacial acetic acid. After dyeing, fasciculate conidiophores were clearly visible with light microscopy.

For further experiments on the study of penetration and condiogenesis, a glimpse on stomata composition of a terminal leaflet of the 6^{th} compound leaf from a two-week old (after transplanting) FMTT260 plants showed the amphistomatous nature of the leaves which corroborates earlier reports of Gay and Hurd (1974). Although the stage of growth, canopy strata and light saturation determine stomatal composition, nearly a double stomatal density was recorded in this research on the lower leaf surface as compared to the upper.

Mendgen *et al.* (1996) reviewed aspects of morphogenesis and mechanisms of penetration by plant pathogenic fungi and underlined that a crucial step for successful parasitism is penetration. This study experimentally complimented many of the earlier assumptions of stomatal mode of penetration of *P. fuligena* (Hartman *et al.*, 1991; Sherf and MacNab, 1986). Moreover, results of SEM ultrasturctural studies of *P. mori* on mulberry and its similarity to *P. fuligena* with many aspects (Babu *et al.*, 2002) serve as supportive evidence of stomatal penetration of the pathogen. Although no other penetration via cuticles or other epidermal cells was observed from the light microscopy of this research, future studies with optimal SEM procedures should substantiate this outcome. Mode of egress of *P. fuligena*, however, was unambiguously and solely stomatal. This was consistently witnessed from the observations of light and scanning microscopy whereby no single erumpent egress was detected. Beilharz (1994) profoundly discussed about the stroma formation and mode of egress of cercosporoid fungi on Australian native plants.

Hartman and Wang (1992) stated that BLM symptoms did not appear until 10-14 DAI in field trials but in controlled conditions at 28°C, lesions were visible already 6 DAI (Wang *et al.*, 1996). Matured conidiophores and conidia from the same experiment of Wang *et al.* (1996) were observed 12 DAI. *In situ* microscopic observations of infection progresses from cleared leaves at 7, 12 and 17 DAI in this research went in agreement with the above findings. While both methods that were used for leaf clearing showed the pattern of egress, secondary infection hyphae and thus *in situ* infection clearly, the one from de Luna *et al.* (2002) was practical from the perspective of time management. It only took 30 minutes (0.05% acid fuchsin solution) to stain the fungal structures after a transparent leaf section was obtained. Though primary and secondary infection hyphae were observed in this research, there was no cross-sectional study undertaken to observe the third type of hyphae, internal hyphae, which according to Babu *et al.* (2002) develop inside leaf tissue and produce stromata in the substomatal chambers.

Deighton (1976) redescribed *Pseudocercospora* Speg., placing great emphasis on the unthickened nature of the abscission scars in that genus. He and many other scientists then transferred a number of *Cercospora* species with unthickened scars to *Pseudocercospora* (Beilharz, 1994). The combined ITS regions including the 5.8S rRNA gene among Cercosporoid taxa examined in this study varied in length from 502 bp in *Paracercospora fijiensis* to 595 bp in *Pseudocercospora* species. Future studies regarding the phylogeny of this fungal pathogen shall be carried out to pinpoint the position of *P. fuligena* within the hierarchical classification system of organisms.

Put in summary, this study has found results ranging from descriptions of macroscopic to microscopic characterizations of *P. fuligena*, the causative agent of BLM. Stomatal penetration and egress as well as progression of the latent infection are very informative for designing disease management strategies.

Appendix: Growth media tested for culturing of *P. fuligena*

1. ***Biomalt agar – BMA*:** 15 g biomalt, 10 g agar powder added to 1 L of distilled water and solution autoclaved 121ºC for 20 minutes.
2. ***Carrot leaf-decoction agar – CLDA*:** 300 g finely ground carrot leaf, boiled in 500 mL distilled water, steamed for 1 hr and strained through a double layer of cheese cloth, filtrate mixed with 500 mL distilled water in which 12 g agar has been dissolved. Solution volume adjusted to 1 L and autoclaved 121ºC for 20 minutes.
3. ***Carrot leaf oatmeal agar – CLOA*:** 50 g oatmeal boiled in 500 mL for 20 minutes and filtered, 50 mL carrot leaf juice, 20 g agar mixed and distilled water added onto the filtrate to adjust the volume to 1 L.
4. ***Malt extract agar – MEA*:** 20 g glucose, 20 g malt extract, 1 g peptone, 1000 mL tap water; 20 g agar dissolved in 1500 mL water in microwave oven and mixed with remaining ingredients and autoclaved at 121ºC for 20 minutes.
5. ***Oatmeal agar – OMA*:** 20 g rolled oats, coarsely ground in a mortar and pestle, boiled in 500 mL water and sieved. 15 g agar added, volume was adjusted to 1 L with distilled water and autoclaved at 121ºC for 20 minutes.
6. ***Potato carrot agar – PCA*:** 15 g potato, scrubbed and diced, 15 g carrot, peeled and diced, all boiled in 500 mL distilled water and sieved. 20 g agar added, volume adjusted to 1 L by adding distilled water and mixture autoclaved at 121ºC for 20 minutes.
7. ***Potato dextrose agar – PDA*:** 39 g of powdered potato dextrose agar mixed and stirred in 1 L of distilled water and autoclave at 121ºC for 20 minutes.
8. ***Tomato oatmeal agar – TOA*:** 50g shredded tomato and 15 g oatmeal boiled separately each in 500 mL water, suspension sieved through two layers of cheese cloth, mixed, 15 g agar powder added, volume of solution adjusted to 1 L by filling distilled water, autoclaved 121ºC for 20 minutes. 1 g of $CaCO_3$ added to the solution mix before autoclaving for TOA amended medium for sporulation tests.
9. ***V_8 juice agar – V_8 Agar*:** 200 mL V_8 juice, 500 mL water, sieved and pH adjusted to 7 to 7.5, 3 g $CaCO_3$ and 10 g agar added and mixture volume adjusted to 1 L by adding distilled water, autoclaved at 121ºC for 20 minutes.
10. ***Water agar (1%) – WA*:** 10 g agar powder added to 1 L distilled water, solution autoclaved at 121ºC for 20 minutes.

Chapter 2: Monocyclic components of black leaf mold (*Pseudocercospora fuligena*) on tomato under ambient and controlled environments

ABSTRACT

Monocyclic components of the black leaf mold (BLM) disease, caused by *P. fuligena*, were investigated in controlled (CE) and ambient environment (AE) experiments conducted at Leibniz Universität Hannover and Asian Institute of Technology. Amongst the factors tested, a temperature (*T*) of 28ºC under CE resulted in incubation period (*IP*) of 11 to 13 days after inoculation (DAI) as compared to inoculation at 24ºC which showed BLM symptoms 15 to 17 DAI. There was a respective delay of about 4 and 5 days of *IP* and latent period (*LP*) from the same CE experiment in growth chambers. Detached 1-week old leaflets in one of the experiments similarly showed maximum symptomatic colonial density (per cm^2 leaflet area) of 4.6 (at 25 DAI), 2.8 (at 30 DAI), 1.8 (at 30 DAI) and 0.5 (at 30 DAI) at incubations of 28, 24, 20 and 32°C respectively. Colony growth was poor at 32ºC and the highest temperature (36ºC) was excluded from analyses as the detached leaves were burnt within 4 to 10 DAI.

Under AE in greenhouses in Thailand with a daily mean temperature (T_{DM}) of 29ºC, *IP* ranged from 9.2 to 11 days but was extended to 14 days when T_{DM} was fallen to 25.6ºC during the cold month of February 2007. Leaf age wise, outputs from all the CE and AE experiments proved that young and fully unfolded old leaves showed more symptomatic (DI_{SY}) and sporulating (DI_{SY}) leaflets than older leaves. For instance, from the CE experiments, leaves of age 1, 3 and 5 weeks had more disease and shorter *IP* and *LP* than leaves that were 7 weeks old. The same trend was observed under AE when 1- and 3-week aged leaves were compared. There was about 50% less DI_{SY} and DI_{SP} on 3 weeks old leaves than on 1 week old ones.

Wetness duration on the other hand proved to be a necessity for germination and penetration of *P. fuligena*. Without wetness duration, for instance, a respective DI_{SY}* and DI_{SP}* proportion of 0.05 and 0.01 was recorded from the growth chamber experiment. These proportions are negligible compared with 94 and 98% less DI_{SY}* and DI_{SP}* as compared to the maximum values achieved from the other wetness durations.

Key words: *Pseudocercospora fuligena*, monocyclic components, temperature, wetness duration, leaf age, incubation and latent period

INTRODUCTION

Under favorable environmental conditions and prevalence of a susceptible host, a pathogen has to complete its life cycle, perpetuate itself and spread to new infection sites in order to cause an epidemic. Pathogen life cycle, synonymously labeled as "infection chain" (Gäumann, 1950), can be studied by descriptive and measurement terms of its biological components such as over-seasoning, infection, latency, lesion formation, infectious period and dispersal (Kranz, 2003; de Wolf and Isard, 2007). Essential biological monocyclic components of biotic diseases are explicitly described by many scientists (Vanderplank, 1963; Zadoks and Schein, 1979; Aust and Hau, 1983; Hau, 1990; Bergamin Filho and Amorim, 1996; Kranz, 2003; Hau and de Vallavieille-Pope, 2006; de Wolf and Isard, 2007). Structures of these events during the infection chain, expressed as their intensity and time elapsed to complete each stage, have a bearing on the course of epidemics and hence on disease management of a particular pathosystem (Kranz, 2003). The same holds true for the hyphomycetous fungus *Pseudocercospora fuligena* (Roldan) Deighton (syn. *Cercospora fuligena* Roldan) causing the black leaf mold (BLM) disease (syn. *Cercospora* leaf mold) of tomato (*Solanum lycopersicum* L.).

P. fuligena causes the polycyclic disease BLM both under field (Blazquez and Alfieri, 1974; Hartman *et al.*, 1991; Hartman and Wang, 1992, 1993; Wang *et al.*, 1996) and protected cultivation systems (Blazquez and Alfieri, 1974; Hartman and Wang, 1992; Wang *et al.*, 1996; Kandziora, personal communication). Despite blanket information on incubation period on tomato plants (Chupp, 1954; Blazquez and Alfieri, 1974; Sherf and MacNab, 1986; Hartman *et al.*, 1991; Wang *et al.*, 1996), there is lack of explicit studies undertaken to pinpoint the monocyclic components under combined circumstances of favorability factors.

With high significance of the disease in protected systems, the need to study the main components of the infection chain of *P. fuligena* vis-à-vis their epidemiological implications is worthless to mention. This study, as part and parcel of a wider epidemiological investigation of the pathosystem, was thus designed with an objective to bridge this gap by incorporating combinations of temperature regimes, wetness durations and host ages and pointing their impacts in determining the two important biological components of the infection chain, *i.e.* incubation (the time duration from inoculation to appearance of BLM symptomatic units) and latent (the time duration from inoculation until appearance of sporulating units) period.

Each component of the infection chain is affected by external factors from the disease square (Vanderplank, 1963; Zadoks and Schein, 1979), *i.e.* the host-pathogen-weather-human

interference interactions. Weather, for instance, affects diseases and their epidemics as open systems most directly during their life cycles (Waggoner, 1965; Rotem, 1978; Kranz, 2003). Kranz (2003) and Hau and de Vallavieille-Pope (2006) listed the more prevalent, primary and directly measured weather parameters in plant pathology. Amongst them, temperature, relative humidity and leaf wetness duration were used in this research.

Temperature is among the most important environmental factors that control plant development, growth and yield. All biological processes respond to temperature, and all responses can be summarized in terms of three cardinal temperatures, namely the base or minimum (T_{min}), the optimum (T_{opt}) and the maximum (T_{max}) temperatures (Yan and Hunt, 1999). Schrödter (1965), Aust and Hau (1983), Hau (1985, 1990) and Hau and de Vallavieille-Pope (2006) descriptively reviewed impacts of temperature and its modeling while studying epidemics of foliar diseases. With respect to *P. fuligena*, an *in vitro* experiment in Taiwan reported 26°C to be optimal and 34°C as detrimental for mycelial growth (Hartman *et al.*, 1991). Another output from Yamada (1951) found the maximum temperature for germination of conidia to be 36°C.

Relative humidity and leaf wetness also influence the fungi during production and transport of inoculum. Whereas relative humidity can be physically well defined, leaf wetness duration, by contrast, is very difficult to define because various portions of leaves and canopies wet and dry at different times (Huber and Gillespie, 1992). Jones (1986), Hau (1990), Duthie (1997) and Hau and de Vallavieille-Pope (2006) have similarly reviewed aspects of relative humidity and wetness duration on epidemics of different pathosystems. From the same *in vitro* experiment of Hartman *et al.* (1991), the minimum relative humidity requirement for *P. fuligena* was determined to be 84.5%. Chupp (1954), Blazquez (1991) and Sherf and MacNab (1986) as well as other research outputs from AVRDC reported some blanket recommendations of temperature and relative humidity requirements of the pathogen.

Moreover, susceptible growth stages of the host also determine the components of the infection chain. The final aspect considered in this research, host age at the time of infection, attempted to develop this relationship for the *P. fuligena* - *S. lycopersicum* pathosystem. Except comparisons and assumptions of BLM epidemics with respect to a growing season and canopy strata, studies focusing on the aspect of host age vis-à-vis epidemics of BLM are rare. Ogawa *et al.* (1967), Hau (1985) and Kranz (2003) have researched this aspect with other biotrophic and necrotrophic pathosystems.

MATERIALS AND METHODS

Experimental overview

Monocyclic components of the black leaf mold (BLM) caused by the hyphomycetous fungus *P. fuligena* were investigated in three experiments under controlled (CE) and ambient environments (AE) that were carried out at the Institute of Plant Diseases and Plant Protection (IPP) of Leibniz Universität Hannover (LUH) in Germany and at the Asian Institute of Technology (AIT) in Thailand. The experimental site for the latter was located 44 km north of Bangkok in Khlong Luang, Pathumthani province, which is the central region of Thailand (14º 04′ N, 100º 37′ E, altitude 2.3 m above sea level).

The first experiment at LUH was carried out in two growth chambers to test effects of temperature regimes (*T*) in °C, wetness duration (*WD*) in days after inoculation (DAI) and age (*A*) of tomato leaves at the time of inoculation (in weeks) on incubation and latent period of BLM. The second experiment entailed three more temperature regimes in growth cabins with the same leaf ages tested on detached leaves in Petri dishes. The third experiment at AIT was performed under ambient growing conditions in a greenhouse (BioNet™, Klayman Meteor Ltd. Petah-Tikva, Israel) with two ages of tomato leaves.

In all experiments, the fresh market tropical tomato (*S. lycopersicum*) cultivar FMTT260, an F1-hybrid from AVRDC (Shanhua, Taiwan), was used as experimental plant. Besides, all experiments were carried out after artificial inoculation of quantified amount of propagules (experiments 1 and 2) or conidia of *P. fuligena* (experiment 3) that were determined using Fuchs-Rosenthal® haemocytometer (Brand GmbH, Wertheim, Germany)**.** The main experimental units in this study were leaflets.

Experimental design and setups

Growth chamber experiment

FMTT260 seedlings were raised in the nursery of IPP for about 10 days and then transferred to a 2-L capacity plastic pot filled with the substrate Frühstorfer Erde (Industrie-Erden Werk, Germany). The transplants were then moved to a growth chamber where all management practices were followed at optimal with a respective temperature and relative humidity setting of 27°C and 65-70% and a light situation of 12/12 h light/dark duration using a fluorescent

lamp. The liquid fertilizer "Wuxal top N®" with 12 - 4 - 6 (N, P_2O_5 and K_2O respectively) was applied at 0.3% v/v concentration at weekly intervals starting 7 days after transplanting (DAT). After full establishment of the transplant, the oldest leaves (represented by the 2nd to 4th compound leaf counted ascending from the base) were marked with multicolored clips wrapped on the petiole followed by successive fortnightly markings of the youngest tips (for three more times after the initial marking) in order to get 4 leaf ages (1, 3, 5 and 7 weeks) at the time of inoculation. These plants were kept in the growth chamber for both non-destructive and destructive (detached leaf experiments) samplings after inoculation.

Inoculation proceeded from fungal propagules (mycelia, mycelial fragments, conidiophores and conidia) of *P. fuligena* that were gently scraped and collected from a three-week axenic culture grown on tomato oatmeal agar (TOA). The incubator for the pure cultures was set at 27°C (± 1°C) and duration of 12/12 h light/dark situation. For the inoculation of plants in growth chambers, the volume of spray was calibrated using distilled water and a final concentration of propagules (mycelia, mycelial fragments, conidiophores and conidia) of 2 x 10^5 mL^{-1} was prepared from a stock solution.

In this experiment, two temperature regimes (24 and 28°C) and four wetness durations (0, 1, 2 and 3 DAI) were tested on four leaf ages (1, 3, 5 and 7 weeks) forming a total of 32 treatments replicated four times (Table 1). Since the leaf ages were in a form of strata across the plant canopy, a total of 32 experimental plants were assessed with the compound leaves considered as experimental units. The experiment was run in two growth chambers, each set to a 24-h temperature of 24 and 28°C respectively. Each growth chamber contained 16 plants that were arranged in a completely randomized design (CRD) with the four wetness durations repeated in four replications. Wetness duration was managed by not covering plants after inoculation (0 DAI) and covering the plants for 1, 2 or 3 DAI (refer to Fig. 1). During inoculation, a 95% relative humidity was automatically programmed in each growth chamber which after a day was reduced to 85%.

Host parameters like plant height (*PH*) and total leaf number (*TLN*) were monitored on a weekly basis from individual plants. Just a day before inoculation, leaf dimensions (length in cm) of those marked leaves (representing the 4 ages in weeks) were measured and then plants were moved to the respective growth chambers for inoculation. Disease was assessed on the basis of a leaflet in 2-day interval for the first 18 days and thereafter in an interval of 4 days until 34 DAI. For each experimental unit, the number of inoculated units was compared with the symptomatic units (DI_{SY}) at monitoring to define the incubation period and to those

sporulating (dark grey to fuliginous lesions observed using a magnifying glass) units (DI_{SP}) to determine the latent period.

Fig. 1. Experimental setup of artificial inoculation in growth chamber experiments at the Institute of Plant Diseases and Plant Protection, LUH, Germany.

Detached leaflet experiment

This was an *in vitro* experiment using detached leaflets from plants that were grown under the same condition as that of the previous experiment. At 7 WAT, the area of the terminal leaflet of those marked leaves (1, 3, 5 and 7 weeks old) was destructively measured using a leaf area meter (model LI-COR LI-3100, Lincoln, NE). A single terminal leaflet was then immediately put to a 9-cm or 15-cm diameter Petri dish (based on the leaflet size) containing 1% water agar amended with 15 mg benzimidazole per litre of medium (added on top of the medium at 50°C put on a shaking water bath). Each Petri dish was thereafter filled with the medium to two-third level of volume to support the detached leaflet for longer time without withering. After labeling the treatment combinations, each leaflet was then inoculated while in the Petri dish with the stock solution of propagules and inserted to the respective growth cabins.

Inoculation was carried out in an aseptically cleaned chamber using a concentration of propagules (mycelia, mycelial fragments, conidiophores and conidia) of 2 x 10^5 mL^{-1} that was prepared in the same way as described above. The Petri plates were thereafter inserted to growth cabins set to the respective temperature regimes. Relative humidity was set to 85% in the cabins where the temperature regimes 20, 24, 28°C were tested, whereas humidity in the cabins of temperature regimes 32 and 36°C was maintained by having concentrated salt solution in an open plastic plate in each cabin. The water in these cabins was refilled every second day and kept in shelves with close proximity to the Petri dishes.

Due to limitations of growth chambers, only the two temperature regimes 24ºC and 36ºC were repeated whereas the other were done only once (Table 1). The experimental setup, with 4 ages and 5 temperature regimes, was a CRD with 20 treatments each replicated five times. For each leaflet, number of growing colonies was counted every day for the first 10 days and then at an interval of 2 to 5 days until 30 DAI. The time from inoculation until 50% of the colonies appeared was considered as incubation period. Adding the days to further appearance of sporulating colonies (with a slight change of grayish to black color as seen through a stereomicroscope (63x magnification) illuminated with external light) was considered as latent period.

Greenhouse experiments in Thailand

FMTT260 seeds were sown in peat moss and raised in a fan and pad equipped nursery for an average of two weeks. A single seedling was then transplanted into a 10-L capacity perforated white plastic pot containing a local commercial potting mix substrate (Textural classes: 30% sand, 31% clay, 39% silt, 28% organic matter and a pH of 5.3; Dinwandeekankasat, Ayutthaya, Thailand). This experiment in a CRD comprised six treatments, namely three inoculum levels, *i.e.* non-inoculated (NI), inoculated with high (I_H) and low (I_L) concentrations of suspension of *P. fuligena* over 2 leaf ages (1 and 3 weeks) replicated three (3A and 3B) and four (3C and 3D) times following a single plant as experimental unit approach of Kranz and Jörg (1989). Whereas the four treatments in experiments 3A and 3B included fungicide application (NI, I_H, I_L and NI_F) over two leaf ages (1 and 3 weeks) replicated three times, fungicide (mancozeb) application was omitted and replication was raised to four in experiments 3C and 3D.

Marking the oldest leaf started 3 days after transplanting and continued 2 weeks later by marking the youngest tip leaves (which were found convenient for wrapping the thread around

the petiole). A week later, data on host parameters like *PH*, *TLN* and length of marked compound leaves (LL_C) and their terminal leaflets (LL_T) in cm were measured. Whereas *PH* was measured every 4th day, *TLN* was counted every other day. Leaf dimension was measured at the time of inoculation, about 10 days later and at the end of the experiment (on average at 25 DAI) to compare dynamics of leaf area growth of both younger (1 week) and older (3 week) leaves. Accordingly, leaf length measurements were fitted to a power function derived from a sample of 250 compounds leaves and their terminal leaflets from those plants grown in the same greenhouse. For simplicity, the two parametric power function was developed based on the relationship between the length (LL_C) and the leaf area of the compound leaf (LA_C), *i.e.* $LA_C = 0.15 \cdot LL_C^{1.91}$. Before transplanting, and until the time of inoculation, a targeted successive weekly fungicide spray of the border plants and of the white plastic floor was performed to mitigate the impact of natural infections.

For inoculation, conidia of *P. fuligena* were prepared from freshly harvested and profusely sporulated lesions of FMTT260 leaves that were cut out using a 6-mm diameter cork borer. Many of these cut pieces were put into a beaker of 500 mL distilled water, the lesion-water mix was stirred for 5 to 10 minutes and later filtered using a double layer of cheese cloth. Two drops of Tween 20 were then added and after vortexing for a minute, conidial density was determined using a Fuchs-Rosenthal® haemocytometer (Brand GmbH, Wertheim, Germany). Accordingly the two conidial densities, *i.e.* high (I_H) and low (I_L) were prepared from a stock solution with a content of 2 x 10^4 and 400-500 conidia mL^{-1} of distilled water respectively. The conidial suspension was then inoculated on three weeks old (after transplanting) tomato plants in the greenhouse. Abaxial sides of all the leaves were kept flat open by supporting each with the palm to ensure complete coverage of finely sprayed conidial suspension. Plants in control treatments were similarly sprayed with distilled water. Microclimate at the vicinity of the experimental plants during the night of inoculation was measured using two Tinytag® Plus data loggers; one of them put on the surface of the substrate and the other suspended at about 75 cm height of the tomato plant. The inoculated plant was thereafter immediately seal-covered in a black plastic sheet. Weather parameters like temperature (°C) and relative humidity (%) as of uncovering the inoculated plants (14 to 16 hours post inoculation) were recorded every 10 minutes using an automated central data logger system.

Leaflets at time of inoculation were counted and data on BLM incidence of symptomatic leaflets (DI_{SY}) were collected every two days. At the same time, inception of sporulation (turning of white and grey yellow lesions to black) was followed similarly by counting the number of sporulating leaflets (DI_{SP}). Ten lesions were randomly selected on the marked

leaflets and the respective diameter was measured four times during each experiment. At transplanting, plants with any visible symptomatic leaves were discarded. Besides, due to the very low natural infection that prevailed within the short period of experimental duration (24 to 26 DAI), the NI treatment was neglected during the final analyses. This experiment was repeated 4 times (Table 1).

Table 1. Summary of experiments conducted, schedule and the three factors tested in each.

Experiment	Experiment duration (Inoculation – last assessment)	Temperature regime (°C)	Leaf age (weeks)	Wetness duration (days/hours after inoculation)
1. Growth chamber	22/04/05 – 26/05/05	24, 28	1, 3, 5, 7	0, 1, 2, 3 days
2. Detached leaf	22/04/05 – 22/05/05	24, 28, 32, 36	1, 3, 5, 7	-
	16/05/05 – 15/06/05	20, 24, 36	1, 3, 5, 7	-
3. Greenhouse in Thailand	30/05/06 – 24/06/06	Ambient	1, 3	14 hours
	08/08/06 – 01/09/06	Ambient	1, 3	14 hours
	07/11/06 – 01/12/06	Ambient	1, 3	16 hours
	02/02/07 – 28/02/07	Ambient	1, 3	16 hours

Data analyses

In all the experiments, progress curves of either BLM incidence of symptomatic leaflets (DI_{SY}) or colony density per leaflet were depicted across time of assessment for each temperature regime, leaf age and wetness duration. In a similar way, the progress curves of the proportion of sporulating leaflets (DI_{SP}) or colonies (sporulating colonies per leaflet) were depicted across the same time frame of assessment. In both cases, trends were interpreted in relation to the onset of each disease parameter, their intensity, steepness of the curves and duration elapsed to achieve maximum levels.

Area under the curves (AUC) of each of the above mentioned progress curves was calculated following the trapezoidal method (Campbell and Madden, 1990) as shown in equation 1. In eq. 1, y represents all the dependent variables of the disease, *i.e.* either proportion DI_{SY}, DI_{SP} or colony densities (symptomatic and sporulating) at assessment time t_j and m is number of assessments.

$$AUC = \sum_{j=1}^{m-1} \left[y\ (t_j) + y\ (t_{j+1}) \right] \cdot \left[(t_{j+1} - t_j) \right] / 2 \qquad (1)$$

Only for the second experiment, *AUC* was modified to represent the area under colony growth curve of symptomatic ($AUCGC_{SY}$) and sporulating ($AUCGC_{SP}$) colonies. Statistical significance of the three main factors (temperature, leaf age and wetness duration) as well as their interactions was tested using PROC GLM procedure in SAS (2003) and further mean separations were done according to Tukey's test of significance at $p < 0.05$. When interactions between the factors were significant, data were usually pooled to the next lower level.

AUC was then standardized by taking into account the total time duration ($t_m - t_1$) over which the summation was carried out. For clarity, all variables standardized in this way were marked with *, *i.e.* DI_{SY}* and DI_{SP}* in cases of the non-destructive experiments (1 and 3) or AUC_{SY}* and AUC_{SP}* for the detached leaf experiment (2). This standardized *AUC* value can be interpreted as a mean proportion of symptomatic or sporulating units.

Incubation period (*IP*) for the respective treatments was calculated by counting number of days from inoculation to the time when 50% of the BLM symptomatic leaflets, in relation to the maximum achieved, were recorded. Latent period (*LP*) too was counted from time of inoculation until 50% of the maximum experimental units (leaflets or lesions) started to sporulate. Although sampling started from the smallest unit (leaflet) and went on to the higher level (individual plant), *IP* and *LP* as well as all the other analyses in this chapter were made only on the leaflet basis considering the small sample size in the later two levels (leaves and plants). Since sampling interval was spaced unequally, a linear interpolation (eq. 2) was used to estimate the time when the 50% level of DI_{SY} and DI_{SP} was achieved. In eq. 2, y_1 is the amount of symptomatic units achieved just before 50% at time t_1, y_{50} refers to 50% of maximal number of symptomatic units and y_2 is the amount of symptomatic units just after surpassing the 50% of the maximum population at time t_2. For latent period, *IP* was changed with *LP* and *y* in all cases represented sporulating units.

$$IP = \left[(y_{50} - y_1) \cdot (t_2 - t_1) \right] / (y_2 - y_1) + t_1 \qquad (2)$$

Furthermore, disease severity at the last assessment date was compared on the terminal leaflets of those experimental plants in both growth chambers during experiment 1. Quantitative aspects like counting and lesion diameter measurement (in mm) of 10 randomly selected lesions (sporulating lesions) were carried out at 10, 16, 20 and 24 DAI in the last experiment.

For the AE experiments, weather parameters at inoculation (from inoculum spray till opening the plastic bags 14 to 16 hours later) in terms of temperature (T_I) and relative humidity (RH_I) were recorded every 10 minutes using Tinytag® Plus 2 (Gemini data loggers Ltd., UK).

Besides, daily mean temperature (T_{DM}) and relative humidity (RH_{DM}) values of the first 15 days after inoculation (DAI) were computed for each experiment from the climatic data record of the automated data logger system. Furthermore, favorability indices (*FI*) based on prevalence of optimal temperature (FI_T) and relative humidity FI_{RH} for the first 15 DAI were computed. The ranges, *i.e.* 26-33.4°C for mean *T* and > 84.5% for mean *RH* were delineated based on earlier reports from Hartman *et al.* (1991) and Wang *et al.* (1996). For each day *i*, the favorability index of each of the weather parameter during the day (FI_{TDi} or FI_{RHDi}) and night (FI_{TNi} or FI_{RHNi}) was set to 1 if the condition was favorable and to 0 if not:

$$FI_{TDi} \text{ or } FI_{TNi} = \begin{cases} 1 \text{ if the mean day } (TD_i) \text{ or night } (TN_i) \text{ time temperature of day } i \text{ is in the interval } 26°C \leq T < 33.5°C \\ 0 \text{ if } TD_i \text{ or } TN_i \text{ of day } i \text{ is } < 26°C \text{ or } \geq 33.5°C \end{cases}$$

$$FI_{RHDi} \text{ or } FI_{RHNi} = \begin{cases} 1 \text{ if the mean day } (RHD_i) \text{ or night } (RHN_i) \text{ time relative humidity of day } i \text{ is } > 84.5\% \\ 0 \text{ if } RHD_i \text{ or } RHN_i \text{ of day } i \text{ is } \leq 84.5\% \end{cases}$$

FI of temperature of the day (FI_{TD}) and night (FI_{NT}) for the first 15 post-inoculation days were then computed using equations 3 and 4, respectively.

$$FI_{TD} = \sum_{i=1}^{15} (FI_{TDi})/15 \qquad (3) \qquad FI_{TN} = \sum_{i=1}^{15} (FI_{TNi})/15 \qquad (4)$$

The same procedure was followed to compute *FI* of relative humidity of the day (FI_{RHD}) and night (FI_{RHN}) as shown in equations 5 and 6, respectively.

$$FI_{RHD} = \sum_{i=1}^{15} (FI_{RHDi})/15 \qquad (5) \qquad FI_{RHN} = \sum_{i=1}^{15} (FI_{TNi})/15 \qquad (6)$$

For each experiment, favorability values for temperature (FI_T) and relative humidity (FI_{RH}) were formed by calculating the mean value of the day and night favorability indices, i.e. $FI_T = 0.5 \cdot (FI_{TD} + FI_{TN})$ and $FI_{RH} = 0.5 \cdot (FI_{RHD} + FI_{RHN})$, respectively. In all the analyses of the weather parameters from the data loggers, the interval from 06:00 to 17:59 and from 18:00 to 05:59 were considered as day and night times, respectively.

RESULTS

Growth chamber experiment

BLM incidence of symptomatic leaflets (DI_{SY}) in growth chambers varied among the two temperature regimes (T_{24} and T_{28}), four leaf ages (A_1, A_3, A_5 and A_7) and four wetness durations (WD_0, WD_1, WD_2 and WD_3) tested (Fig. 1). Those leaflets inoculated and incubated at 28°C (Fig. 1 right) showed earlier onset of BLM symptoms and reached the maximum proportion of 1.0 faster than those at 24°C (Fig. 1, left) across the two other factors tested. Besides, the steeper slopes from onset of BLM incidence to its maximum were indicative that *P. fuligena* prefers incubation under 28°C than 24°C. Whereas days from inception of symptom appearances to the maximum level of 1.0 for the respective leaflet ages of 1, 3, 5 and 7 weeks were 14 to 22, 12 to 22, 12 to 22 and 14 to 30 at 24°C, these durations were shortened to 10 to 14, 10 to 16, 10 to 18 and 10 to 22 days at 28°C.

For successful infection and substantial epidemics of BLM to take place, a certain duration of wetness was a necessity, as was shown from comparisons of disease progresses without wetness duration (Fig. 1a and b) to the others with wetness durations. Without wetness duration, for instance, there was no disease development on the oldest leaves (7 weeks old). On the other three younger leaf age groups (1, 3 and 5 weeks old), however, low levels of DI_{SY} were observed as *WD* increased from 1 to 3 days (Fig. 1a and b). These low levels of DI_{SY} on leaflets 1, 3 and 5 weeks old could be attributed to those few conidia which managed to penetrate and cause symptom, aided with the confounding high *RH* during the night of inoculation. While the differences with and without wetness were conspicuous, a similar trend of DI_{SY} progress was observed amongst the other three wetness durations, *i.e.* 1 (Fig. 1c and d), 2 (Fig. 1e and f) and 3 (Fig. 1g and h) days.

Leaf age at inoculation also influenced DI_{SY} across the temperature regimes and wetness durations. There was less disease on leaflets of the oldest age (7 weeks old) compared to the other three ages (1, 3 and 5 weeks old). This was not only expressed in quantity but also through late onset in treatments with wetness durations of 1 to 3 days. At 24°C, for instance, DI_{SY} started at 12 DAI on those fully expanded young leaflets of ages 3 and 5 weeks but at 14 days on the oldest (7 weeks) and youngest (1 week) leaflets.

Proportion of sporulating leaflets (DI_{SP}) on the other hand showed a similar trend as that of DI_{SY} in all the three factors tested (Fig. 2). Latency was highly favored and thus shorter at 28°C (Fig. 2, right) compared to 24°C (Fig. 2, left). Besides, younger leaves (1, 3 and 5 weeks old) showed better sporulation in terms intensity and early onset than 7 weeks old leaves.

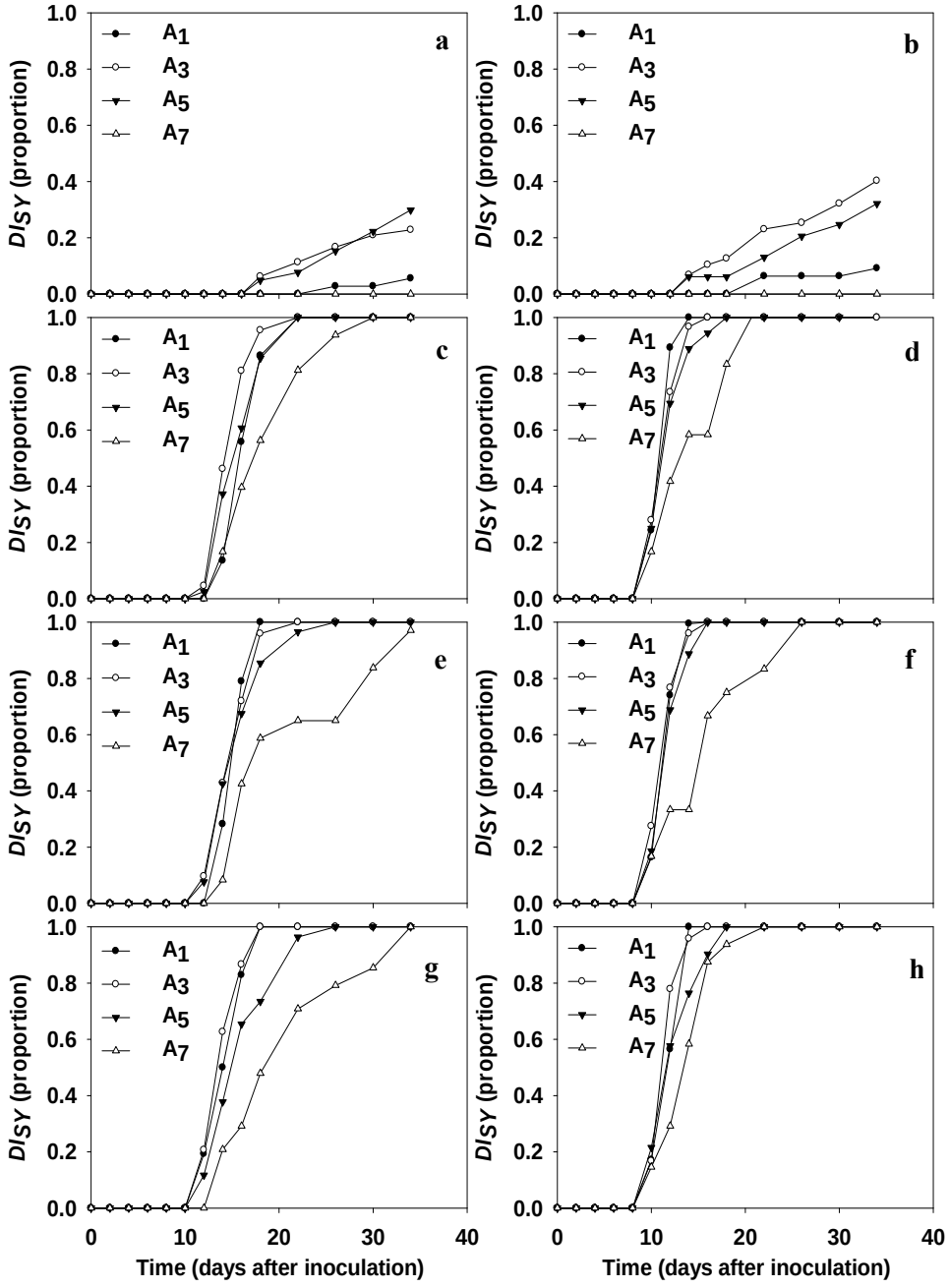

Fig. 1. BLM incidence of **symptomatic** leaflets **(DI_{SY})** artificially inoculated at four different ages (A_i = *i* weeks old) in growth chambers with temperature regimes of **24 (left)** and **28°C (right)** and exposed to wetness duration of **0** (a and b), **1** (c and d), **2** (e and f) and **3** (g and h) days (after inoculation).

More importantly, sporulation responded very much to wetness during infection. There was a clear tendency of increased steepness in slopes of DI_{SP} progress curves as wetness duration increased from 0 (Fig. 2a and b) to 1 (Fig. 2c and d), 2 (Fig. 2e and f) and 3 (Fig. 2g and h) days. DI_{SP} in the growth chamber, nonetheless, was lower and later in onset as compared to DI_{SY}.

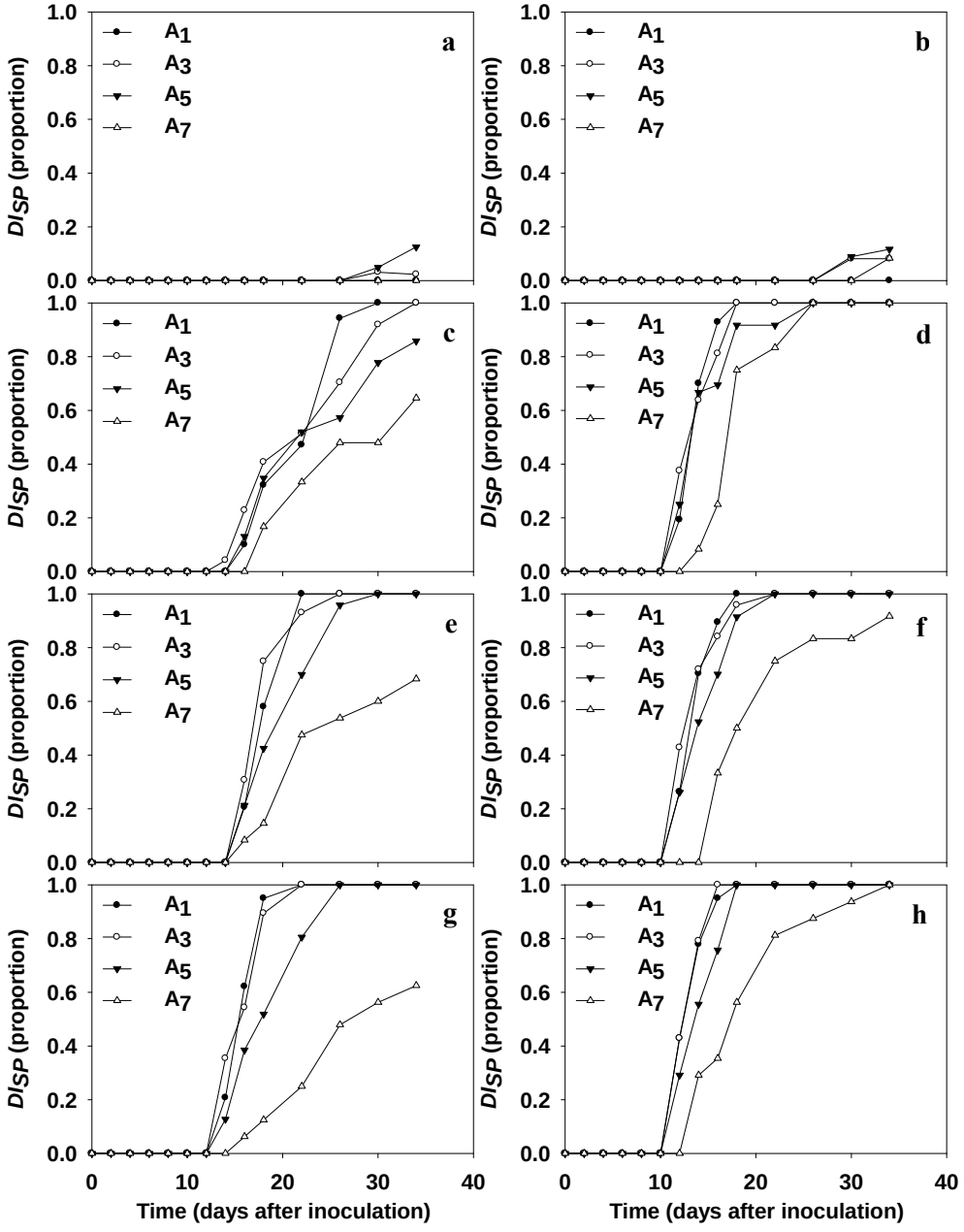

Fig. 2. BLM incidence of **sporulating** leaflets **(DI_{SP})** artificially inoculated at four different ages in growth chambers with temperature regimes of **24 (left)** and **28°C (right)** and exposed to wetness duration of **0** (a and b), **1** (c and d), **2** (e and f) and **3** (g and h) days after inoculation across the four leaf ages (A_i = *i* weeks old).

To acquire an elaborative and in-depth understanding of the impacts of the three factors, standardized areas under progress curves of BLM incidence of both symptomatic and sporulating leaflets were computed. After test of significances amongst the interactions, data were pooled to a one-way ANOVA and mean separations were performed according to Tukey's test at $p < 0.05$. The standardized area under the curves of symptomatic (DI_{SY}*) and sporulating (DI_{SP}*) leaflets (Fig. 3) reaffirmed all the aspects discussed above with respect to the progress curves.

The overall trend of the two temperature regimes proved a statistically significant difference with higher incidence of sporulating leaflets under 28°C (0.52a ± 0.05) than 24°C (0.34b ± 0.03) (Fig. 3a). Despite less visible symptomatic disease observed under 24°C, the difference to 28°C in terms of the DI_{SY}*, however, was not significantly different from 0 (Fig. 3a).

When comparing the effect of leaf age on the integral variables DI_{SY}* or DI_{SP}* (Fig. 3b), BLM incidence on leaflets 1 (0.82a ± 0.09), 3 (0.64ab ± 0.06), and 5 (0.57bc ± 0.05) weeks old at inoculation was visibly different from the oldest leaflets inoculated at the age of 7 weeks (0.38c ± 0.04). Accordingly, there were 30.5 and 53.7% less BLM symptomatic leaflets when ages 5 and 7 respectively were compared to the youngest leaflet age of 1 week. DI_{SP}* across the four leaf ages followed a similar decreasing trend (Fig. 3b).

Wetness duration, without doubt, was shown to be a prerequisite for germination and penetration of *P. fuligena* and hence for the onset of BLM infection. This was witnessed from the statistically significant difference of the three wetness durations of 1 (0.77a ± 0.04), 2 (0.79a ± 0.05) and 3 (0.80a ± 0.05) days (after inoculation) compared to the no wetness (0.05b ± 0.001) situation in terms of DI_{SY}* (Fig. 3c). DI_{SP}* was even negligible without wetness duration whereby 98.4% more sporulation was recorded when it was compared with that of the maximum wetness duration of 3 days (Fig. 3c).

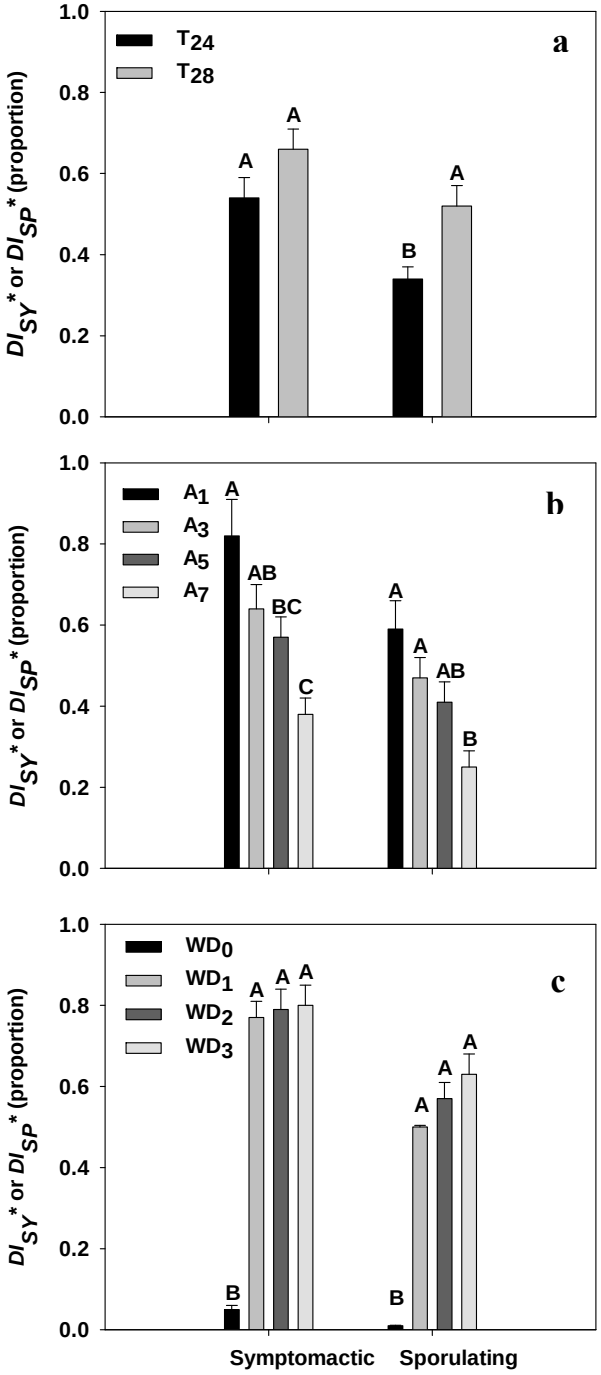

Fig. 3. Standardized **areas under curves** of BLM incidence of **symptomatic** (***DI*$_{SY}$*****) and **sporulating** (***DI*$_{SP}$*****) leaflets compared across a) temperature regimes of 24 (T_{24}) and 28°C (T_{28}), b) leaf ages of 1 (A_1), 3 (A_3), 5 (A_5) and 7 (A_7) weeks old and c) wetness durations of 0 (WD_0), 1 (WD_1), 2 (WD_2) and 3 (WD_3) days after inoculation. NB: Data were pooled into one-way ANOVA after hierarchical test of interactions between the three factors and means were separated using Tukey's test at $p < 0.05$. NB: Bars indicated with the same letters are not statistically different at $p < 0.05$ according to Tukey's test.

Separate analysis of incubation (*IP*) and latent (*LP*) periods (eq. 3) across the three factors is presented in Table 2. Obviously, both *IP* and *LP* were longer when leaflets were left without any further wetness duration (WD_0) after inoculation than otherwise (WD_1, WD_2 and WD_3). Accordingly, WD_0 resulted in as long as 30 days for *IP* and up to 32 days for *LP* (Table 2). Within the range of 1 to 3 days of wetness, however, both *IP* and *LP* were 2.2 to 5 days and 3 to 9.6 days shorter at 28°C than at 24°C. Generally, the shortest periods recorded were on fully unfolded leaves 3 and 5 weeks old (Table 2). Although increased wetness durations showed a general pattern and resulted in shortening both periods, the effect on *LP* was more pronounced at 24°C. Sporulating leaflets appeared 3.8 to 6.9 days earlier when wetness duration was increased from 1 day to 3 days, except a single case of the oldest leaf age (Table 2).

Table 2. Incubation (***IP***) and latent (***LP***) period (in days) of BLM on tomato leaves inoculated with *P. fuligena* at four ages (1, 3, 5 and 7 weeks) exposed to three wetness durations (1, 2 and 3 days after inoculation) in **growth chambers** set to 24 and 28°C.

Temperature (°C)	Leaf age (weeks)	Wetness duration (days)							
		0		**1**		**2**		**3**	
		IP	LP	IP	LP	IP	LP	IP	LP
24	**1**	26.0	-*	16.0	22.3	15.2	17.6	13.9	15.4
	3	30.0	28.0	14.4	21.6	14.6	16.7	13.8	15.6
	5	26.0	30.8	15.1	21.5	14.6	19.0	14.9	17.7
	7	-	-	17.3	24.3	16.9	23.3	17.0	27.0
28	**1**	21.0	-	10.9	13.2	11.2	13.1	11.7	12.4
	3	20.8	28.0	11.0	12.9	10.9	12.5	11.1	12.4
	5	23.5	28.7	11.1	13.2	11.2	13.8	11.6	13.6
	7	-	32.0	13.0	17.0	12.6	18.0	12.0	17.4

* No symptomatic or sporulating leaflets observed

BLM severity on a leaflet basis reached a proportion of 0.76 on 3 weeks old leaves inoculated in a growth chamber with 28°C (Fig. 4). This proportion was statistically non-significant as compared to the next leaf age (5 weeks old) which on average showed a severity proportion of 0.66 (Fig. 4). Lowest severity (0.17c ± 0.05) was recorded on the oldest leaves (7 weeks old). There was less disease and thus a lower proportion of BLM severity at 24°C. For instance, the reduction was 8, 43, 45 and 76% on leaves 1, 3, 5 and 7 weeks, old respectively. The difference between leaflets 1, 3 and 5 weeks old was non-significant but the comparison with the oldest leaflet showed about 91% less disease on the latter (Fig. 4). Considering optimal growth of *P. fuligena* on 1 week old leaflets (expressed in terms of incidence of symptomatic and sporulating units, refer Fig. 3b), nonetheless, the low severity estimated was a surprise.

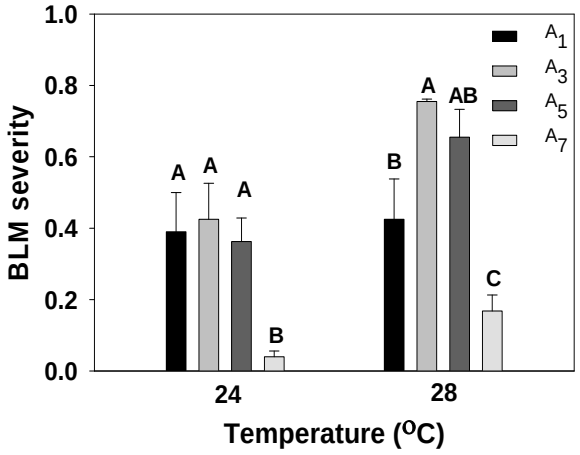

Fig. 4. Proportion **BLM severity** estimated (34 DAI) on artificially inoculated FMTT260 leaflets aged 1 (A_1), 3 (A_3), 5 (A_5) and 7 (A_7) weeks and incubated at 24 and 28°C during growth chamber experiment. NB: Bars indicated with the same letters are not statistically different at $p < 0.05$ according to Tukey's test.

Detached leaflet experiments

Growth of *P. fuligena* on detached leaflets at 36°C was tested twice in Petri dishes but ended without success since the leaflets could not stand the high temperature and got burnt 4 to 7 DAI. All analyses hereafter thus were only from the other four temperature regimes.

Density of BLM colonies, standardized to cm^2 leaflet area at the time of inoculation, showed a clear difference in terms of the two factors tested, *i.e.* leaf age and temperature (Fig. 5, left) in detached leaf experiments. Leaflet area was measured using a leaf area meter (model LI-COR LI-3100, Lincoln, NE) and standardized to sampled measurements at time of inoculation

(table not presented) and all density expressions were then computed on the basis of cm^2 leaflet area. Despite a nearly equal leaflet area of the youngest and oldest leaflets, 1 and 7 weeks old respectively, the count of symptomatic colonies on the former was exclusively higher across the four temperature regimes (Fig. 5, left). For instance, at the optimal temperature of 28°C, a maximum density of 4.64 colonies was recorded 18 days after inoculation (DAI) from the youngest 1 week old leaflets which was substantially higher compared to only 1.94 (at 25 DAI), 1.67 (at 25 DAI) and 1.36 (at 22 DAI) of 3, 5 and 7 weeks old ones (Fig. 5e). Appearance of BLM colonies also responded highly to the four temperature regimes. This could be illustrated from comparison of maximum colony densities of 4.64 (at 25 DAI), 2.78 (at 30 DAI), 1.82 (at 30 DAI) and 0.49 (at 30 DAI) at 28, 24, 20 and 32°C respectively (Fig. 5 left). This led to a conclusion that *P. fuligena* colonies on detached leaflets grew well at a temperature of 28°C (Fig. 5e) followed by that of 24°C (Fig. 5c) but less to few colonies were observed in Petri dishes incubated at 20°C (Fig. 5a) and 32°C (Fig. 5g).

Since the number of sporulating colonies were determined based on the total number of colonies grown (whitish representing symptomatic colonies) in a Petri dish, only the onset and speed of achieving the maximum saturation point were affected by the temperature regimes. Accordingly, the onset of sporulation was earlier and reached the saturation point in shorter time at temperature regimes of 24 (Fig. 5d) and 28°C (Fig. 5f) as compared to 20°C (Fig. 5b). There was, however, a distinct difference between the densities of those symptomatic (Fig. 5g) and sporulating (Fig. 5h) colonies at the highest temperature regime of 32°C. With regard to sporulation, there was a noticeable delay as compared to the observations from the growth chamber experiments. Overall, there was a delay of 2 to 6 days when detached leaflet and growth chamber experiments were compared. The progress curves of colony densities showed some decreases, specifically during the last assessment dates in the temperature regimes 20, 24 and 28°C due to the fast radial growth and coalescing of the colonies (Fig. 5a, c and e). A similar trend was observed for the same temperature regimes in terms of sporulating colonies, too (Fig. 5, right).

Statistical analyses of the two factors on the detached leaves were very much supportive to the above trends of the progress curves and helped in quantitative analyses in terms of average symptomatic (AUC_{SY}*) and sporulating (AUC_{SP}*) colony densities (Fig. 6). The overall trend was a general decrement of number of symptomatic as well as sporulating colonies as the leaflets became older (Fig. 6a). Whereas the average colony density on the youngest leaflet (1 week old) was statistically different from others in terms of the symptomatic and sporulating colonies, a similar significance was recorded from comparison of maximum colony density

per leaflet (Fig. 6a). The lowest value in terms of all the variables was always recorded from 7 weeks old leaflets (Fig. 6a).

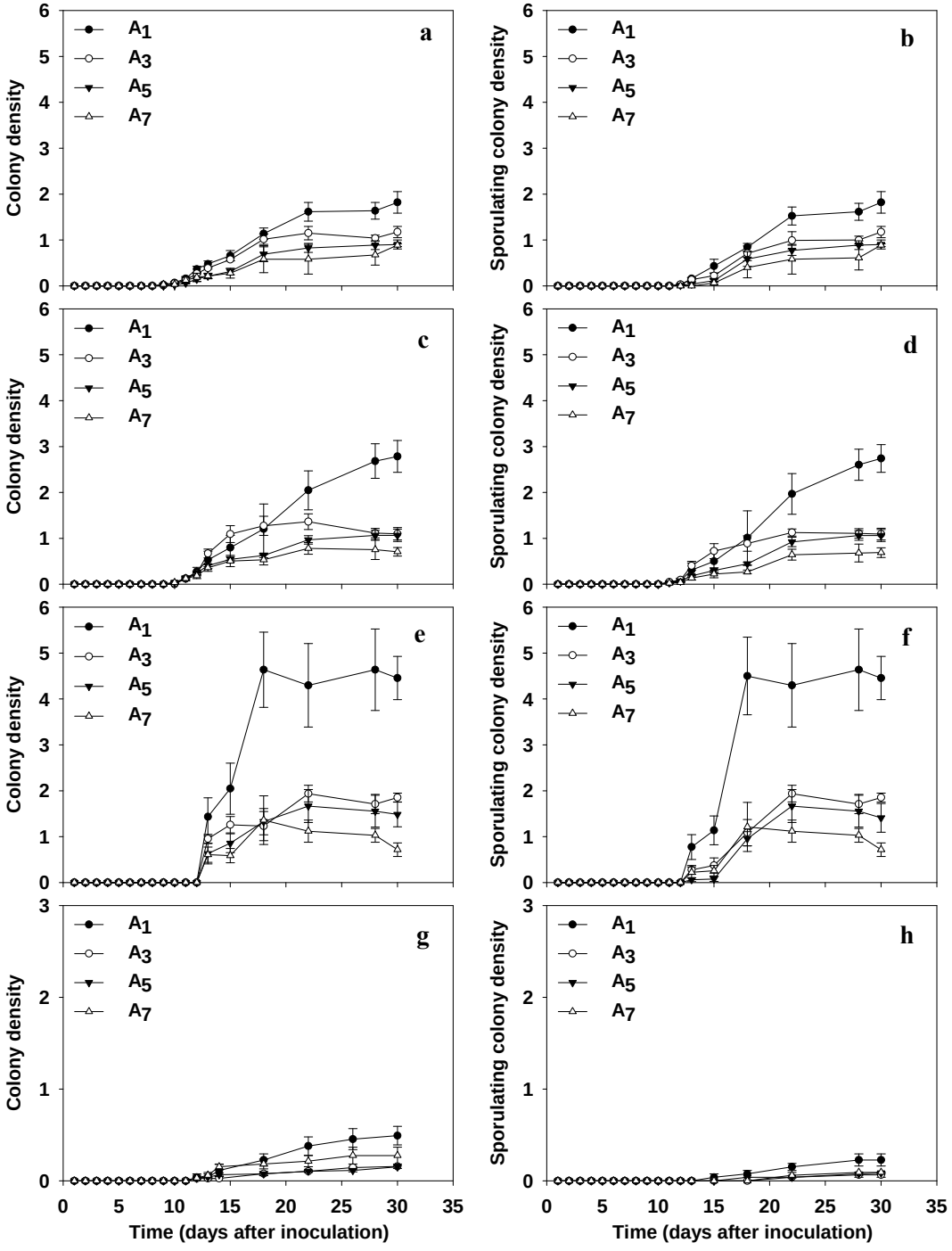

Fig. 5. BLM **symptomatic (left)** and **sporulating (right) colony densities** (number per cm^2 leaf area) on detached leaflets of FMTT260 at four different ages of 1 (A_1), 3 (A_3), 5 (A_5) and 7 (A_7) weeks at temperature regimes of **20** (a and b), **24** (c and d), **28** (e and f) and **32°C** (g and h). NB: Scaling of the colony densities at 32°C was reduced by half for clarity (Fig. 5g and h).

In contrast to the leaflet age, comparison of temperature regimes showed more distinct variations in all the assessments of AUC_{SY}*, AUC_{SP}* and maximum colony densities (Fig. 6b). In all comparisons, 28°C was found to be the most favorable temperature for incubation of *P. fuligena*, followed by 24, 20, and 32°C in descending order (Fig. 6b). Quantitatively, for instance, there was 35.3, 45.1 and 39.4% less disease in 24°C as compared to 28°C with respect to AUC_{SY}*, AUC_{SP}* and maximum colony density, respectively. The difference in terms of these three values was huge when comparison were made further to 32°C with 91.1, 96.1 and 89.6% less disease in this temperature regime compared to 28°C.

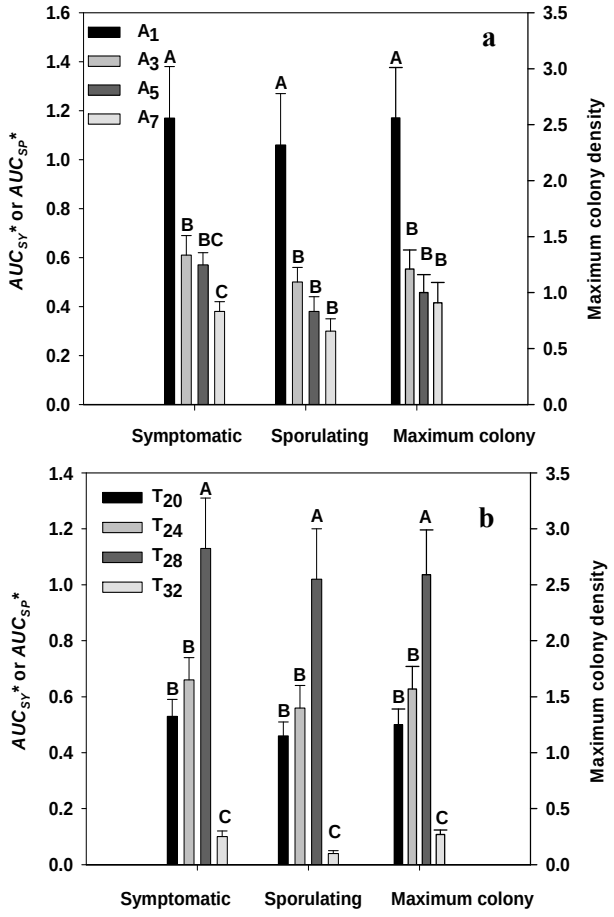

Fig. 6. **Standardized areas under colony density growth curves** in terms of symptomatic (***AUC*$_{SY}$****) and sporulating (***AUC*$_{SP}$****) colonies as well as maximum colony density (per cm^2 leaflet area) of BLM on artificially inoculated detached leaflets of four different **ages (a)** of 1 (A_1), 3 (A_3), 5 (A_5) and 7 (A_7) weeks and **temperature regimes (b)** of 20 (T_{20}), 24 (T_{24}), 28 (T_{28}) and 32°C (T_{32}). NB: Bars indicated with the same letters are not statistically different at $p < 0.05$ according to Tukey's test.

Unlike in the growth chamber experiment where symptomatic and sporulating units were analyzed in relation to the fixed number of inoculated units, the analyses in case of the detached leaflets was variable as it was based on the dynamic count of colonies on a single leaflet. This variation was partly corrected by standardizing each count as colony density (per cm^2 leaflet area) instead of using colony proportions. There was still no consistency in terms of incubation and latent period as the time to achieve maximum colony density as well as its intensity varied highly. Besides, the fluctuations in colony number due to coalescence of colonies affected the analysis of the above-mentioned periods presented in Table 3. Accordingly, the incubation period ranged between 13.0 and 18.9 days whereas the latent period was 13.6 to 24.1 days long (Table 3). In the same way as in the growth chamber experiments, the shortest incubation period was recorded on those leaves incubated at 28°C followed by 24°C. *IP* and *LP* were generally shorter on those fully unfolded leaflets of age 3 and 5. Nevertheless, there were some inconsistencies because of consideration of maximum colony count during analyses (Table 3). Overall, incubation period preceded latency by 0.5 to 9.3 days (Table 3).

Table 3. Incubation (***IP***) and latent (***LP***) period of BLM on **detached tomato leaflets** inoculated with propagules of *P. fuligena* at ages of 1, 3, 5 and 7 weeks and temperature regimes of 20, 24, 28 and 32°C.

Leaflet age (weeks)	Temperature (°C)							
	20		**24**		**28**		**32**	
	IP	*LP*	*IP*	*LP*	*IP*	*LP*	*IP*	*LP*
1	16.6	18.6	18.9	19.5	15.9	17.5	18.9	20.0
3	14.9	16.5	13.6	13.9	13.0	17.3	14.8	24.1
5	16.1	17.1	15.2	19.0	14.8	17.1	16.0	22.5
7	16.7	18.9	15.8	18.6	15.7	18.0	13.8	19.8

Greenhouse experiments in Thailand

Disease progress curves and integral variables

In a similar way as in the growth chamber experiment, the progress curves of BLM incidence of symptomatic (DI_{SY}) and sporulating (DI_{SP}) leaflets revealed a visible difference mainly among the two leaf ages tested (Fig. 7). Whereas the trends during the first three repetitions (experiments 3A to 3C) were very similar, the last repetition in February 2007 showed a slight variation, both in terms of onset of the disease and steepness of the slope. In all cases, however, younger leaflets at the time of inoculation (1 week old) showed earlier onset and reached the maximum proportion of 1.0 faster than those inoculated at the age of 3 weeks. For that matter, leaflets on 3 weeks old leaves, in all instances, never reached the maximum point during the observation time of the experiment, *i.e.* 24 days after inoculation (Fig. 7).

Generally, it took only 8 to 12 days for the appearance of BLM symptoms (Fig. 7, left) and sporulating lesions were seen about 2 to 6 days later (Fig. 7, right). BLM incidence on the leaflets in terms of both DI_{SY} (Fig. 7g) and DI_{SP} (Fig. 7h) during the last repetition in February 2007, however, was longer and required more time was to reach the maximum proportion of 1.0. This was depicted by the gentle slope of the progress curves (Fig. 7g and h), as compared to the top three repetitions (Fig. 7a to f).

Unlike the obvious distinction caused by the leaf age, the effect of the inoculum density was not substantially distinct. Considering leaves of age 1 week at inoculation, the proportion of symptomatic as well as sporulating leaflets inoculated with lower inoculum density (I_L) arrived at the maximum saturation level 2 to 4 days later than those inoculated with higher inoculum density (I_H).

This difference among the treatments across the four repetitions could also be seen from the quantitative and statistical comparison of the standardized areas under disease progress curves of symptomatic (DI_{SY}*) and sporulating (DI_{SP}*) leaflets (Fig. 8). Statistical analyses of the two factors in all cases showed no significant effects of the inoculum density alone and its interaction with the leaf age. As a result, the data were pooled to a one-way ANOVA and separation of the means using Tukey's test (at $p < 0.05$) proved a statistically significant difference between the leaf ages in all the cases.

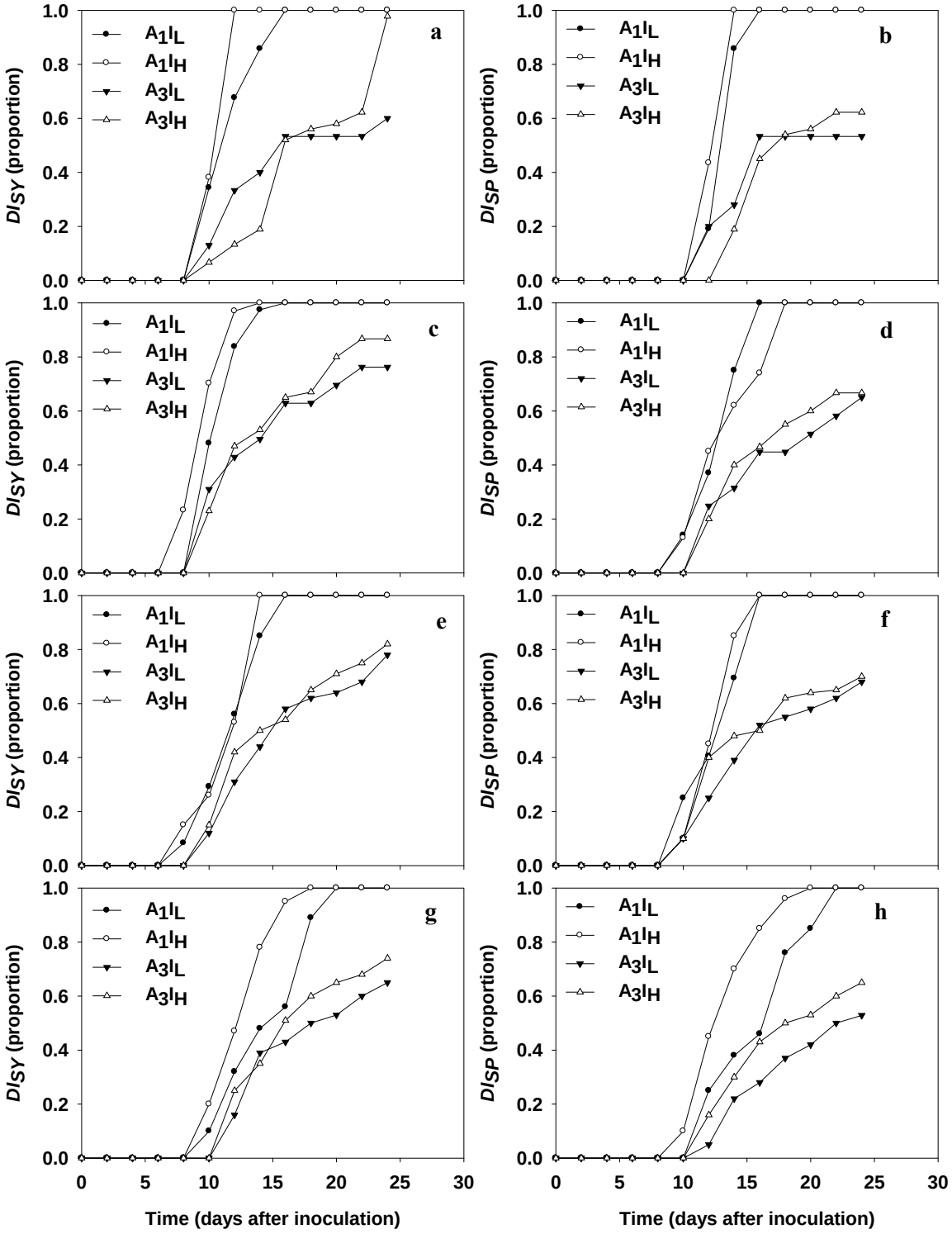

Fig. 7. BLM incidence of **symptomatic** (***DI*$_{SY}$, left**) and **sporulating** (***DI*$_{SP}$, right**) leaflets 1 **(A_1)** and 3 **(A_3)** weeks old when artificially inoculated with low **(I_L)** and high **(I_H)** inoculum density of *P. fuligena* under ambient environment experiments **3A** (a and b), **3B** (c and d), **3C** (e and f) and **3D** (g and h) at AIT.

In terms of DI_{SY}, for instance, there were 45 (Fig. 8a), 37 (Fig. 8b), 38 (Fig. 8c) and 44% (Fig. 8d) less symptomatic leaflets in 3 weeks old leaves as compared to the 1 week old. The differences were similar in case of DI_{SP} with 43, 41, 38 and 47% less leaflets with sporulation for the respective repetitions of experiments 3A to 3D.

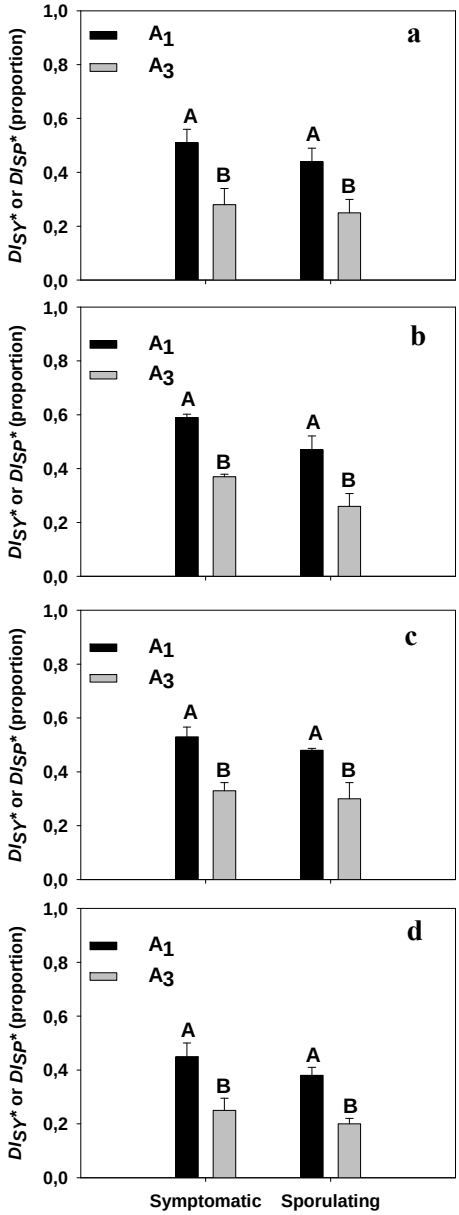

Fig. 8. Comparison of **standardized areas under disease progress curves** of BLM incidence of **symptomatic** (***DI*$_{SY}$****) and **sporulating** (***DI*$_{SP}$****) leaflets across leaf ages of 1 ($\mathbf{A_1}$) and 3 ($\mathbf{A_3}$) weeks from the four repetitions (a to d) of **the greenhouse experiments**. NB: Treatments with low (I_L) and high (I_H) inoculum density of *P. fuligena* were pooled after non-significant effect from ANOVA. Bars indicated with the same letters are not statistically different at $p < 0.05$ according to Tukey's test.

Data from count of total (*TLC*) and sporulating lesions (*SLC*) as well as radial measurement of individual lesions on terminal leaflets in one of the greenhouse experiments (3A) are presented in Table 4. *TLC* on the basis of a terminal leaflet was significantly different when compared across ages of 1 and 3 weeks and inoculum concentrations of low and high (Table 5). There were about 4 to 9 fold symptomatic and sporulating lesions on the younger leaflets than on older ones. Inoculum concentration was also directly related to the lesion count whereby about 3 fold symptomatic and sporulating lesions were produced from the high concentration as compared to the low (Table 4). Lesion diameter (mm) was found to be significantly different among the leaf ages (A_1 and A_3) but not among the inoculum concentrations (I_L and I_H) tested. Lesions were wider in diameter in younger leaves inoculated with low conidial density and vice versa (Table 4).

Table 4. Means (± SE) of lesion diameter (***LD***) in millimeter, total (***TLC***) and sporulating lesion count (***SLC***) on leaflets sprayed with low ($\mathbf{I_L}$) and high ($\mathbf{I_H}$) inoculum density of *P. fuligena* at ages of 1 (A_1) and 3 (A_3) weeks during experiment 3A.

Age	***LD***	***TLC***	***SLC***
A_1	7.3a ± 0.23	35.5a ± 4.48	23.9a ± 4.38
A_3	5.1b ± 0.22	3.8b ± 0.37	1.9b ± 0.29
Inoculum density	***LD***	***TLC***	***SLC***
I_L	6.7a ± 0.26	10.8b ± 2.53	7.5a ± 2.40
I_H	6.5a ± 0.27	28.4a ± 5.39	18.2b ± 4.65

NB: Means indicated with the same letter within a column are not statistically different according to Tukey's test at $p < 0.05$.

Weather parameters at inoculation and analyses of incubation and latent periods

Weather parameters in the greenhouse were highly favorable with respect to temperature (T_I) and relative humidity (RH_I) at inoculation, with an average T_I of 26.5°C and RH_I of 95.0% (Table 5). Since plants were tightly covered and sealed with black plastic sheets immediately after inoculation, long durations of favorability (14 to 16 hours) have resulted in similarities for the onset and intensity of the disease in all the repetitions. Except the last repetition in

February 2007, the daily mean temperature (T_{DM}) and relative humidity (RH_{DM}) for the first 15 days after inoculation were 29°C and 78% respectively. This was correspondingly reflected in the favorability index of temperature (FI_T). Since the BioNet™ greenhouse, where this experiment was run, was naturally ventilated, relative humidity during the day was considered negligible (refer to chapters 5 and 6) and hence only favorability of the night relative humidity (FI_{NRH}) was chosen for comparison (Table 5). FI_{NRH} was high during experiments 3C, medium in experiment 3B but low in the two others.

Table 5. Weather parameters at inoculation in terms of temperature (T_I) and relative humidity (RH_I) as well as daily mean temperature (T_{DM}) and relative humidity (RH_{DM}) and favorability indices of temperature (FI_T) and relative humidity (FI_{RH}) for the first 15 days after inoculation of each experiment **in a greenhouse in Thailand**.

Experiment	Weather parameters					
	T_I (°C)	RH_I (%)	T_{DM} (°C)	RH_{DM} (%)	FI_T	FI_{NRH}
3A	26.8	95.0	29.9	75.0	0.90	0.33
3B	27.1	95.2	28.9	78.6	1.00	0.47
3C	26.5	94.9	28.8	77.6	0.83	0.80
3D	26.2	94.6	25.6	77.3	0.33	0.33

Consistently, high favorability at the time of inoculation and the post-inoculation period in the greenhouse experiment resulted not only in a quick appearance of BLM symptoms but also in early onset of profuse sporulation as compared to the growth chamber and detached leaflet experiments. For instance, incubation period (*IP*) ranged between 9 to 12 days when 1 week old leaves were inoculated with the high inoculum density (A_1I_H) (Table 6). Latent period (*LP*) similarly ranged between 12 to 13 days for the same treatment (Table 6). There was a distinct difference between the two leaf ages with respect to *IP* and *LP*. Irrespective of inoculum density, there was a delay of 3.5 to 5 and 3.3 to 4.8 days in terms of *IP* and *LP* respectively when older leaves were compared to younger leaves.

Linear regression of both favorability indices (FI_T and FI_{NRH}) in all cases proved a reverse relationship with that of *IP* and *LP* as was revealed from negative slope term. FI_T for instance

fitted well with a moderate to high R^2 values with that of *IP* for inoculation of 1 (*IP* = 15.2 ± 0.98 – 5.02·FI_T, p = 0.0061, R^2 = 0.74) and 3 (*IP* = 18.4 ± 1.19 – 4.28·FI_T, p = 0.0270, R^2 = 0.59) weeks old leaves. The respective R^2 values in case of *LP* were 0.34 (*LP* = 15.2 ± 1.3 – 2.81·FI_T, p = 0.1290) and 0.49 (*LP* = 19.9 ± 1.2 – 3.67·FI_T, p = 0.0523). The overall comparison indicated that the first three repetitions were with comparatively higher values of favorability indices and resulted in shorter *IP* and *LP* as compared to the last experiment during the cold season of February 2007.

Table 6. Incubation (*IP*) and latent (*LP*) periods of BLM on tomato leaflets inoculated with low (I_L) and high (I_H) inoculum density of *P. fuligena* on leaf ages of 1 (A_1) and 3 (A_3) week old **in a greenhouse in Thailand**.

Exp.	Treatment							
	A_1I_L		**A_1I_H**		**A_3I_L**		**A_3I_H**	
	IP	*LP*	*IP*	*LP*	*IP*	*LP*	*IP*	*LP*
3A	10.9	12.9	10.4	12.3	15.5	15.8	15.9	16.8
3B	10.1	12.7	9.2	12.6	14.0	17.7	13.0	16.8
3C	11.6	12.7	11.8	12.3	14.9	15.7	14.0	16.0
3D	14.5	16.3	12.2	12.4	18.0	20.0	15.9	18.0

Host growth parameters like plant height (Fig. 9a) and total leaf number (Fig. 9b) were not substantially affected by the artificial inoculation during the observation time of all the repetitions of experiment 3. Since the time interval between inoculation and data assessment of the experiments coincided with the period of linear growth, the actual host progress curves both in terms of plant height (Fig. 9a) and total leaf number (Fig. 9b) fitted well to a linear function with $R^2 > 0.993$ (data not presented). While considering the dynamics of host growth over the whole period of observations in other components studies (refer to chapter 3, 5 and 6), however, *PH* and *TLN* fitted well to logistic function.

More interesting was the comparison of leaf area growth dynamics between leaves 1 and 3 weeks old. Right at the time of inoculation, the younger leaves at the tip were smaller but as time progresses this situation was reversed since the fast growth of younger leaves took over

the higher position. Actual levels of epidemics on young leaves under natural conditions and even after artificial inoculations sometimes get misinterpreted due to such high enlargement of the leaf area which dilutes the effect of the disease. This was clearly demonstrated in Fig. 9c whereby the average leaf area at time of inoculation (in cm^2) of the older leaves (49.5a ± 3.25 cm^2) did not significantly differ from the leaf area of the same leaves 24 days after inoculation (61.3a ± 3.97 cm^2). On the other hand, 1 week old leaves grew nearly 12 fold as they expanded from only 18.5c ± 1.86 cm^2 to 222.3a ± 1.24 cm^2 within 24 DAI (Fig. 9c).

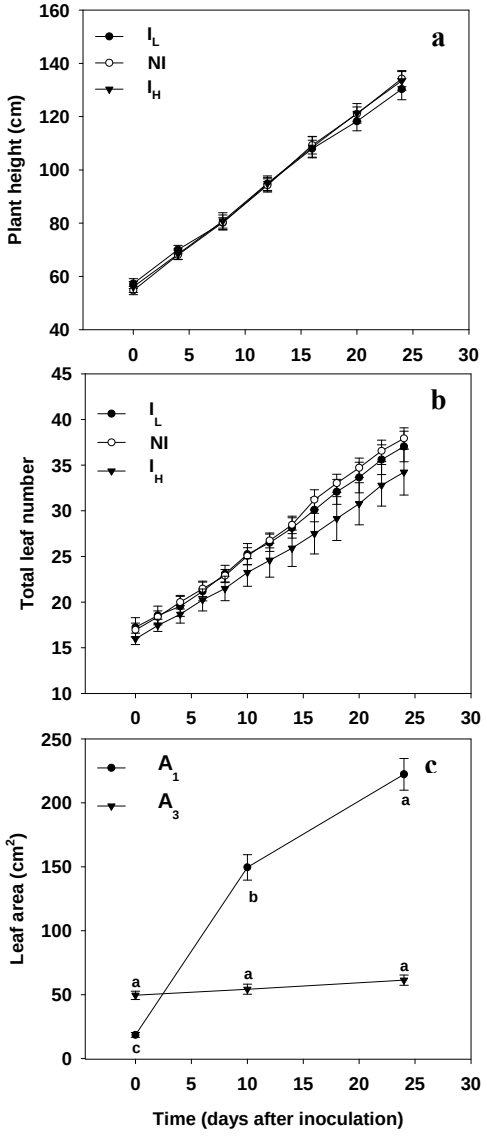

Fig. 9. Host growth parameters of FMTT260 plants during inoculation experiments under ambient environment at AIT indicating the dynamics of plant height (a) and total leaf number (b) from the treatments low (**I_L**), high (**I_H**) and no inoculum (**NI**) density of *P. fuligena* and comparison of leaf area progresses of 1 (**A_1**) and 3 (**A_3**) weeks old leaves within the assessment period of 24 days after inoculation (c).

DISCUSSION

Results from the non-destructive as well as detached leaflet experiments were in agreement with earlier reports of the same pathosystem. For instance, the observed relationships between the temperature regimes tested (20, 24, 28, 32 and 36°C) and onset of symptomatic and sporulating units was consistent with that of the *in vitro* experiments of Hartman *et al.* (1991) as well as other similar *Cercospora* pathosystems. Earlier onset of BLM symptomatic as well as sporulating units was optimally observed at 28°C compared to the others. Despite differences of the temperature regimes tested in this research and that of Hartman *et al.* (1991), *i.e.* 10, 14, 18, 22, 26, 30 and 34°C, the optimum temperature during their research (26°C) goes in agreement with our findings. Chupp and Sherf (1960) as well as Sherf and MacNab (1986) compiled the same range of preference for *P. fuligena*, *i.e.* between 26 and 28°C, in their books which was based on earlier outputs from Roldan (1938), Yamada (1951) and Mohanty and Mohanty (1955). Moreover, Blazquez (1991) indicated favorability of 27°C for BLM development. Incubators for growing *P. fuligena* on artificial media (refer to chapter 1 for the list of media) in this research were also set to this temperature (27°C ± 1°C).

The maximum temperature limit from earlier *in vitro* experiments ranged between 34°C (Hartman *et al.*, 1991) and 37.7°C (Chupp and Sherf, 1960). Although detecting any fungal growth at the highest temperature in this research (36°C) was impossible due to drying out of the detached leaves, there was no further confirmation whether growth of the fungus culminated at this temperature. Considering prevalence of high temperature particularly during noontimes in greenhouses in Thailand, the upper limit of temperature for the growth of the fungus could be assumed to be as high as that mentioned by Chupp and Sherf (1960). The *in vitro* experiments from Hartman *et al.* (1991) on growth media implicated survival and growth of the fungus even at temperatures as high as 40°C for a duration of less than 45 minutes. Running similar experiments on detached tomato leaflets, on the other hand, was found impossible as they could not withstand the heat stress from such a high temperature.

Regarding the lower temperature limit, growth of *P. fuligena in vitro* on artificial media was observed as low as 10°C. Considering the prevailing high day temperature which usually surpasses 28°C in the greenhouses (refer to the hourly temperature range from chapter 6, Fig. 4), however, disease development should most certainly assumed to be fairly favored by a temperature regimes between the optimal (28°C) and the maximum range (34 to 36°C). Though the daily mean temperature recorded from the ambient experiments at AIT was less due to the mild night temperature, BLM development could be optimally favored between 24

and 30°C. The colony counts from *in vitro* experiments at AVRDC (Hartman *et al.*, 1991) and the incubators set to 28 ± 2°C for incubating *P. fuligena* (Wang *et al.*, 1996) went in agreement with our observations.

Appearance of sporulating lesions too was highly affected by the temperature regimes tested in this research. This was shown with other *Cercospora* pathosystems like for instance in the model CERCOPRI developed by Rossi and Battilani (1991) for *Cercospora*-Sugar beet pathosystem. The effect of temperature in this model was based on principles of integrated values of degree-days. A typical approach that they used to model the relationship between temperature and latent period was by establishing latency rate as a function of temperature. On the other hand, a rate summation scheme (Hau *et al.*, 1985) was used to sum the rates and when the accumulated rates reached one, the latent period is declared to end.

Incubation (*IP*) and latent period (*LP*) in this research were defined by the time when 50% of the maximum appearance of BLM symptom or sporulating lesions, on the smallest unit inoculated, *i.e.* the leaflets, were recorded. Linear interpolation was used to pinpoint the exact time when this point was reached. Roumen and Boef (1993) used the same methodology in their study of leaf blast disease on rice. Depending on the wetness duration, *IP* of BLM after artificial inoculations varied between 11 to 13 days in growth chambers (non-destructive) and 14 to 16 days in growth cabins (on detached leaflets) at 28°C. Under artificial inoculations in the greenhouse in Thailand, however, *IP* and *LP* were shorter than observations from the aforementioned experiments at LUH. *IP* for instance was between 9 to 12 days and *LP* was between 12 to 13 days at high inoculum density in the greenhouse. This goes in agreement with earlier outputs of Yamada (1951), Hartman *et al.* (1991) and Hartman and Wang (1992) who reported a fairly long *IP* of 10 to 14 days. In contrast to the artificial inoculations, results from natural epidemics under ambient environment indicated longer *IP* which could on average start from about 11 days but extended up to 25 days (refer to chapter 4).

The fact that BLM is of a major concern in humid tropical countries where temperature for most parts of the year favors its epidemics, however, shifted the interest of our experiments to test other favorability factors like relative humidity (*RH*) and wetness duration (*WD*). This was similarly highlighted by Hartman and Wang (1992) who reported no temperature restrictions for the fungus (*P. fuligena*) within the normal range of tomato production. Wang *et al.* (1996) stated that the highly significant correlation between AUDPC and the number of dew days clearly showed that conditions conducive to dew formation and periods of prolonged leaf wetness play a key role in BLM development. Long periods of high *RH* have been shown to be instrumental to the rapid disease build-up of related diseases like *Cercospora* leaf spot of

peanuts (Hsieh and Goh, 1990), *Cercospora* blight of celery (Berger, 1969) and *Cercospora zeae-maydis* (Paul, 2005).

Since high relative humidity was recommended in many of the above-mentioned literature of our pathosystem, wetness duration was taken as an option to get an insight to the other favorability factor in this research. Both non-destructive experiments at LUH (wetness durations of 0, 1, 2 and 3 days) and AIT (a wetness duration of 14 to 16 hours) unambiguously proved importance of certain period of leaf wetness for BLM disease epidemics. The attempt to repeat the same wetness durations by covering the plants for more days in the greenhouse was impossible due to burning effect of the strong solar radiation. Without wetness duration, BLM developed negligibly and thus *IP* and *LP* were longer. In well-ventilated growth chamber experiments at LUH, it was noted that the fine spray droplets of inoculum lasted only about 20 minutes to 1 hour on the leaflet before drying out. Correlating this phenomenon from our study with that of lowest stomatal penetration (9.8%) observed even three hours after inoculation (Wang *et al.*, 1996), this was expected. In this research, despite progressive increment of symptomatic and sporulating units, there was no significant effect between them when the wetness duration was increased from 1 to 3 days. Many artificial inoculations during this research were successful only after wetness durations of 14 to 16 hours and thus the assumption of a minimum requirement of one day wetness duration for onset of BLM symptom under humid tropical greenhouses could be valid. Hartman *et al.* (1991) reported similar results but under different experimental setups of relative humidity during inoculation and transfers to greenhouse.

The final aspect, *i.e.* impact of age of tomato leaves on BLM epidemics, has not been reported in earlier studies. During both experiments at LUH, leaves 1, 3 and 5 weeks old at inoculation time were found to be more susceptible than 7 weeks old leaves. Particularly, *IP* and *LP* were delayed on these oldest leaves when compared to the fully unfolded leaves at a time of inoculation, *i.e.* 3 and 5 weeks old ones. There was a delay of 0.5 to 2 and 4 to 5 days of *IP* and *LP* respectively when the former were compared to the young and fully unfolded leaves as was witnessed from the growth chamber experiments. The detached leaves too showed a similar but less delay of 1.5 and 1.0 days for *IP* and *LP* respectively. One week old leaves at the time of inoculation in greenhouses similarly showed earlier appearance of BLM symptoms (3.5 to 5 days) and sporulating units (3.3 to 4.8) when compared to three week old leaves. A case in point from other pathosystems with similar approaches of determining the monocyclic components include that of *Colletotrichum coccodes* (Byrne *et al.*, 1998) whereby a direct progressive relationship between temperature regimes (15, 20 and 25°C), wetness duration (0,

4, 8, 12, 16, 20 and 24 hours) and leaf age (top, middle and bottom) with that of colony count per leaf disk was observed.

An important aspect that was noticed while considering host age at time of inoculation was the interactive dynamics of host growth in relation to disease severity. Observed low disease severity on 1-week-old leaves 34 DAI from growth chamber and ambient environment experiments (data not presented) highlighted this contrast when compared to results of detached leaf experiments. Faster growth dynamics of younger leaves within the time interval of inoculation and disease assessment certainly had a diluting effect on disease severity. Considerations of fixed host and misleading interpretations of impacts of plant diseases has been outlined among others by Vanderplank (1963) and Campbell and Madden (1990). Recently, Mersha and Hau (2008) have reported influence of such dynamic host growth situation on bean rust pathosystem.

Chapter 3: Effects of black leaf mold (*Pseudocercospora fuligena*) epidemics on host growth parameters of a fresh market tropical tomato (*Solanum lycopersicum*)

ABSTRACT

The effect of black leaf mold (BLM) epidemics on host dynamics of a fresh market tropical tomato grown under protected cultivation was studied from four plantings (A, B, C and D) starting June 2005 in central Thailand. BLM epidemics were highest during the plantings in August and September 2005 followed by that of November 2005. An epidemic during the planting in June 2005 was the lowest. The highest peak during the plantings of August and September 2005 and that of low disease epidemic from the planting in November 2005 corroborated with favorability based on seasonal classifications as hot-wet and cool-dry seasons respectively. On the other hand, it was most likely that level of inoculum played a role for the low epidemics of the planting in June 2005.

Disease incidence (*DI*) and severity (*DS*) fitted well to a logistic function and the sigmoidal shapes suggested that multiple cycles of infection occurred on leaves season-long. At times of heavy disease epidemics, a clear effect of reduced host growth particularly in terms of healthy leaf area (*HLA*) was discernible from a comparison of treatments with (F) and without (NF) fungicide application. A standardized disease severity (*DS**) proportion of 0.3 from those two undisturbed peak-epidemics plantings resulted in a 68% loss of *HLA* when treatments *F* and *NF* were compared. Healthy leaf index (*HLAI*) comparison of healthy plants ($HLAI_{HP}$) with that of plants with F ($HLAI_F$) and NF ($HLAI_{NF}$) treatments showed a respective loss of 11% and 50%. BLM, however, did not significantly affect other host parameters like plant height, total leaf number and even total leaf area formed for some of the plantings as heavily infected leaves remained hanging on the plant.

BLM epidemics in Thailand under protected cultivation substantially reduced *HLAI* of tomato during times of high weather favorability like the hot-wet season. In addition, level of inoculum at the time of planting influenced the epidemics making phytosanitary efforts obligatory. Two applications of the fungicide mancozeb at 4 and 6 weeks after transplanting (WAT) brought a highly significant reduction of 60 to 90% BLM epidemics during this research. Besides, one more application of mancozeb at 2 WAT could be optional at times of heavy epidemics as well as other inoculum situations of a particular planting season.

Key words: Black leaf mold, *P. fuligena*, FMTT260, host growth, healthy leaf area index, mancozeb

INTRODUCTION

Vegetable production under protected cultivation in humid tropical countries like Thailand presents not only opportunities to seize upon but also many challenges to confront. On the one hand, the long prevailing warmth and high humidity in these environments provide fertile grounds for faster and higher rates of photosynthesis and thereby biomass accumulation than temperate climate. On the other hand and to the dismay of the producer, many biological organisms, like plant pathogens and phytophagous insects, which are major biotic threats to vegetable production, are also favored by the same weather parameters. The case in point in this research is the foliar pathogen *Pseudocercospora fuligena* (Roldan) Deighton (syn. *Cercospora fuligena* Roldan) and epidemics of the black leaf mold (BLM) disease it causes on tomato (*Solanum lycopersicum* L.). The yearlong warmth and high humidity in the confined protected cultivation systems favors perpetuation of the pathogen thereby exacerbating the epidemic problem.

BLM was reported as a disease causing damage to tomato in Asia (Roldan, 1938; Yamada, 1951; Chupp, 1954; Mohanty and Mohanty, 1955; Hsieh and Goh, 1990; Blazquez, 1991; Hartman *et al.*, 1991), Africa (Chupp, 1954; Hsieh and Goh, 1990), Australia and Oceania (Chupp, 1954), North America (Blazquez and Alfieri, 1974) and was also recently detected at Roraima, Brazil (Halfeld-Viera, 2006). The hyphomycetous fungus *P. fuligena*, causing BLM, is highly favored by high humidity, moderate to high temperatures and low night temperatures which result in extended periods of leaf wetness (Blazquez, 1991; Wang *et al.*, 1996). Initial symptoms of the disease appear as small, pale yellow lesions with no definite margin on either the upper or lower leaf surface. These lesions have white fungal growth that turns gray to black as the fungus sporulates. Later, black sooty fungal growth will occur on both the upper and lower leaf surfaces.

The host, tomato (*Solanum lycopersicum* L.) on the other hand is a highly cherished and most widely grown solanaceaous vegetable next to potato globally (Rubatzky and Yamaguchi, 1997). Despite the steady increment of tomato production in Thailand (FAO, 2007), information about BLM is scarce, with only a single report from Chandrasrikul (1962), until a recent survey of its threat under protected cultivation (Kandziora, personal communication).

Regardless of the biochemical changes that take place as a result of host-pathogen interaction, if the changes are to affect yield, they must be reflected as changes in one or more of the physiological determinants of crop growth at the level of the whole plant as categorized by

Charles-Edwards (1982). One of these determinants, proposed as overridingly important (Waggoner and Berger, 1987), is radiation interception by the crop canopy. Except those diseases which destroy the yield bearing organs directly, for most groups of diseases that severely impair precursor physiological aspects of yield formation, an approach that accurately accounts for all the interactive effects of the disease on a host is crucial (Rouse, 1988). Such effects of a disease on a host have been reviewed and further elaborated by many scientists (Boote *et al.*, 1983; Rouse, 1988; Hau and Kranz, 1990; Burdon, 1993; Kranz, 2003). Waggoner and Berger (1987) have proposed using healthy leaf area (HLA) or absorbance of photosynthetically active radiation (PAR) by HLA over the course of crop development as a predictor of yield. Seem (1988) similarly used the concept of healthy area duration (HAD). Besides, de Jesus Junior *et al.* (2003) and Mersha and Hau (2008) have followed similar trends in showing the respective effects of angular leaf spot and bean rust on host dynamics of *Phaseolus* beans. With this respect, BLM as a foliar pathogen obviously affects photosynthesis since carbon uptake is hindered in the affected lesion area resulting in a loss of photosynthesizing tissue. Eventually, the effect of BLM is also reflected in affecting the yield component as reported causing substantial yield loss to tomato in countries like Taiwan (Hartman and Wang, 1992), India (Mohanty and Mohanty, 1955), Japan (Yamada, 1951) and Thailand (Mersha and Hau, unpublished).

Except very few yearly reports and earlier peer-reviewed research outputs, the last of which dated more than a decade back, there is no study undertaken to quantify effects of BLM on the host growth parameters. One of the objectives of this research was thus to analyze the impact of *P. fuligena* on host growth parameters of *S. lycopersicum* from four plantings at different times in a naturally ventilated greenhouse in Thailand. Moreover, despite earlier frequent spray recommendations (Blazquez and Alfieri, 1974; Hartman and Wang, 1992; Anonymous 2007) of fungicides to control BLM, no concrete recommendations were so far available as to timing and frequency of their application. As epidemiological principles become important in determining the time of fungicide application with greater precision (Royle, 1994; Cook and Yarham, 2006), this study covered the aspect by comparing values of maximum disease levels with and without application of the fungicide mancozeb.

MATERIALS AND METHODS

Experimental overview

An experiment comprising four successive plantings of the indeterminate fresh market tropical tomato (*S. lycopersicum* L.) cultivar FMTT260 (AVRDC, Shanhua, Taiwan) in a greenhouse was carried out to study the natural epidemics of the foliar fungal disease BLM caused by *P. fuligena*. The plantings began with a single stem in June and August 2005 (plantings A and B) but continued with double stemmed plants in September and November 2005 (plantings C and D). All plantings of the experiment were run in a 50-mesh naturally ventilated greenhouse (BioNet^{TM}, Klayman Meteor Ltd. Petah Tikva, Israel) with an area of 200 m^2 (20 m long, 10 m wide, and 7 m high). Computer automated active ventilation was provided by two exhaust fans installed on one gable, which were calibrated to be on at temperatures exceeding 30ºC and 33 ºC for the first and second fans respectively. As the experiment involved undisturbed BLM epidemics, plants were left untouched for natural infection except in treatments when fungicide was sprayed to create a different level of epidemics. To curb the impact of neighbor rows and the carry-over of conidia from one planting to the other, border rows were selectively sprayed with the fungicide mancozeb (80% WP). The white plastic floor was disinfected by recurrent sweeping of the floor with 95% Ethanol and fungicide (mancozeb) spray at the start and finish up of each planting.

The experimental site was in the campus of the Asian Institute of Technology (AIT) at the greenhouse construction area of the "protected cultivation project" located 44 km north of Bangkok, in Khlong Luang, Pathumthani province which is the central region of Thailand (14º 04′ N, 100º 37′ E, altitude 2.3 m above sea level).

Production and management of experimental plants

FMTT260 seeds were sown in peat moss and raised in a fan and pad equipped nursery for an average of two weeks. A single seedling was then transplanted into a 10 liter capacity perforated white plastic pot containing a local commercial potting mix substrate (Textural classes: 30% sand, 31% clay, 39% silt, 28% organic matter and a pH of 5.3; Dinwandeekankasat, Ayutthaya, Thailand). The pots were arranged in 6 rows at an interval of 1.3 m in the greenhouse. Within a row, 50 or 60 pots were spaced 35 or 30 cm apart resulting in a total population of 300 or 360 plants for double and single stemmed plantings respectively. Automated fertigation was scheduled based on a radiation sum and growth stage

whereby dripper intervals were regularly adjusted producing an average of 9 irrigation cycles per day (J. Max, personal communication).

Experimental setups and data collection

Amongst the six rows in the greenhouse, the two exterior and two most interior rows were left as boarder rows and thus were fortnightly sprayed with the fungicide mancozeb. Only the 2nd and 5th rows were used as experimental plots for the study of BLM epidemics. In these rows, twelve plants were arranged in a split plot design with planting time considered as a main plot and the two treatments, *i.e.* fungicide spayed (F) and non-sprayed (NF) as sub-plots. The sub-plots were set as blocks that were separated with a gap of 0.6 to 1 m. Two consecutive plantings were separated with a 2 m gap whereby a space was left empty after removing 6-7 pots. There were six replications for each treatment considering the single plant as experimental unit following the approach of Kranz and Jörg (1989). Accordingly, in the treatment F, six plants were sprayed with the fungicide mancozeb at the age of 4 and 6 weeks after transplanting (WAT) whereas the rest half (NF treatment) were left untouched for natural infection. Within a row, two plants at both edges and between treatments were left as boarder plants. Two plants of the 3rd planting were discarded due to mechanical damage and viral infection.

Plant height (*PH*) was measured from the base of the stem to the tip of the youngest leaf and number of leaves was counted on a weekly interval to represent host growth parameters. Each week, diseased leaves were marked with multi-colored threads loosely wrapped around the petiole and disease incidence was computed on a plant basis. Besides, disease severity was visually estimated on a plant basis on a weekly interval for 14 to 16 WAT. To mitigate subjective errors, disease was assessed solely by the researcher.

Moreover, destructive samplings and quantification of severity on a plant and leaf basis were performed using the microscopic software AxioVisionAC (version 4.2.0, Carl Zeiss Vision, GmbH, Germany). Total leaf area (*TLA*) formed by individual experimental plants was measured using a leaf area meter (model LI-COR LI-3100, Lincoln, NE). For modeling the host area, three plants from the boarder rows (those frequently sprayed with mancozeb) were destructively sampled on a weekly basis and their leaf area was measured using the leaf area meter.

Weather parameters, among others, temperature (*T*) and relative humidity (*RH*) inside and outside the greenhouse were recorded every 5 minutes using a central automated data logger system attached to two sets of sensors for each weather parameter.

Data analyses

Statistical analyses of disease intensities

Disease incidence of leaves (*DI*), on a plant basis, was calculated as a proportion of the number of symptomatic leaves on a plant as compared to total number of leaves at a time of assessment. Disease severity (*DS*) per plant on the other hand was done by visual estimation of percentage of diseased leaf area in comparison with the total leaf area (100%) of the plant following the method of Hartman and Wang (1992, 1993). All *DS* and *DI* values in this study are presented as proportions. Data from the temporal progresses of *DI* and *DS* were fitted to the three parametric logistic function (eq. 1).

$$y(t) = y_{\max} / 1 + (y_{\max} / y_0 - 1) \cdot e^{-r_y \cdot t} \qquad (1)$$

The parameters y_0 and $y_{\max}$ are the respective initial and maximum disease intensities (*DS* or *DI*), respectively, while r_y refers to rate of infection (1/day) for the logistic function.

Host growth analyses

Host growth parameters like plant height (*PH*) and total leaf number (*TLN*) scouted for 14-16 weeks after transplanting (WAT) on a weekly basis were fitted to a logistic function (eq. 2). To detect prevalence of any significant difference between the fungicide (F) and non-fungicide (NF) treatments, the y_{max} was compared pairwise for each planting following the methodology of Campbell and Madden (1990).

$$H(t) = H_{\max} / 1 + (H_{\max} / H_0 - 1) \cdot e^{-r_H \cdot t} \qquad (2)$$

The parameters H_0 and $H_{\max}$ are the initial and maximum host growth parameters of either plant height (*PH*) in cm or count of total leaf number (*TLN*), respectively, while r_H refers to rate of host growth (1/day) for the logistic function.

Besides, healthy leaf area (*HLA*) in cm^2/plant was calculated by subtracting the diseased leaf area (*DLA*) from the total leaf area (*TLA*) formed at harvesting. *DLA* was calculated as a product of the visually estimated *DS* in proportion and *TLA*. Consequently, healthy leaf area index (*HLAI*) was calculated by considering the area of the greenhouse (200 m^2) and the respective plant population of 360 or 300 for single and double stemmed plantings respectively, *i.e.* plant density of 1.8 or 1.5 m^{-2}.

Furthermore, in planting 3 (September, 2005), three healthy plants (border rows sprayed with fungicides 7 times fortnightly) were destructively sampled for leaf area index (LAI_{HP}) measurement, a parameter which is considered to be equal to healthy leaf area index of healthy plants ($HLAI_{HP}$). Dynamics of $HLAI_{HP}$ was linearly regressed to time (t in WAT) according to the function $HLAI_{HP} = 0.22 \cdot t - 0.25$ ($R^2 = 0.96$). The same function was used for those experimental plants which were twice sprayed ($HLAI_F$) or non sprayed ($HLAI_{NF}$) but by reducing the values of DLA.

Integral variables

For comparisons of epidemics, the area under the disease progress curve ($AUDPC$), in proportion-days, was calculated both for DS and DI using the trapezoidal integration method (eq. 3). Similarly, area under host growth curve ($AUHGC$) was also computed using the same method for both PH and TLN by replacing all y by H in eq. 3 to represent host growth resulting in cm^2-days or count-days respectively. In eq. 3, y represents all the dependent variables of the disease (DS or DI) or host (PH or TLN) parameters, at assessment time t_j, and m is number of assessments.

$$AUDPC = \sum_{j=1}^{m-1} \left[y\ (t_j) + y\ (t_{j+1}) \right] \cdot \left[(t_{j+1} - t_j) \right] / 2 \qquad (3)$$

Since the observation periods were 106, 110, 105 and 103 days during the plantings A, B, C and D, respectively, the integral variables were divided by the total time duration ($t_m - t_1$) of the epidemic or host growth, over which the summation was carried out. For clarity, all variables standardized in this way were marked with *. For $AUDPC$, this standardized value can be interpreted as the mean disease severity (DS) or incidence (DI) per plant over the assessment period. Data from standardized area under curves were then subjected to ANOVA and multiple tests of mean separation using the statistical analysis package, SAS 9.1 (SAS, 2003). All regression analyses of host and disease epidemic parameters were performed using SigmaPlot (2006).

RESULTS

Disease and host progress curves

BLM epidemics of the four plantings showed a conspicuous difference in terms of both disease incidence (Fig. 1, left) and severity (Fig. 1, right) across the four plantings and fungicide treatments. Actual disease incidence (*DI*) values of NF treatments at the last assessment date (average of 106 DAT) were 0.45, 0.98, 0.84 and 0.85 for plantings A, B, C and D respectively (Fig. 1, left). The difference was even more pronounced in terms of disease severity (*DS*) with respective proportions of 0.05, 0.72, 0.73, and 0.38 for plantings A to D (Fig. 1, right).

General pattern of epidemics during these four plantings as depicted in Fig. 1 reveals two scenarios: horizontally, the *DI* (left) for each planting was higher compared to the respective *DS* (right). Vertically, a clear variation in disease epidemics was discernible along the plantings in terms of *DS* with the two BLM peak-epidemic plantings, *i.e.* B (August, 2005) and C (September, 2005), followed by less epidemics of planting D (November, 2005) to the lowest in planting A (June, 2005).

Three parametric logistic function (eq. 1) fitted well to all the four epidemics in terms of *DI* and *DS* with R^2 values ranging between 0.98 and 0.99 (Fig. 1, Table 1). The disease progress curves, in all cases of the fitted epidemics in Fig. 1, further depicted clear differences between F and NF treatments within each planting. Accordingly, when y_{max} values of *DS* were considered, there was 2.1, 3.9, 3.1 and 14.5 fold more disease in the undisturbed epidemics of the non-fungicide treatments (NF) as compared to those sprayed with the fungicide mancozeb (F). The respective y_{max} estimates of the NF treatments ranged from 0.60 to 0.96 for *DI* and 0.05 to 0.72 for *DS* at times of lowest and highest epidemics (Table 1).

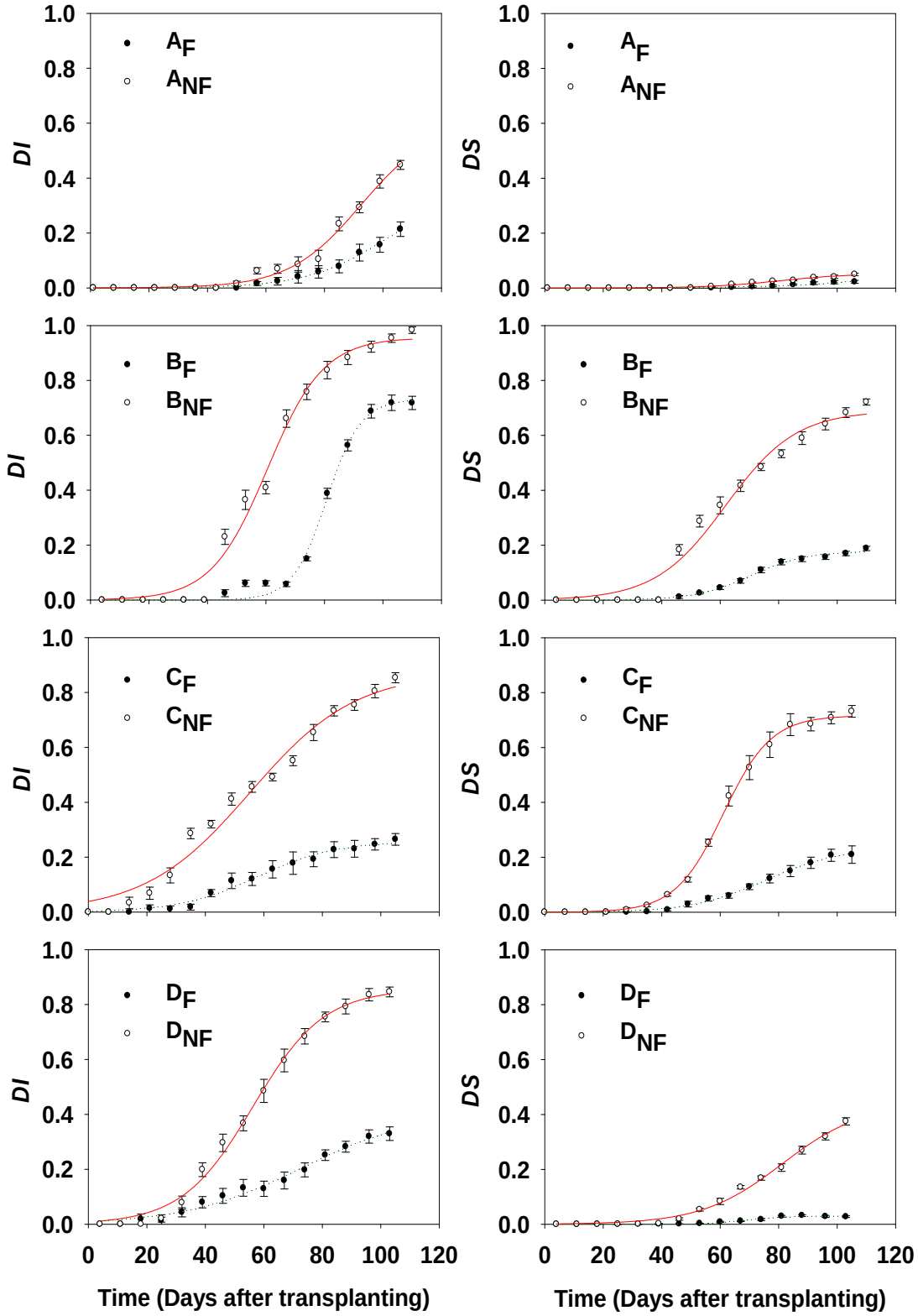

Fig. 1. Non-linear regression analyses of proportion disease incidence (left) and severity (right) of BLM epidemics with (F) and without (NF) fungicide spray fitted to logistic functions in plantings A to D.

In consequence, pairwise comparisons of all epidemics in both disease intensity categories (*DS* and *DI*) showed statistically significant difference at $p < 0.05$. Due to the inherent negative autocorrelation of the parameters y_0 and r_y in the logistic function, however, comparison of rate parameters within the plantings could not produce biologically plausible meanings as r_y in some F treatments was higher than in NF (Table 1).

Table 1. Estimated parameter values and coefficients of determination (R^2) of the logistic function (eq. 1) fitted to BLM epidemics expressed as disease incidence and severity proportions and comparison of the y_{max} parameter between fungicide (F) and non-fungicide (NF) sprayed treatments.

Exp. (Trt.)		BLM incidence				BLM severity			
		Parameters				Parameters			
		y_{max}	y_0	r_y	R^2	y_{max}	y_0	r_y	R^2
A_{NF}	Coeff.	0.6037a*	0.0003	0.0815	0.989	0.0530a	0.00007	0.0816	0.985
	SE	0.0760	0.0002	0.0097		0.0046	0.00005	0.0112	
A_F	Coeff.	0.3702b	0.0002	0.0717	0.995	0.0247b	0.000001	0.1144	0.960
	SE	0.0612	0.0001	0.0063		0.0006	0.000001	0.0052	
B_{NF}	Coeff.	0.9559a	0.0017	0.1042	0.989	0.6896a	0.0040	0.0831	0.980
	SE	0.0266	0.0011	0.0108		0.0324	0.0027	0.0116	
B_F	Coeff.	0.7307b	0.00007	0.1722	0.995	0.1787b	0.0007	0.1053	0.995
	SE	0.0164	0.00006	0.0151		0.0044	0.00006	0.0081	
C_{NF}	Coeff.	0.8728a	0.0384	0.0556	0.982	0.7165a	0.0004	0.1223	0.999
	SE	0.0495	0.0110	0.0065		0.0072	0.0001	0.0049	
C_F	Coeff.	0.2578b	0.0034	0.0748	0.987	0.2343b	0.0006	0.0794	0.996
	SE	0.0109	0.0015	0.0083		0.0084	0.0002	0.0050	
D_{NF}	Coeff.	0.8564a	0.0082	0.0824	0.996	0.4400a	0.0012	0.0727	0.994
	SE	0.0194	0.0022	0.0053		0.0311	0.0005	0.0064	
D_F	Coeff.	0.4053b	0.0099	0.0509	0.986	0.0302b	0.000001	0.1477	0.978
	SE	0.0405	0.0031	0.0060		0.0015	0.000001	0.0264	

* Values of y_{max} with the same letters in a column are not statistically different according to Tukey's test at $p < 0.05$ in pairwise comparisons of F and NF treatments.

Actual progress curves for the host growth in terms of *PH* and *TLN* are shown in Fig. 2. Except the obvious differences in *TLN* of the single and double stemmed plants (Fig. 2b), the growth pattern of most of the progress curves was very uniform. Data from these progress curves were later used in calculating the *AUHGC* (refer section about integral variables).
FMTT260 plants grew linearly to a certain time of the growth stage (until about 2 m height) in terms of *PH* which was an obvious expectation as the cultivar used in this research was an

indeterminate type (Fig. 2a). Since the plantings started with single stem, this linear growth went until about 30 leaves in terms of *TLN* whereas it was further extended to about 50 leaves in case of double stem (Fig. 2b). Except slight deviation during late growing season, the progress curves in all cases of host dynamics showed a trend implicating no substantial differences between fungicide sprayed (F) and non-sprayed (NF) plants (Fig. 2).

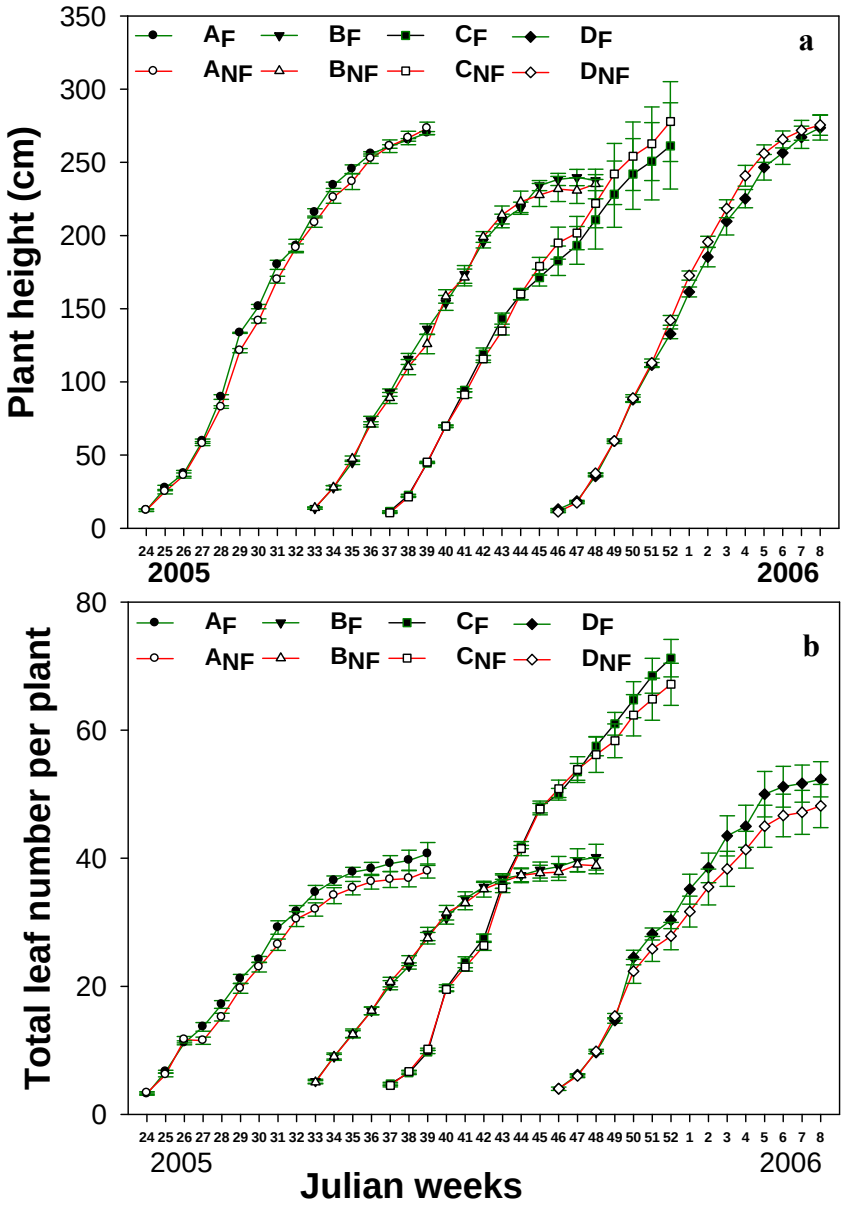

Fig. 2. Actual progress curves of the host growth dynamics of FMTT 260 plants a) expressed as plant height and b) total leaf number under the influence of *P. fuligena* with (F) and without (NF) fungicide treatments during the four plantings (A to D).

Table 2. Estimated parameter values and coefficients of determination (R^2) of logistic functions (eq. 2) fitted to host growth expressed as plant height in cm and total leaf number and comparison of the H_{max} parameter between fungicide (F) and non-fungicide (NF) treatments.

Experiment (Trt.)		Plant height (cm)				Total leaf number			
		Parameters				Parameters			
		H_{max}	H_0	RH	R^2	H_{max}	H_0	RH	R^2
A_{NF}	Coeff.	271.6a*	13.1	0.43	0.996	38.3b	3.22	0.41	0.995
	SE	4.20	1.6	0.02		0.59	0.33	0.02	
A_F	Coeff.	268.2a	12.9	0.46	0.996	40.8a	3.46	0.41	0.998
	SE	4.21	1.80	0.02		0.49	0.28	0.02	
B_{NF}	Coeff.	241.1a	0.37	0.42	0.996	38.9b	0.09	0.43	0.998
	SE	3.44	0.09	0.02		0.27	0.01	0.01	
B_F	Coeff.	246.0a	0.49	0.40	0.996	39.9a	0.12	0.41	0.998
	SE	3.96	0.12	0.12		0.32	0.02	0.01	
C_{NF}	Coeff.	278.8a	0.23	0.35	0.989	66.2a	0.03	0.39	0.993
	SE	9.3	0.11	0.03		1.47	0.01	0.02	
C_F	Coeff.	259.1a	0.23	0.35	0.984	71.2a	0.06	0.35	0.996
	SE	9.68	0.11	0.03		2.19	0.03	0.02	
D_{NF}	Coeff.	282.1a	0.001	0.45	0.998	49.3a	0.001	0.38	0.989
	SE	3.76	0.0003	0.02		1.37	0.0007	0.03	
D_F	Coeff.	281.6a	0.002	0.41	0.997	53.3a	0.0005	0.41	0.989
	SE	5.2	0.001	0.02		1.47	0.0004	0.03	

* Values of H_{max} with the same letters in a column are not statistically different according to Tukey's test at $p < 0.05$ in pair wise comparisons of F and NF treatments.

Integral variables and data at harvesting

The integral variables for the epidemic *DI** and *DS**, calculated as standardized area under disease progress curve (*AUDPC**), reconfirmed the difference between F and NF treatments as well as between all the plantings of the undisturbed epidemics (Table 3). *DS** comparison of the natural epidemics in the four plantings showed a significantly lower value in planting A (0.013) as compared to the other three (Table 3). Similarly, the *DI** values of 0.414, 0.408 and 0.376 in plantings B, C and D, respectively were statistically different from that of planting A (0.097). Due to variations in use of single and double stem as well as the prevailed external variations of heat stress problem during the last planting (D), comparison across plantings was not carried out.

Total leaf area formed at harvesting from fungicide sprayed and non-sprayed FMTT260 plants was only significantly different during single stem plantings but did not show any statistically significant difference during double stemmed plantings (Table 3). FMTT260 plants with lower BLM disease, however, showed higher leaf area formation as compared to these plants grown under heavy disease epidemics. Due to the impact of BLM, however, healthy leaf area at the time of harvesting was significantly different among F and NF treatments in three out of the four plantings (Table 3). During these three plantings (B, C and D) consequently, a loss of *HLA* up to 69% was recorded as compared to the 6% loss during the first planting (A) in June 2005.

Table 3. Standardized *AUDPCs* in terms of incidence (*DI**) and severity (*DS**), the respective *AUHGCs* of plant height (*PH**) and total leaf number (*TLN**) as well as total leaf area (*TLA*) formed until harvesting of FMTT260 plants grown under the influence of BLM disease with (F) and without (NF) fungicide application.

Experiment (Trt.)		Integral variables					Harvesting parameters		
		Epidemics (*AUDPC**)		*DS** reduction (%)	Host growth (*AUHGC**)		*TLA*	*HLA*	Loss of HLA (%)
		*DI**	*DS**		*PH**	*TLN**			
A_F	Coeff.	0.040b[1]	0.005 b		163.2a	26.3a	7976.2a	7791.1a	
	SE	0.009	0.002		0.74	0.50	401.1	372.6	
A_{NF}	Coeff.	0.097a	0.013 a	61.5	158.6a	24.5b	7703.7a	7320.9a	6.03
	SE	0.010	0.001		1.92	0.48	163.1	178.7	
B_F	Coeff.	0.195b	0.062 b		145.1a	26.9a	6228.3a	5047.8a	
	SE	0.006	0.004		1.55	0.58	265.0	182.2	
B_{NF}	Coeff.	0.414a	0.288 a	78.5	143.3a	26.7a	5952.8a	1648.6b	67.34
	SE	0.007	0.007		2.78	0.51	187.1	42.4	
C_F	Coeff.	0.114b	0.067 b		151.2a	40.4a	18150.5a	14283.2a	
	SE	0.016	0.008		9.64	0.92	610.0	186.0	
C_{NF}	Coeff.	0.408a	0.298 a	77.5	155.9a	39.5a	16492.6b	4410.0b	69.12
	SE	0.006	0.013		8.92	1.09	465.0	335.5	
D_F	Coeff.	0.129b	0.010 b		145.5a	31.0a	15041.8a	14631.9a	
	SE	0.013	0.001		3.36	1.52	528.5	536.8	
D_{NF}	Coeff.	0.376a	0.098 a	89.8	151.1a	28.5a	14336.3b	8989.1b	38.60
	SE	0.013	0.004		2.85	1.89	650.2	539.5	

[1] Means indicated with the same letters are not statistically different at $p < 0.05$ according to pairwise comparison of F and NF treatments of the integral variables (*AUDPC** and *AUHGC**) and harvesting parameters (*TLA* and *HLA*).

HLAI comparisons in healthy, fungicide sprayed (F) and non-sprayed (NF) plants

There was a distinct difference in *HLAI* progress between $HLAI_{HP}$, $HLAI_F$ and $HALI_{NF}$ (Fig. 3). Whereas $HLAI_{HP}$ reached 3.23 at the last assessment date, $HLAI_F$ and $HLAI_{NF}$ had only 2.53 and 0.68 respectively. Equivalently, the area under the curve of *HLAI* for $HLAI_{HP}$, $HLAI_F$ and $HLAI_{NF}$ treatments was 23.8, 21.2 and 11.8, respectively. The progress curves of the different *HLAI* in Fig. 3 clearly depicted the narrow window of epidemic boost period which later on influenced the dynamics of healthy leaf area components. They also portrayed the late onset and thereby the time after which the disease under natural epidemics started to have an impact on *HLAI*, *i.e.* about after 6 to 8 WAT.

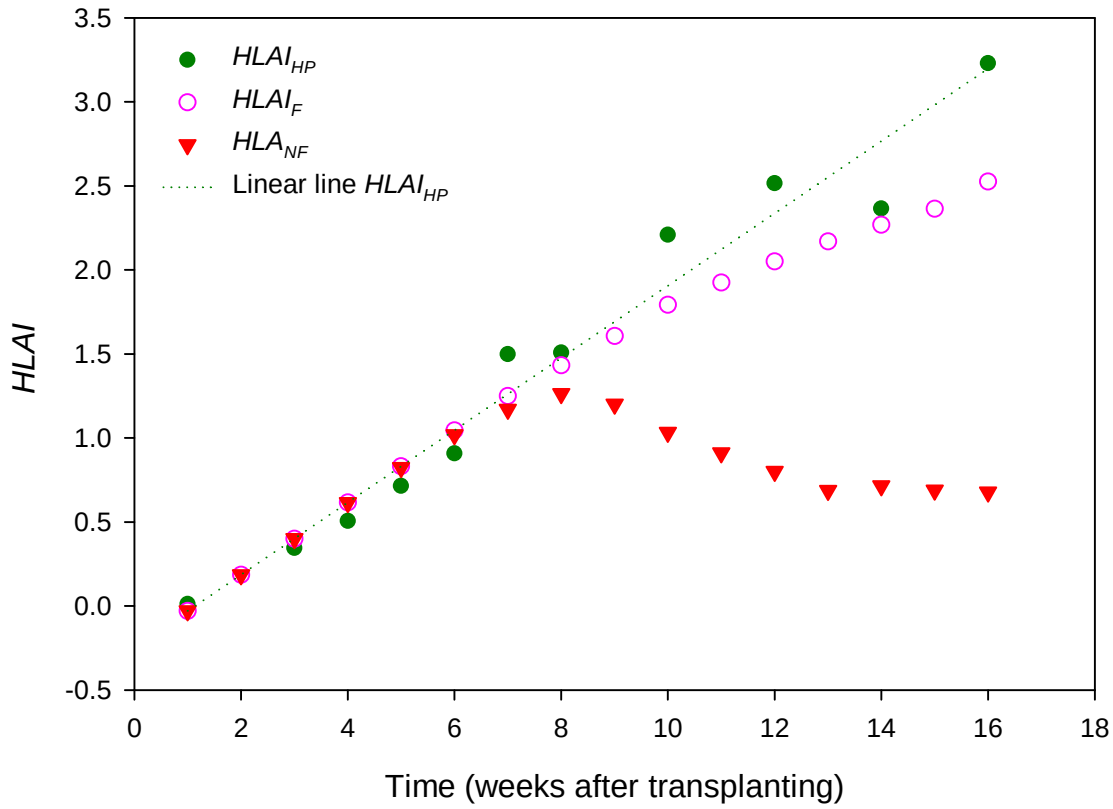

Fig. 3. Comparison of the dynamics of healthy leaf area index (*HLAI*) between destructively sampled healthy boarder plants ($HLAI_{HP}$), twice fungicide sprayed ($HLAI_F$) and non-fungicide treated ($HLAI_{NF}$) plants in experiment C of September 2005 at AIT, Thailand.

DISCUSSION

Distinct variation of disease intensities (BLM severity and incidence) between the four different plantings was a clear indicator of the association of BLM with the prevailing weather parameters in central Thailand area where the experiments were carried out. Kleinhenz *et al.* (2006) categorized the climatic conditions into three seasons: cool-dry (November to February), hot-dry (March to May) and hot-wet (June to October). The high disease epidemics in this research were observed in the August and September 2005 plantings which directly fell in the time frame of hot-wet season unlike that of the November 2005 planting during the cool-dry season that showed low BLM epidemics. This went in agreement with earlier findings which put optimum requirements of *P. fuligena* in terms of temperature, relative humidity and wetness durations (Blazquez, 1991; Hartman *et al.*, 1991; Hartman and Wang, 1992; 1993; Wang *et al.*, 1996). Drepper *et al.* (1993) found a similar trend of seasonality for maize-downy mildew pathosystem whereby plantings in November (cool and dry winter) showed no systemic infection but high severity was recorded during the rainy seasons. The low epidemic during the planting in June 2005, however, cannot be explained by the favorability since this season conventionally belongs to the hot-wet season whereby the weather favorability for BLM was assumed to optimal. This low epidemic at the beginning of the hot-wet season in 2005 may be attributed to low initial inoculum around the experimental site as all the greenhouse construction area was renovated and the inception of the research coincided with this planting time.

Maximum disease severity per individual plant at the last assessment date (about 106 DAT on average) were in a proportion range of 0.04 and 0.41 during the first (planting A) and fourth (planting D) plantings in contrast to 0.68 and 0.81 during the peak seasons of the plantings B and C. These results corroborated with the results of Wang *et al.* (1995) who stated that BLM severity can range from 0.1 to 0.4 for individual plants of resistant parents and 0.5 to 0.9 for plants of the susceptible parents in their screening trials. Though from artificial inoculations, Hartman *et al.* (1991) similarly reported a severity proportion of 0.87 on a plant basis after exposing the plants for two days (24 hrs. after inoculation) of high relative humidity. While the year round field results of Wang *et al.* (1996) at AVRDC centre in Taiwan proved similar with *AUDPC* values this research at times of high disease epidemic, November plantings from our research at the Asian Institute of Technology (AIT) in Thailand, however, were not found to be a high time for severe epidemics.

The non-significant differences between treatments in terms of plant height and total leaf number are indicators that these parameters are only slightly or none affected by BLM. Since, even heavily infected leaves curl upward, die prematurely but remain on the plant (Wang *et al.*, 1995), number of leaves may not be affected by the disease. Same phenomenon was observed in this research which is also extended to result partly in non-significant difference of total leaf area formations between fungicide treatments in plantings of June and August 2005. Hartman and Wang (1992), on the other hand, mentioned late defoliation as a possible reason for less impact of BLM which was not the case in this research.

Except at times of very low epidemics during the first planting, with mean *DI** and *DS** values of about 0.1 and 0.01, respectively, healthy leaf area was found to be significantly affected by BLM during the other three plantings. Under natural undisturbed epidemics, the two BLM peak-epidemics in plantings B and C, for instance, resulted in an average reduction of 68% of *HLA* from a respective *DI** and *DS** values of 0.4 and 0.3. Low values of *DI** and *DS** in contrary to high disease level at the last assessment dates of the plantings could mainly be attributed to the late onset of the disease.

Considering static situations, most crops are particularly vulnerable to diseases during the time of germination in the nursery, at a time of transplanting and establishment and during crop senescence at harvest. In temporal perspectives, however, there are usually high epidemics late in the growing season mainly due to canopy closure providing optimum conditions for the spread of pathogens. This eventually leads to increment of disease levels within the crop during the growing season. In case of protected cultivation, the cladding material and restricted air flow within a greenhouse exacerbates the situation and makes the window of epidemic boost narrow. The comparison of progress curves of *HLAI* between the three treatments ($HLAI_{HP}$, $HLAI_{F}$, and $HLAI_{NF}$) depicted this scenario very well. Besides, the progress curves portrayed the time after which the disease under natural epidemics started to have an impact on *HLAI*, *i.e.* about 6 to 8 WAT in this situation. Substantial decrement of *HLAI* was, however, visible as of 8 WAT. This had an implication for the time of fungicide application to control the disease. Earlier application of mancozeb at 4 and 6 WAT in this research in overall ensured a good control of BLM. Depending on the season and existing level of inoculum during planting, however, another earlier application at the 2nd week could be taken as an option.

Heavy disease epidemics like those of plantings B and C could inflict damage to the final yield (Mersha and Hau, unpublished). The yield loss attributable to BLM, however, may not be as great as other tomato foliar diseases such as bacterial spot, early and late blight or

Septoria leaf spot (Hartman and Wang, 1992) mainly because of late onset of epidemics as well as confinement of the disease to the lower stratum of the canopy which contributes less to yield compared to the upper most layer. Acock *et al.* (1978) indicated that the upper most layer which accounted for 23% of the total leaf area of tomato was the main interceptor of light and thus assimilated 66% of the net CO_2 fixed by the canopy. Such aspects of gradients in disease epidemics across plant canopy and the concentration of disease on the lower stratum led us to the next question of this research (refer to chapter 4).

In conclusion, the effect of BLM was clearly reflected in a significant reduction of host growth dynamics, particularly in that of the healthy leaf area at a time of heavy disease epidemics. Estimates of maximum disease level (y_{max}) from the logistic function were significantly different between the F and NF treatments in all the plantings. With y_{max} estimate of 0.69 and 0.72 for NF treatments during plantings B and C, 68% reduction in healthy leaf area was recorded. This output corroborated earlier works, that were mainly done at AVRDC, which were indicative for the economic damage of the disease at times of heavy disease epidemics. Besides, an effective two-time spray of the fungicide mancozeb at times of heavy epidemics could serve as part of integrated approaches of managing BLM. This could be witnessed from the two times mancozeb 80% WP (manganese ethylenebis, a dithiocarbamate polymeric complex with zinc) application and the respective disease reduction of 62 to 90% from this research. Hartman and Wang (1992) used a biweekly spray of benomyl (Benlate 50WP, 0.5 kg a.i./ha and maneb (Dithane M-45, 1.6 kg a.i./ha) during their inoculation trials at AVRDC. Furthermore, list of a wide range of fungicides against many fungal pathogens on tomato in Japan are listed by Arie *et al.* (2007). Seasons of low disease epidemics, however, could be left without fungicide, but only under a scrutinized surveillance and augmented with such novel approaches as step-wise pruning the lower portion of tomato canopy. This aspect would be dealt in depth in the coming chapter.

Chapter 4: Spatio-temporal dynamics of black leaf mold (*Pseudocercospora fuligena*) across the tomato (*Solanum lycopersicum*) canopy in natural and artificial epidemics under protected cultivation in Thailand

ABSTRACT

The vertical distribution of black leaf mold (BLM), caused by *Pseudocercospora fuligena*, was investigated across the canopy of the tomato cultivar FMTT260 under protected cultivation in Thailand. After 16 weeks of a natural epidemic, BLM severity (*DS*) of the lower canopy layer (0-50 cm) was 42% and significantly higher compared to 26% of the middle (51-150 cm) and 5% of the upper (> 150 cm) layer. During the growing period of another natural epidemic, a similar distribution of BLM was observed, but higher *DS* and bigger lesions were detected on leaf positions 5 to 10 (in ascending order from bottom up) than the first four leaves borne in the nursery. Further non-destructive samplings until 65 days after transplanting revealed the earliest BLM symptoms on leaf positions 5 to 8. When cohorts of 5 leaves were formed starting from the bottom, BLM incidence of leaves of the 1st cohort was only 79% while the next two cohorts reached 100%. In artificially inoculated plants, however, BLM was clearly more prevalent in the middle layer of the plant canopy as compared to the lower and the top part. Plants inoculated at 4 weeks after transplanting and monitored 10 days later, showed a *DS* of 43, 67 and 21% on the 1st, 2nd and 3rd cohorts, respectively. Thus, given equal chance of *P. fuligena* inoculum infect all the leaves of a tomato plant at one time, more BLM developed on fully expanded younger leaves than older once. High BLM severity of the lower canopy is not related to the age of tomato leaves, but attributed to proximity to substrate evaporation coupled with the down-hanging nature of FMTT260 leaves that created a confounding microclimate which led to higher relative humidity within the range of about 50-70 cm.

Key words: ***P. fuligena, FMTT260, canopy strata, leaf position, leaf cohort, canopy morphology, microclimate, leaf age***

INTRODUCTION

Profitable production of fresh market tropical tomatoes in the humid tropics under protected cultivation relies among others on effective management of foliar fungal diseases as the prevailing microclimate favors perpetuation and spread of the microbial organisms that cause these diseases. More importantly, the microclimate at the phyllophane that directly impacts plant growth also favors the phytopathogenic organisms (Jewett and Jarvis, 2001). Consequently, microclimate on and around a living plant can have a significant impact on the epidemics of fungal pathogens and the population dynamics of pests (Zhang *et al.*, 2002a). Black leaf mold (BLM) caused by *Pseudocercospora fuligena* (Roldan) Deighton (syn. *Cercospora fuligena* Roldan) is one of those fungal diseases reported to be favored by such microclimates in greenhouses in the humid tropics and hence leads to substantial yield loss on tomato (*Solanum lycopersicum* L.) in countries like Taiwan (Hartman and Wang, 1992), India (Mohanty and Mohanty, 1955), Japan (Yamada, 1951) and Thailand (Mersha and Hau, unpublished). Regional yield trials from Taiwan (Hartman and Wang, 1992) reported 32% yield loss due to BLM from a level of disease severity up to 53% on a plant basis for fresh market tropical tomato (FMTT) hybrids implicating a high susceptibility of these varieties. Mersha and Hau (unpublished) also a recorded an average marketable yield loss of 32% from an undisturbed high epidemics (mean severity of 30%) on an F1-hybrid FMTT260 under protected cultivation in Thailand.

The causative agent of BLM, *P. fuligena*, is a hyphomycetous fungus which is favored by high humidity, moderate to high temperatures and extended periods of leaf wetness (Blazquez, 1991; Hartman *et al.*, 1991; Wang *et al.*, 1995, 1996). Initial symptoms of the disease appear as small, pale yellow lesions with no definite margin on either the upper or lower leaf surface. These lesions have white fungal growth that turns gray to black as the fungus sporulates. Later, black sooty fungal growth will occur on both the upper and lower leaf surfaces of tomato.

Late onset of BLM epidemics and concentration of the disease at the lower canopy of tomato plants are mentioned as some of the reasons for the relatively lower impact of the disease on tomato as compared to other bacterial and viral diseases (Hartman and Wang, 1992). In their experiments in Taiwan, Hartman and Wang (1992) reported slow development of the disease on younger inoculated plants but a rapid increment was detected as the plants aged. There is, however, no study done so far to pinpoint the factors attributing to much confinement of the disease at the lower canopy as well as the rapid increment at the late growing season. Other

experiments in this study, for instance that on monocyclic components as affected by leaf age, in contrast, resulted in more susceptibility of younger leaves than older ones (refer to chapter 2). This aspect of resistance, which is mentioned as the phase-dependent (ontogenic) resistance within the framework of the genetically determined resistance (Hau and de Vallavieille, 2006) of a cultivar could also change the monocyclic parameters due to partial resistance, expressed after the seedling stage or at the adult plant stage. More evidence of preference of the pathogen to grow on younger leaves is mentioned in Blazquez (1991) from an indirect observation of ceasing of lesion expansions on matured leaflets as compared to younger leaves.

Moreover, factors like canopy morphology and growth habit of host plants and their cultivars also can affect the severity of epidemics since biomass of a host plant is not homogenous in its susceptibility and contribution to yield formation (Kranz, 2003). Some research works on plant and leaf age as well as leaf position have been done in some pathosystems by Hau (1985), Jörg *et al.* (1987), Shaw and Royle (1993), Lovell *et al.* (1997), Byrne *et al.* (1998), Schlösser *et al.* (2000), Arnold and Herre (2003), Visker *et al.* (2003) and Mersha and Hau (unpublished). Nevertheless, Royle (1994) underscoring the relevance of canopy architecture in affecting disease progresses in many pathosystems stated that this influence has often been ignored or considered simplistically in many instances and thus aspects of vertical disease niche of pathogens at a point in time and across the growing period are barely investigated.

Moreover, identifying inoculum source of a disease within a canopy profile of an individual plant as well as at the level of plant population is of high concern in studying epidemics of foliar fungal pathogens in protected cultivation systems since disease levels at certain point in the course of the growing period can abruptly increase and cause an irreversible damage. Such information on the spatio-temporal dynamics of the disease across the plant canopy serves as a foundation for designing integrated strategies of combating the disease at the right time before the epidemic boost gets out of hand. In this research thus a number of experiments from undisturbed natural epidemics of BLM as well as artificial inoculations were carried out to extract information on host tissue susceptibility and to use it for future disease management strategies of BLM and other foliar diseases with similar epidemiological requirements.

MATERIALS AND METHODS

Overview of experiments

Four experiments were carried out on the fresh market tropical tomato (*S. lycopersicum*) cv. FMTT260 (AVRDC, Shanhua, Taiwan) planted in two naturally ventilated greenhouses from June 2005 to March 2007. While the first two experiments focused on investigating the distribution of the disease across the canopy from natural epidemics, the third one was carried out with the same objective but after artificial inoculation of *P. fuligena* conidial suspension. The last experiment was performed to envisage the impact of canopy morphology and its microclimate on the disease zonation of BLM. A detailed summary of the experiments with the calendar of events is presented in Table 1. Canopy of FMTT260 was subdivided in experiment 1 into three strata (L: 0-50 cm, M: 51-150 cm and U: > 150 cm) and in experiments 2 and 3 based on leaf positions or cohorts. For the latter, the count was made based on insertion points of internodes from base in ascending order upwards.

Table 1. List and description of experiments with their respective calendar.

Exp.	Objective (BLM epidemics)	Begin of experiment	Stem (plant population)
1	Spatial distribution of BLM across plant canopy from natural epidemics at harvesting with and without fungicide	Sep. 2005 to Mar. 2007	Double (50 x 6 = 300)
2	Spatio-temporal dynamics of BLM across plant canopy from natural epidemics with and without destructive samplings of single and double stemmed plants	May 2006 to Nov. 2006	Single (60 x 6 = 360) Double (50 x 6 = 300)
3	Spatio-temporal dynamics of BLM across plant canopy from artificial inoculation of single and double stemmed plants	Jul. 2006 to Dec. 2006	Single (60 x 6 = 300) Double (50 x 6 = 300)
4	Impact of microclimate and host canopy architecture on BLM epidemics	Jun. 2006 to Jul. 2006	Double (50 x 6 = 300)

All experiments were run in two greenhouses each constructed on an area of 200 m^2 (20 m long, 10 m wide, 7 m high) with polyethylene roof and passively ventilated side walls made of 50-mesh UV absorbing insect proof net screens (BioNet^{TM}, Klayman Meteor Ltd., Petah Tikva, Israel). Computer automated active ventilation was provided by two exhaust fans installed on one gable, which were calibrated to be on at temperatures exceeding 30 and 33ºC for the first and second fan respectively. The experimental site was in the campus of the Asian Institute of Technology (AIT) at the greenhouse construction area of the "protected cultivation project" located 44 km north of Bangkok in Khlong Luang, Pathumthani province, which is the central region of Thailand (14º 04′ N, 100º 37′ E, altitude 2.3 m above sea level).

Production and management of experimental plants

Seeds of the indeterminate tomato (*S. lycopersicum* cv. FMTT260) were sown in peat moss and seedlings were raised in an evaporative cooled nursery (fan and pad equipped) for an average of two weeks. A single seedling was then transplanted into 2 or 10 liter capacity perforated polyethylene pots containing a local commercial potting mix substrate (Textural classes: 30% sand, 31% clay, 39% silt, 28% organic matter and a pH of 5.3; Dinwandeekankasat, Ayutthaya, Thailand). The pots were arranged in 6 rows at an interval of 1.3 m in the greenhouse. Within a row, 50 or 60 pots were spaced 35 or 30 cm apart resulting in a planting density of 1.5 or 1.8 plants m^{-2} for double and single stemmed experiments respectively. Daytime irrigation frequency was dependent on solar radiation integral whereby the dripper duration interval was regularly adjusted according to plant age, increasing from 1 minute at the beginning to 12 minutes at the end of the trial. On average, 9 irrigation cycles per day (33 mL min^{-1}) were delivered with an average over drain of 25% of the supply in order to avoid salt accumulation in the substrate (Mutwiwa, 2007; J. Max, personal communication). Temperature (*T*) and relative humidity (*RH*) were recorded every 5th minute using a central automated data logger system attached to two sets of sensors for each weather parameter inside and outside the greenhouses.

Inoculum source and inoculation of *P. fuligena*

For those experiments involving undisturbed natural BLM epidemics, plants were left untouched for the duration of the experiment. To curb the impact of neighbor rows and the carryover of conidia from season to season, however, plants in the border rows were fortnightly sprayed with the fungicide mancozeb. Besides, the white plastic floor was

disinfected by sweeping the floor with 95% Ethanol and spraying mancozeb at the start and finish up of each planting. To mitigate inoculum build-up, each greenhouse was separately used for either a study of natural epidemics or artificial inoculation.

Artificial inoculations, on the other hand, were performed after harvesting conidia of *P. fuligena* from diseased tomato leaves with profuse sporulating lesions. Pieces of lesions were cut using a 6-mm diameter cork borer, thoroughly stirred in distilled water using a magnetic stirrer for 30 minutes and filtered through double layered cheese cloth. Spray requirement of individual plants was calibrated with water ahead of time and conidial density of 2×10^4 mL^{-1} of water suspension was measured using Fuchs-Rosenthal chamber (Brand GmbH, Wertheim, Germany) in a total volume of spray required. All artificial inoculations started late in the afternoon (after 17:00 hour) and were extended further to evening time. Inoculated plants were tightly covered with black plastic sheets for 12 to 16 hours depending on the prevailing solar radiation during the post-inoculation day.

Experimental setups and data collection

Spatial distribution of BLM across plant canopy from natural epidemics at harvesting

The proportion of plant height that showed BLM symptom on leaves was measured from the base to the height at which the last symptomatic leaf was observed. From a total of 183 plants inspected at harvesting from September 2005 until January 2007, 92 plants were from fungicide treated (F) and 91 non-fungicide treated (NF). The last harvesting date, which on average was 102 days after transplanting (DAT), differed amongst the plantings which were carried out on a fortnightly or monthly interval. Individual plants were cut with a sharp scissor at the base, total plant height (PH) as well as height to the last symptomatic leaf (PHDI) was measured and further partitioned into pieces comprising the L, M and U strata. Number of infected leaves in each stratum was then counted and disease incidence (DI) was computed accordingly. Moreover, visual estimation of disease severity (DS) was performed for each stratum separately.

Spatio-temporal dynamics of BLM across plant canopy from natural epidemics

Weekly destructive samplings

Quantification of natural epidemics from double stemmed FMTT260 plant including lesion count was accomplished through destructive sampling of individual plants grown in big pots

(10 L capacity). Three plants were destructively sampled on a weekly intervals (11th, 19th and 27th October to 3rd November 2006) representing ages of 4, 5, 6 and 7 WAT, in order to quantify host growth including leaf area formation and the respective BLM symptom incidence. Measurement of lesion dimension and more precise estimation of disease severity, however, were done using the software AxioVision AC (Carl Zeiss Vision, GmbH.). After making digitized photos of the diseased leaves, the software was calibrated for the scaling of lesion sizes and eventually the diseased area on each leaflet was quantified. All leaves were cut, leaf area measured with a leaf area meter (model LI-COR LI-3100, Lincoln, NE) and glued as flat as possible (though tomato leaf shape was asymmetrical and wrinkled after about the 4th WAT). There were 9-12 leaves (an average of 11 leaves reaching until about 54 cm height) from the base to the branching of the two stems. Leaf position was assigned based on the primary stem, the stem extending from the base to the one bearing the earliest inflorescence. To grasp the concept of leaf position clearly, leaves from the secondary stem were matched to the nearest insertion node of the primary stem. Accordingly, leaf position (as of the 12th) was assigned with a variation of 0 – 3 cm from the exact leaf position on the primary axis.

Non-destructive samplings

BLM epidemics across plant canopy were thoroughly characterized by studying vertical disease spread of natural epidemics in an experiment repeated three times (May, July and August 2006) on single stemmed FMTT260 plants. In each of these experiments, 52 plants were randomly selected from the greenhouse and marked for inspection of host growth and BLM symptom appearance. Disease appearance on each leaf was strictly monitored on a daily basis right after transplanting (any visible symptomatic plants at transplanting were discarded) until the greenhouse showed 100% BLM incidence of plants. The interval was widened to 5-7 days thereafter for a time duration of 65 days after transplanting (DAT). During each monitoring, newly appearing diseased leaves were marked by loosely wrapping multicolored threads on the petiole and date of the first BLM symptom appearance in DAT was recorded. Final values of days of first symptom appearance were computed as a mean value of 52 plants. Number of sampled plants for each day varied according to plant age and whether leaves showed symptoms. The extent of plant height to which the BLM symptom reached during the last few assessment days varied and thus slightly affected number of samplings and the associated standard error computation.

Host growth parameters like plant height (*PH*) in cm and total leaf number (*TLN*) were recorded on a plant basis starting from 8 DAT at an interval of 4 to 7 days for the same duration of 65 DAT (10 assessments). In order to synchronize the discrepancy in assessment intervals and thereby predict the daily host growth, *TLN* was regressed to *DAT* from the total population of 1236 sampled plants during all the assessment dates of the same repetitions of the experiment. Accordingly, the power function $TLN = 2.65 \cdot DAT^{0.61}$; $R^2 = 0.91$ was used to fit the leaf count data. From this function, it was possible to estimate the number of leaves that were newly appearing on each date and later on to compute age of the leaves at the time of symptom appearance. To get an in-depth understanding of BLM incidence of leaves across the plant canopy, cohorts of 5 leaves were formed and proportion of symptomatic leaves at the last three assessment days (52, 59 and 65 DAT) were analyzed.

Spatio-temporal dynamics of BLM across plant canopy from artificial inoculations

Single stemmed plants

This experiment was carried out after artificial inoculation of single stemmed FMTT260 plants grown in small pots (2 L capacity) at the age of 4 WAT. The inoculm density was 2 x 10^4 conidia mL^{-1} of water suspension as measured using a Fuchs-Rosenthal chamber (Brand GmbH, Wertheim, Germany). Ten days after inoculation (DAI), three plants were randomly selected for the destructive sampling and quantification of the disease. Each leaf position was labeled and then cut at the base using a scissor. After capturing a digitized picture of all the leaves on each plant, quantification of the leaf area components (total and diseased) as well as disease severity and total lesion count were performed using AxioVision AC (Carl Zeiss Vision GmbH).

Double stemmed plants

Doubled stemmed FMTT260 plants grown in big pots (10 L capacity) were artificially inoculated with inoculum density of 2 x 10^4 conidia mL^{-1} at the age of 4 or 6 WAT. Just before inoculation, every other third leaf (2^{nd}, 5^{th}, 8^{th} etc.) was systematically selected, marked and its leaf length (LL_C, in cm) was measured. Leaf area before inoculation (LA_{BI}) and 14 DAI ($LA_{14\ DAI}$) were accordingly computed using the power function $LA = 0.154 \cdot LL_C^{1.931}$. At 14 DAI, three plants were randomly selected for the destructive sampling and for quantification of the disease. In the same way as in the previous experiment on single stemmed plants, quantification and further analyses were done using AxioVision AC (Carl Zeiss Vision

GmbH), but only on those marked leaves. During this experiment, however, besides the leaf area components and severities, sporulation on a leaf basis was quantified. Each leaf was cut from the base of the petiole and leaf area was measured using a leaf area meter (model LI-COR LI-3100, Lincoln, NE). Consequently, each leaf was chopped into pieces on a cutting board and put into 500 mL autoclaved and distilled water. The mix was then vortexed on a magnetic stirrer for 30 minutes and then sieved in double layered cheese cloth, diluted in series of distilled and autoclaved water (when deemed necessary). Number of conidia in the mix was counted using Fuchs-Rosenthal chamber.

Confounding impact of canopy morphology and microclimate vis-à-vis BLM epidemics

The confounding impact of canopy architecture was assessed through weekly sampling of leaf area of individual compound leaves for 7 successive weeks starting June 2006. This was performed by destructive sampling of three randomly chosen healthy plants. Each week, all leaves were first numbered (labeled) and then leaves hanging down were counted and marked separately. Heights of insertion points of all the individual leaves from the lower (LIH) and middle (MIH) canopy strata as well as the actual difference in height to the tip of hanging leaves were measured using a metric ruler. Later on, points of insertion of leaf internodes were adjusted according to the height of the hanging-down leaves at each stratum, i.e. LIH* and MIH*. Alongside the destructive sampling, the microclimate around plant vicinity was measured using Tinytag® Plus 2 (Gemini data loggers Ltd., UK), one positioned on the surface of the substrate (SS) and another suspended on both branches of a double stemmed FMTT260 at a height of 1 m from the base of the plant, i.e. the surface of substrate. To curb the direct impact of solar radiation, the data loggers at 1 m were hang inside cube boxes with open windows.

Data analyses

Host growth parameters like plant height (*PH*) and leaf internode insertion points were measured using a metric ruler. Total number of leaves (*TLN*) per plant were counted and recorded for the duration of the respective experiments. All regression analyses of host dynamics and disease epidemics as well as regression analyses of total leaf count were done using SigmaPlot (2006). All disease parameters in this research, *i.e. DS*, *DI*, *DS**, *DI** and PH_I are given as proportion and statistical analyses like ANOVA and mean separations were carried out using SAS (2003).

RESULTS

Vertical distribution of BLM across the canopy from natural epidemics at harvest

Stratified assessment of disease intensities (severity and incidence) across plant canopy during harvesting time of the natural epidemics is summarized in Fig. 1. Under naturally undisturbed BLM epidemics, *i.e.* without any fungicide (NF) application, disease severity (*DS*) was more concentrated at the lower (L) stratum (0.42a ± 0.035) as compared to the middle (M) (0.26b ± 0.025) and upper (U) (0.05c ± 0.005) (Fig. 1a). The same trend was observed with respect to disease incidence (*DI*), whereby nearly all of the leaves in the two lower strata, *i.e.* L (0.98a ± 0.005) and M (0.88a ± 0.02), became BLM symptomatic as compared to only 40% leaves of the U stratum (0.41c ± 0.03) (Fig. 1b). *DS* and *DI* represent the average of all plantings; looking at the minimum and maximum values from each planting across the seasons, it was found out that leaves at both L and M strata could be symptomatic up to > 0.9 in both disease intensities, but the maximum *DS* recorded on the upper stratum did not exceeded 0.2.

Fungicide applications at 4 and 6 WAT, on the other hand, led to a reduction in *DS* of 89.1 and 77.6% on the L and M strata when non-fungicide (NF) treatments were compared pairwise to fungicide (F) treatments (Fig. 1a). This suppression of the disease on the two strata in turn impacted the U stratum on which only a mean *DS* of 0.008 was recorded on F treated plants as compared to a 0.046 ± 0.005 for the NF treatment. *DI* too was similarly influenced as reflected by the statistically significant difference between the L and M stratum in case of NF treatments (Fig. 1b). The proportion of plant height to which the last symptomatic leaf was observed (PH_{DI}) differed significantly between F (0.74b ± 0.012) and NF (0.85a ± 0.009) treatments.

Spatio-temporal dynamics of BLM across plant canopy in natural epidemics

Weekly destructive samplings

Leaf area distributions: Total leaf area distribution along leaf positions became right-skewed as the plant grew (Fig. 2a). Within the four weeks of assessments, however, comparatively more leaf area was formed in leaf positions 6 to 12. Diseased leaf area, on the other hand, was concentrated at the leaf positions 5 to 10 (Fig. 2b). 79, 48, 53 and 42% of total and 100, 88, 98 and 87% of diseased leaf area was observed in the first two cohorts, *i.e.* leaf positions 1 to 10,

at 4, 5, 6 and 7 WAT respectively (Fig. 2a and b). Percentages of leaf area on the next two cohorts of canopy level (leaf positions 11 to 20) on the other hand were 21, 37, 39 and 43% for total and 0, 11, 2 and 13% for diseased leaf area at 4, 5, 6 and 7 WAT respectively.

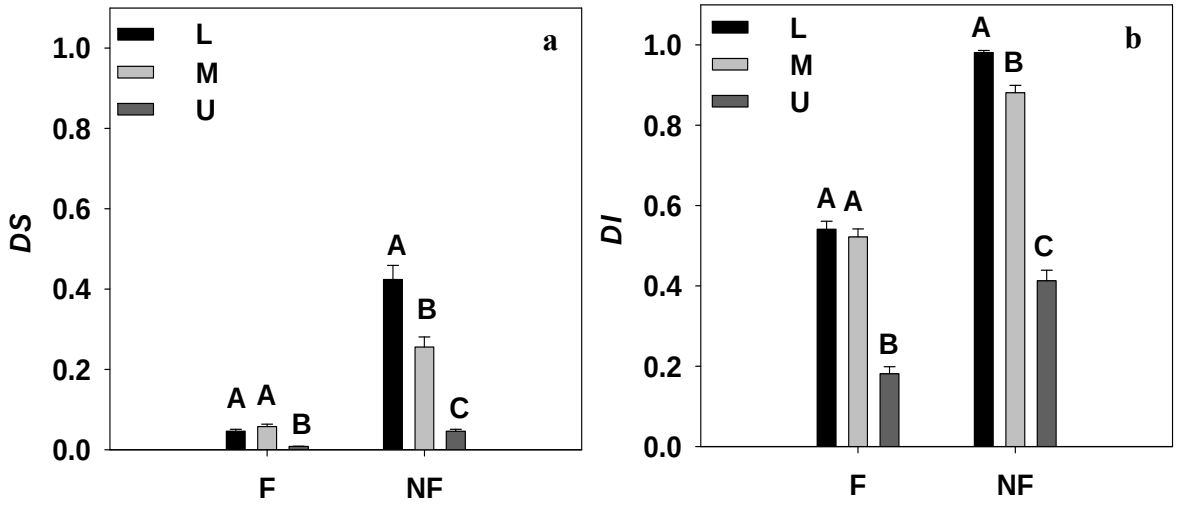

Fig. 1. **Severity** (a) and **incidence** (b) of BLM measured at **harvest** (≈ 102 DAT) across lower (**L: 0-50 cm**), middle (**M: 51-150 cm**) and upper (**U: > 150 cm**) canopy strata of FMTT260 plants with (F) and without (NF) fungicide application. NB: Bars designated by different letters within the F/NF treatments are significantly different at $p < 0.05$ according to Tukey's test.

Disease severity (DS) and incidence (DI): A general trend of right-skewed gradient of *DS* (Fig. 2c) and *DI* (Fig. 2d) was observed across leaf position from base to apex in ascending order. *DI* from the beginning (4 WAT) to the end (7 WAT) of the assessment period in this experiment ranged between 69.3 to 98.6% and 0 to 21.6% in the leaf positions 1 to 10 and 11 to 20 respectively (Fig. 2d). *DS*, on the other hand, was between 4.3 to 60.7% and 0 to 6.2% in the above mentioned cohorts of the leaf positions.

Lesion quantification: The number of lesions on a leaf basis (Fig. 2e) followed a similar trend to that of diseased leaf area distribution whereby the maximum number of lesions was observed at the third assessment (6 WAT). Out of 125, 592, 947 and 794 lesions counted on a plant basis at 4, 5, 6 and 7 WAT respectively, 125, 449, 873 and 602 of them were from the first two cohorts (leaf positions 1 to 10) but the rest from the upper cohorts (leaf positions 11 to 20). Lesion size, on the other hand, showed a similar pattern like the disease intensities

particularly that of *DS* whereby lesions on leaf positions 5 to 10 were bigger in size compared to those up in the canopy and the first four leaves at the lower canopy (Fig. 2f).

Non-destructive samplings

While following incidence of BLM from undisturbed natural epidemics for a time span of 65 days (DAT), the leaf positions 5 to 8 (counted at insertion point from base to apex) showed the symptoms at earliest as compared to those leaves which were even borne in the nursery (Fig. 3a). Whereas BLM symptom appeared about 30 to 38 DAT on the first three leaves, which were borne while still in the nursery, the next 7 leaf positions showed symptoms about 25 to 28 DAT (Fig. 3a). The time to symptom appearance measured as plant age rose thereafter according to the chronology of the leaf appearance (Fig. 3a). BLM symptom appearance on those three leaves borne in the nursery was at the same date as that of leaf positions 11 to 16 which were borne much later (Fig. 3a). Besides, incorporating plant age at leaf formation to that of plant age at symptom appearance showed the widest gap for those older leaves at the bottom (leaf positions 1 to 5) and narrowed down thereafter (Fig. 3a).

On the other hand, while considering the leaf age at time of symptom appearance, three scenarios were observed as shown in Fig. 3b. Obviously those 3 to 4 leaves at the lower leaf positions (that were borne in the nursery) showed BLM symptoms later as compared to the rest of the leaves. This could be attributed either to the adult leaf resistance or to less opportunity of inoculum as the nursery is assumed to be low to no risk of pathogen inoculum or a combination of both. On the next 18 leaf positions (5 to 22), however, leaf age at time of symptom appearance was nearly the same ranging between 18 to 22 days. As of leaf position 23, time to symptom appearance showed a tendency of becoming shorter which implicated preference of younger leaf tissues for BLM infection (Fig. 3b).

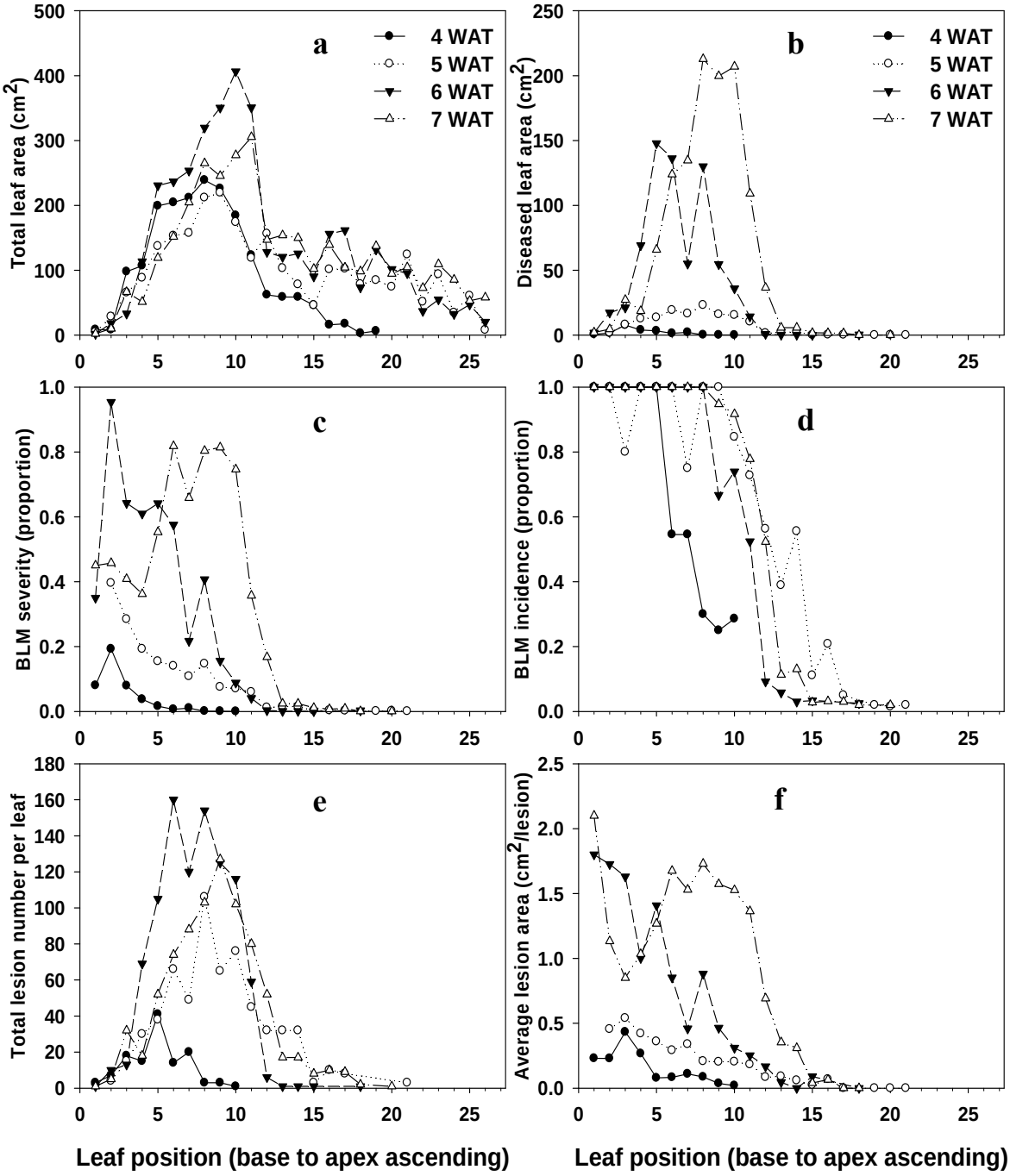

Fig. 2. Total (a) and diseased (b) leaf areas (in cm^2), BLM severity (c) and incidence (d), lesion number (e) and size (f) of individual lesions from destructive sampling of double stemmed FMTT260 plants at four assessments (4 to 7 weeks after transplanting) from undisturbed natural epidemics of *P. fuligena* across leaf positions on the plant canopy.

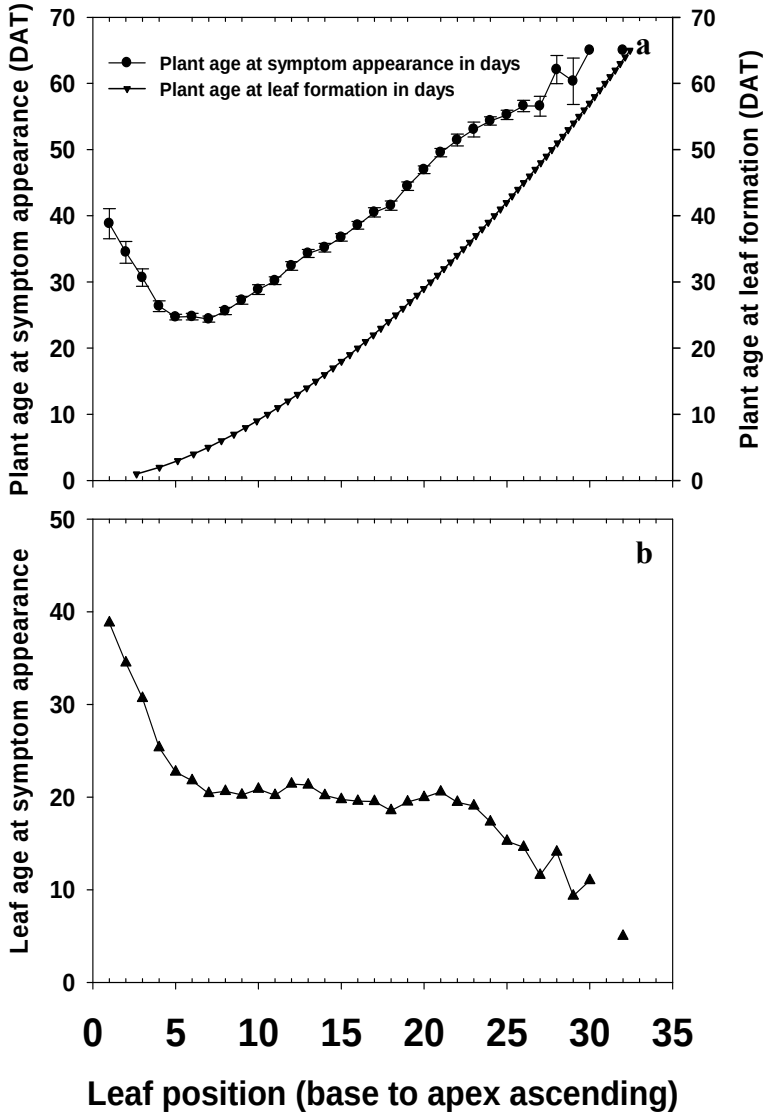

Fig. 3. Time when symptoms appeared on individual leaves, measured (a) as plant age (days after transplanting) or (b) as leaf age (days) in undisturbed natural epidemics.

Temporal assessment of disease incidence of leaves across cohorts, each consisting of 5 leaves from experiment 2 is presented in Table 2. During the first assessment at 52 DAT, *DI* of leaves was only 0.61 and 0.93 for the first (leaf positions 1 to 5) and second (leaf positions 6 to 10) cohort respectively. Whereas 100% of leaves of cohort 2 became symptomatic during the next two assessments (59 and 65 DAT), only 79.1% of the leaves at cohort 1 showed BLM symptoms (Table 2). Thus, the cohort classification similarly highlighted the influence of young tissue susceptibility since less disease symptoms appeared on older leaves. For instance, while the disease incidence of leaves of cohort 3 increased from 0.62 at 52 DAT to

1.00 at 65 DAT, the progress of cohort 1, which commenced with a similar proportion of 0.61 at 52 DAT, reached only to a level of 0.79 at 65 DAT (Table 2). Considering the late chronological appearance of the top leaves (leaves above the 21st internode position) and early harvest of the experiments (at 65 DAT), only relatively few symptomatic leaves (a proportion of 0.01 to 0.59) were recorded on them (Table 2).

Table 2. Vertical distribution of BLM *DI* (proportion) across cohorts of tomato leaves and their dynamics monitored at 52, 59 and 65 days after transplanting (DAT) from the non-destructive natural epidemic in experiment 2.

Cohort	Leaf positions	Disease incidence (*DI*) of leaves at		
		52 DAT	59 DAT	65 DAT
1	1-5	0.61 ± 0.030	0.79 ± 0.030	0.79 ± 0.034
2	6-10	0.93 ± 0.030	1.00 ± 0.001	1.00 ± 0.001
3	11-15	0.62 ± 0.100	0.97 ± 0.005	1.00 ± 0.001
4	16-20	0.31 ± 0.050	0.90 ± 0.014	0.93 ± 0.010
5	21-25	0.03 ± 0.007	0.47 ± 0.060	0.59 ± 0.030
6	26-30	-	0.04 ± 0.009	0.08 ± 0.003
7	31-35	-	-	0.01 ± 0.001

Spatio-temporal dynamics of BLM across plant canopy from artificial inoculation

Single stemmed plants

From a maximum of 18 leaves artificially inoculated at the age of 4 weeks (28 DAT), proportion of 0.25, 0.54 and 0.21 of symptomatic leaves were recorded for the first 3 cohorts, *i.e.* leaf positions 1 to 5, 6 to 10 and 11 to 15, counted from base to apex in ascending order, respectively (Fig. 4a). The respective mean disease severities on these leaf cohorts were 0.43, 0.67 and 0.21 (Fig 4b). Mean lesion count too followed exactly the same trend whereby 32.0, 70.1 and 26.9 lesions per leaf were counted on the 3 cohorts respectively (Fig. 4b).

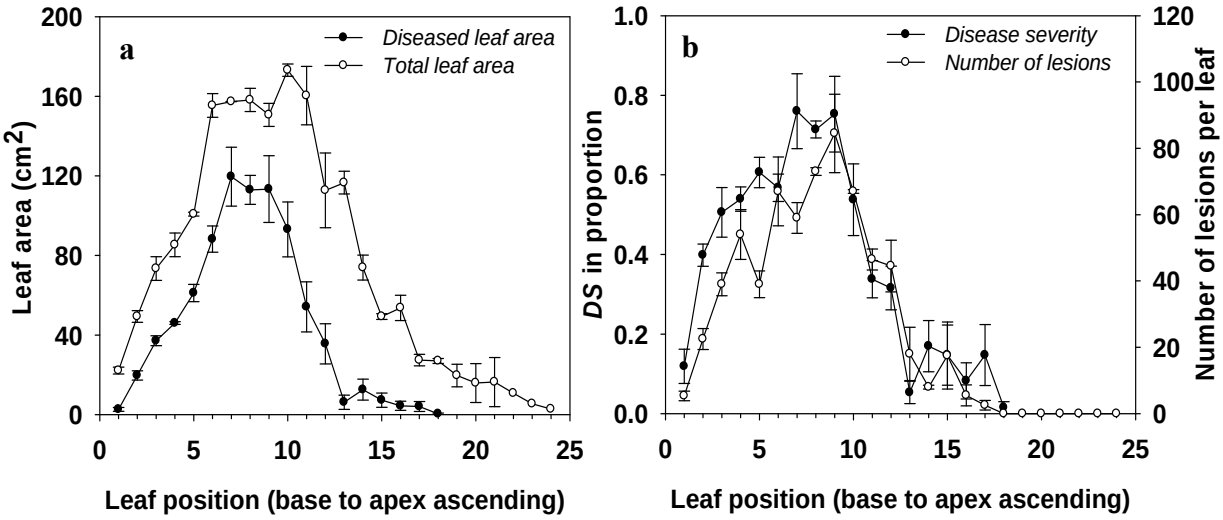

Fig. 4. Total and diseased leaf area (a) and severity (*DS*) and number of lesions/leaf (b) of individual leaves of single-stemmed FMTT260 plants, artificially inoculated 4 weeks after transplanting and quantified 10 days later.

Double stemmed plants

After artificial inoculations, the proportion of total (TLA) and diseased (DLA) leaf area (Fig. 5a and b) as well as incidence (*DI*) and severity (*DS*) of the disease (Fig. 5c and d) were higher at leaves positioned in the middle canopy layer than in the lower and upper ones. Obviously, all leaf area measurements before (LA_{BI}) and after ($LA_{14\ DAI}$) inoculation (at time of assessment) were higher at 6 than 4 WAT. Although most leaves on young plants (4 weeks old) showed rapid growth within 14 DAI whereby leaves up to the 5th compound leaf grew less compared to the young leaves in the middle canopy (Fig. 5a). The observation was different in case of those plants inoculated at 6 WAT. Leaves until about leaf position 8 had nearly a negligible growth within 14 DAI as compared to those leaves borne beyond this position (Fig. 5b). Besides the young age of leaves as one goes to the top of the plant, the newly borne leaves on the second stem also contributed to the difference in leaf area. The case in point for instance could be the six fold leaf area growth of the last leaf on the main stem (4.1 to 24.7 cm^2) and more than 8 times growth on the last leaf of the second stem (4.9 to 41.2 cm^2) between time of inoculation and assessment (14 days).

DI was nearly the same for the leaf positions up to 11 for those plants inoculated at the age of 4 WAT (Fig. 5c), but up to 14 on those inoculated at 6 WAT (Fig. 5D). *DS* on leaf positions 5 to 11, on the other hand, was higher and statistically different from that of leaves on both

extremes (older ones and newly growing leaves on top) when plants were inoculated at 4 WAT (Fig. 5c). Inoculation at 6 WAT showed a similar result for leaf positions 5 to 20 (Fig. 5d). Older leaves as well as the newly growing leaves on top of the plant showed less severity. The former could be attributed to factors related to host tissue susceptibility whereas the later mainly rests on prevalence of small leaf area during time of inoculation.

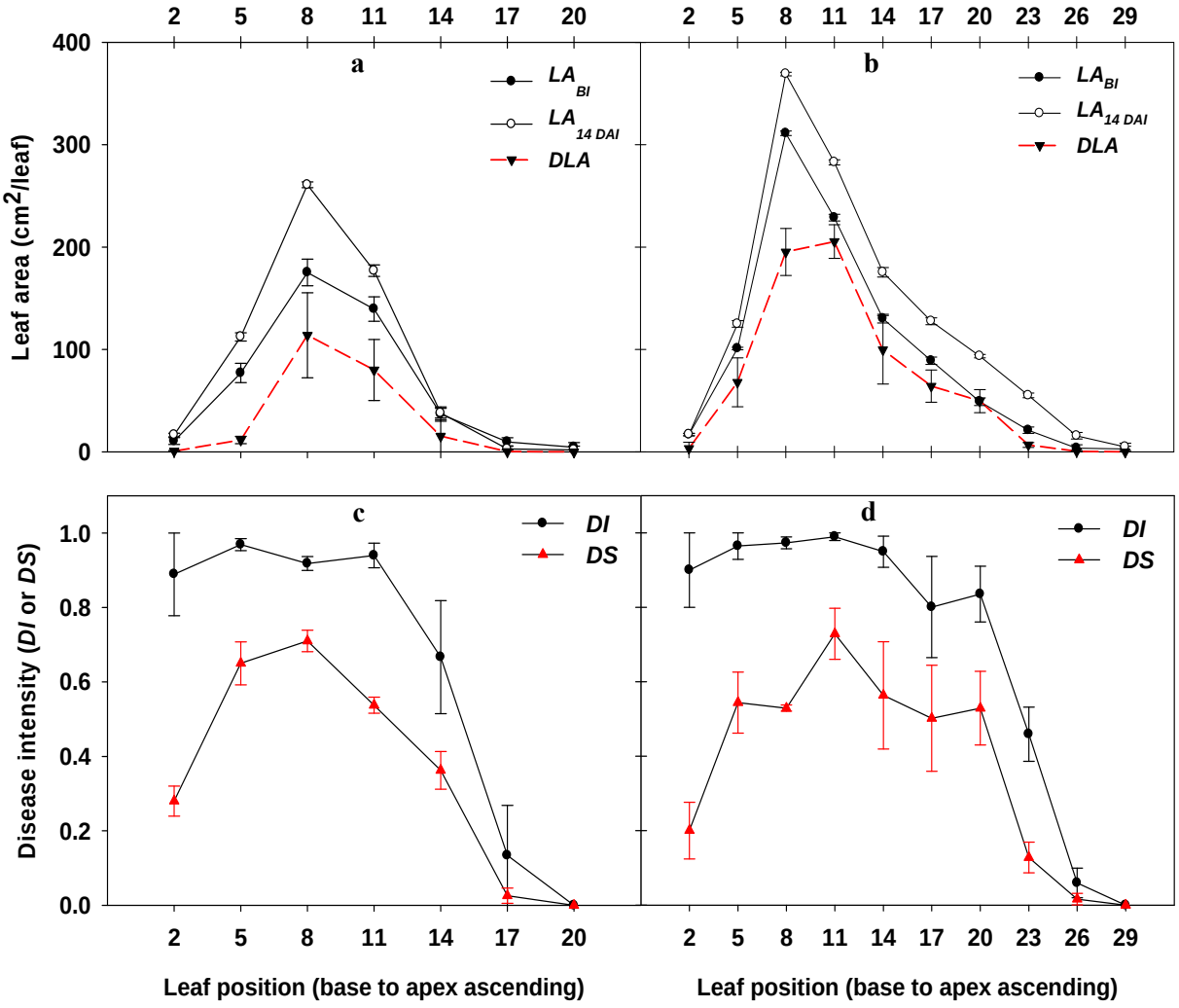

Fig. 5. Leaf area components (top) and quantification of epidemics of black leaf mold (bottom) on the basis of a leaf position across the canopy of FMTT260 plants after artificial inoculation of 2×10^4 conidial suspension on the whole plant at the age of 4 (a and c) and 6 (b and d) weeks after transplanting. NB: Disease assessment and quantification was carried out 14 days after inoculation.

Conidial count on individually diseased leaves revealed a similar result of spore production in the middle part of the canopy. For instance, on the main stem, leaf positions 8 to 20 had high conidial count per cm^2 leaf area (mean 2.1 x 10^3) as compared to the leaf positions 2 and 5 (4.3 x 10^2) as well as the youngest two inoculated leaves, *i.e.* leaf positions 23 and 26 which had only 8 x 10^2 (Fig. 6). Similarly, a higher number of conidia were counted from leaves that were positioned at the middle canopy (2 x 10^2 conidia per 500 mL aqueous solution) as compared to 3.4 x 10^1 of the lower (2^{nd} and 5^{th}) and 2.2 x 10^1 of the upper (23^{rd} and 26^{th}) compound leaves (Fig. 6).

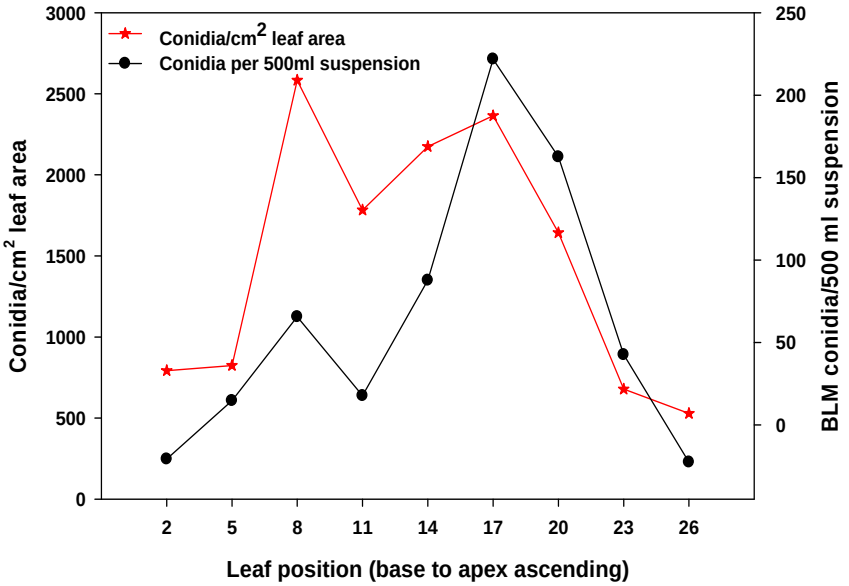

Fig. 6. Quantitation of conidial count in aqueous suspension and further analyses on a leaf area basis after destructive sampling of artificially inoculated leaves of 6 weeks aged plants (at a time of inoculation). NB: Quantification was carried out 14 days after inoculation.

Confounding impact of canopy morphology and microclimate on BLM epidemics

In most of the observations, relative humidity (*RH*) measured at the base (surface of the substrate) was higher during the daytime and lower during the night than at a height of 1 m (Fig. 7a). *RH* near the substrate at any time was on average ≥ 50% whereas at 1 m level it went as low as 25% during mid day (Fig. 7a). Temperature (*T*) was at its peak during noontime and lower during the nighttime (Fig. 7b). Hourly temperature at a height of 1 m was about 3°C higher (a minimum of 0.4 and maximum of 3.6°C) during daytime but about 0.4°C lower (minimum difference of 0.8 and maximum of 0.4°C) than temperature at the surface of the substrate during nighttime (Fig. 7b).

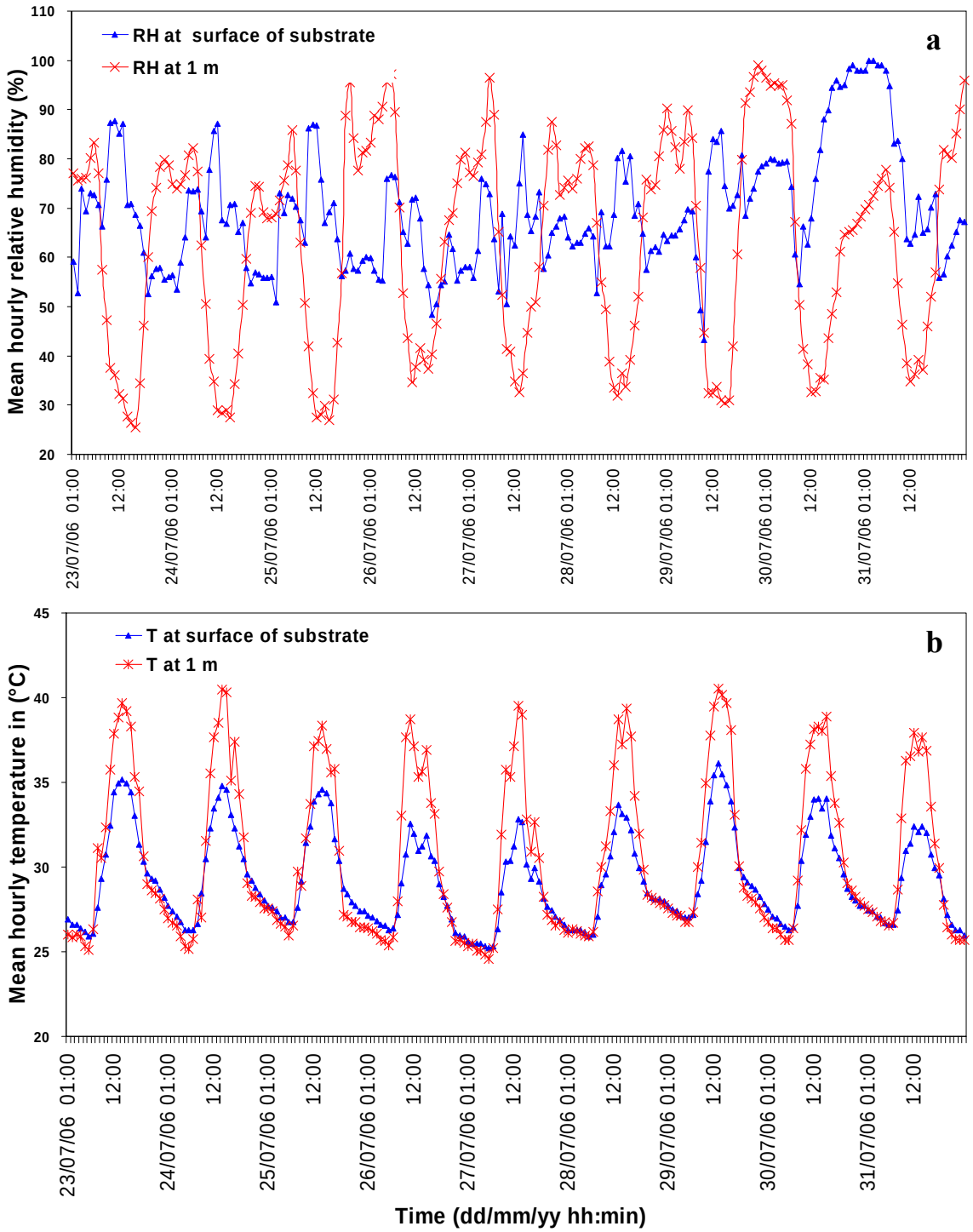

Fig. 7. Microclimate in terms of mean hourly a) relative humidity (*RH*) and b) temperature (*T*) around the vicinity of FMTT260 plants recorded at the surface of the substrate and at 1 m height using Tinytag® data loggers at 10 minutes interval.

The difference within the canopy was distinctively clear in that *RH* at the surface of the substrate during nighttime (*RHN*) was mostly above 80% (mean value of 81.2a ± 2.2) as compared to a nearly 70% (68.7b ± 3.1) at a height of 1 m (Fig. 8). Wider differences were

recorded during daytime (*RHD*) with an average of 71.9a ± 1.7 at the surface of the substrate to 49.1b ± 3.5 at 1 m height (Fig. 8). Temperature at the two canopy strata was only significantly different during daytime (*TD*) with mean hourly values of 33.4a ± 0.7 and 30.7b ± 0.5 at 1 m height and surface of the substrate respectively but not (26.8a ± 0.2 and 27.2a ± 0.3) when the mean nighttime temperatures (*TN*) were compared (Fig. 8). Moreover, despite daily differences in both weather parameters, a gradual build-up of *RH* was recorded when averaged values of the 2nd day and 10th day were compared. In an interval of 8 days, both day and night *RH* values at the surface of the substrate increased from 81.6 to 84% and 95.3 to 96.9% respectively. On the other hand, while *RHD* at 1 m height showed similar increment from 46.3 to 48.3%, *RHN* more or less remained 81% during both time intervals.

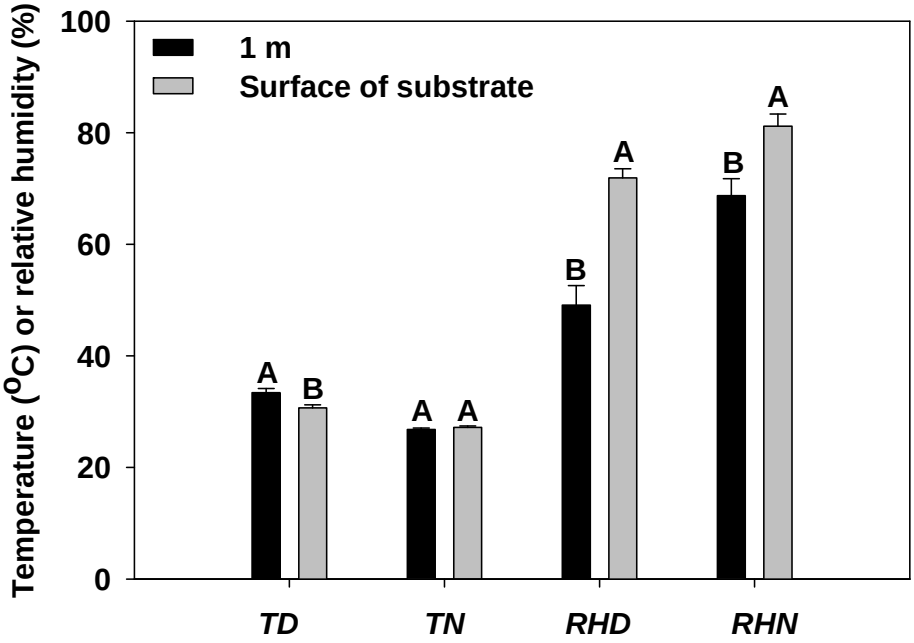

Fig. 8. Mean values of day (*TD*) and night (*TN*) temperature and relative humidity (*RHD* and *RHN*, respectively) measured at 1 m height and surface of the substrate of FMTT260 plants grown in 10 L capacity plastic pots in a BioNet™ greenhouse. NB: Bars designated by different letters within the day and night temperature (*TD* and *TN*) and relative humidity (*RHD* and *RHN*) treatments are significantly different at $p < 0.05$ according to Tukey's test.

Proximity to evaporation from surface of the substrate obviously contributed partly to the observed variation in microclimate within the two strata. Moreover, intuitive observations of growth habits of FMTT260 implicated the part played by canopy morphology towards this variation in microclimate across the plant strata. Destructive sampling of plants for 7 consecutive weeks in September 2006 revealed that nearly all leaves of FMTT260 stayed

upright until 3 WAT because each node was in about acute angle junction between the stem and the petiole (Fig. 9). Afterwards, however, some leaves started to hang down and architectural angle of the stem-petiole junction became nearly perpendicular to obtuse, and to the extent of pendulous leaf attachments in case of many leaves at the lower canopy (0-54 cm). For instance, during the samplings at 4, 5, 6 and 7 WAT, the proportion of hanging-down leaves (as compared to total leaves on a plant) were 0.73, 0.56, 0.68 and 0.59 respectively. While considering proportion of leaf area in each stratum, within the same time of observation, it decreased for that of the lower canopy (1.00 to 0.47) and increased for the middle canopy layer (0.00 to 0.23) during the destructive samplings of weeks 1 to 7 respectively. Area under the curve (AUC) without adjustment of the internode height (IH) at the insertion point was 4.41 and 1.59 proportion-weeks for the lower (L_{IH}) and middle (M_{IH}) canopy respectively. When IH was adjusted taking into account the pendulous leaves (IH*), the AUC of lower canopy (L_{IH*}) increased to 5.48 and that of the middle stratum (M_{IH*}) reduced to 0.52 (Fig. 9).

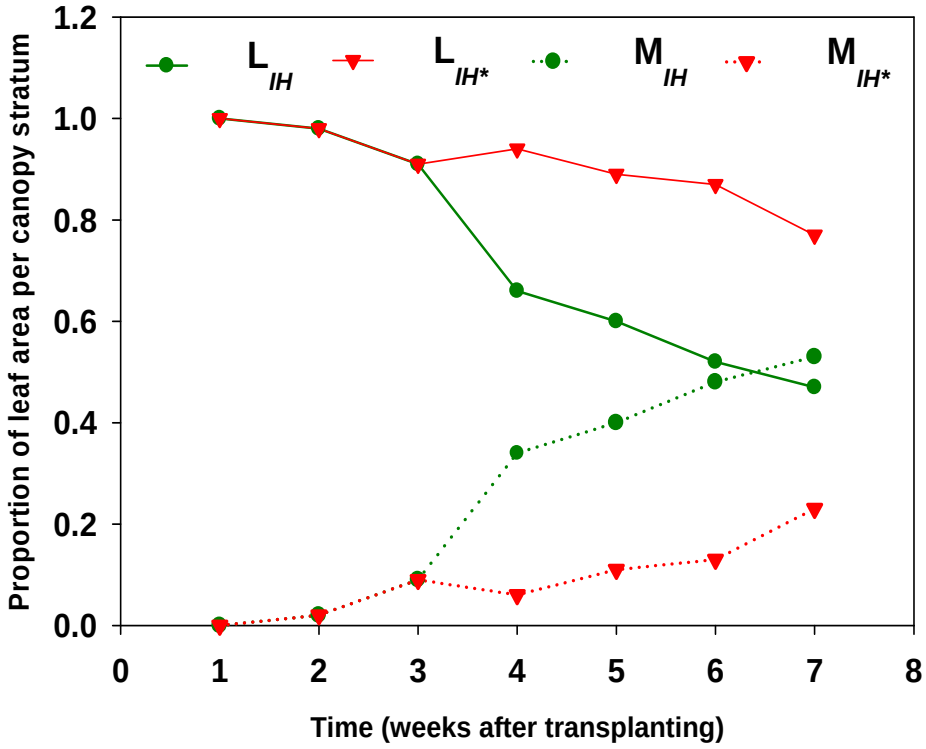

Fig. 9. Confounding impact of down-hanging canopy of FMTT260 expressed in terms of proportion leaf area of the lower (L, 0-50 cm, solid lines) and middle (M, 51-150 cm, dotted lines) canopy stratum with actual internode height (*IH*) of a leaf position compared with that of the adjusted internode height (*IH**).

DISCUSSION

Despite the lack of any explicit research on aspects related to age of host tissue vis-à-vis BLM epidemics, observations of disease level at harvesting time from this research corroborate with earlier visual reports of more disease concentration at the lower part of the canopy as compared to the middle and upper parts. This was mentioned among others by Hartman and Wang (1992) who observed slow progress of the disease on younger inoculated plants but noticed a rapid disease increment later in the growing season as plants get aged. Wang *et al.* (1995) similarly reported inception of the disease from the lower to the upper part of the plant. Final BLM severity assessment at harvesting from the lower canopy stratum (42%) proved to have nearly 2 and 10 fold more disease as compared to the middle (26%) and upper (5%) canopy strata respectively.

Similar results were reported in various pathosystems regarding this instantaneous spatial gradient of the disease across the canopy. For instance, in their study of the pathosystem tomato-*Alternaria*, Mills *et al.* (2002) found a pattern of foliar disease reduction in tomatoes grown in beds with mulch compared with beds with an exposed soil surface. In all the four bedding strategies they tested, however, disease level expressed as *AUDPC* at the lower canopy stratum was 2 to 3 and 20 to 40 fold higher as compared to the middle and upper canopy strata respectively. Some more reports to mention are from Lovell *et al.* (2004) in wheat-*Septoria*, Hau (1985) in barley powdery mildew and Ojiambo and Scherm (2005) in rabbiteye blueberry-*Septoria* pathosystems.

To verify whether such an instantaneous observation of disease concentration at the lower canopy directly implicate tissue susceptibility, more experiments under natural epidemics (with temporal aspects incorporated) were carried out in this research. The weekly destructive sampling during the growing period (4 to 7 WAT) highlighted prevalence of more diseased leaf area and high expansivity of lesion size on leaf positions 5 to 10 as compared to those leaves borne in the nursery. Further detailed spatio-temporal analyses of BLM across leaf positions from the non-destructive experiment of another natural epidemics similarly revealed that the older leaves, especially those borne in the nursery, did not show any preference for symptom appearance. Instead, only 79% of all the leaves from the first cohort (comprising five leaves) showed symptoms at the last assessment date (65 DAT) which was by far less than the next three cohorts (until leaf position 20). Surprisingly, but in agreement with earlier reports, incubation period of the disease on most of the leaves was found to be between 11 to 25 days. Blazquez (1991) reported a little more than two weeks for BLM symptom

development and Hartman and Wang (1992) similarly stated fairly long incubation period for the disease. Considering earlier results from monocyclic component studies in chapter 2 and that of the aforementioned experiments on natural epidemics further concerns were raised with respect to tissue age and BLM epidemics. On top of all, those experiments which incorporated the temporal dynamics implicated contradictions to the instantaneous observations of the natural epidemics at harvesting. To clarify such contradictions, two further experiments with artificial inoculations were carried out in this research.

Despite the paradox of noticeable visual perceptions of disease concentration at the lower canopy of *S. lycopersicum* var. FMTT260 under natural epidemics, fully unfolded leaves at the middle canopy layer were found to be the most vulnerable (as compared to the extreme younger leaves at the tip and older ones at the bottom), when given equal chance for infections after artificial inoculations. Whereas the recorded wide variation of leaf area measurements between inoculation and symptom appearance could partly be considered as a reason for low disease observation from the leaves at the tip, those leaves at the lower canopy grew slowly to negligibly indicating no dilution effect of the host growth and thus tissue resistance to be the possible reason of showing less disease. More importantly, the lower number of lesions and smaller sized lesions at leaves from the lower canopy stratum as compared to the middle and the upper once (refer to chapter 2) reinforced the assumption of host tissue resistance for BLM as the leaves become older. Blazquez (1991) similarly indicated ceasing of lesion expansion of *P. fuligena* as leaflets mature, thereby implicating the best growth of the pathogen on young tissue. Prevalence of more disease severity on leaves in intermediate positions compared with those in upper positions, nonetheless, were mainly due to the greater leaf area available for infection as was also stated in the *Septoria*-rabbiteye blueberry pathosystem (Ojiambo and Scherm, 2005). This aspect of epidemics under dynamic host growth conditions is elaborated in many earlier works (Hau, 1990; Campbell and Madden, 1990; Mersha and Hau, 2008) and also on monocyclic component study of this research. Nonetheless, it is worthwhile to mention circumstances where susceptibility of the older leaves from artificial inoculations of other pathosystems on tomato as reported from Byne *et al.* (1998) in *Colletotrichum*-tomato, Elmer and Ferrandino (1995) in *Septoria*-tomato and others like in *Phytophthora*-potato pathosystems from Visker *et al.* (2003).

Such contradictory phenomenon of instantaneous observation of aggregated disease at the lower canopy under natural epidemics versus spatio-temporal and artificial inoculation evidences of susceptibility of younger leaves, led us to further insightful investigations. Focus points were searching for those factors attributing for disease aggregations at the lower

canopy. The microclimate around the vicinity of the leaves at the lower canopy was one of the reasons for such disease concentration at the lower canopy.

Microclimate on and around a living plant can have a significant impact on the epidemiology of fungal pathogens and the population dynamics of pests (Zhang *et al.*, 2002a). It is well known that differences occur between the microclimate around a leaf surface and the ambient air due to photosynthesis, transpiration, and vapor condensation processes that occur on plant surfaces. These differences may be greater in the greenhouse for two reasons, first, the use of greenhouse cladding greatly reduces local air velocities and traps both absorbed energy and transpired water (Bot and van de Braak, 1995) and subsequently restricts heat and mass energy exchange between vegetation and ambient air; second, the use of heating and cooling equipment in the greenhouse increases the spatial variability of the greenhouse climate (Kempkes *et al.*, 2000). More detailed investigation at the level of a leaf microclimate indicated that leaf is in constant change, particularly variable are relative humidity, temperature, leaf wetness and UV radiation. These factors also change depending on leaf position with the plant canopy. Organisms on the leaf are frequently in direct competition both for very limited nutrients and for shelter from UV and infrared radiation which causes heating and drying of the leaf surface (Sutton, 1995). Result from this experiment, unambiguously proved these differences in microclimate within the canopy of a plant stratified at the level of the substrate surface and at a height of 1 m. Both weather parameters, *i.e.* temperature and relative humidity, showed a difference in these two canopy levels.

Temperature was distinctively higher at 1 m during noontime as compared to surface of the substrate. The range of maximum temperature during this time exceeded the optimal requirement of the pathogen, *i.e.* 34°C (Hartman *et al.*, 1991) and could possibly be a reason for the less chance of the upper leaves to show symptoms. This goes in agreement with Elmer and Ferrandino (1995) and Ferrandino and Elmer (1996) who have reported inhibition of spore germination in conditions of high light intensity from a *Septoria*-tomato pathosystem. Relative humidity, a weather parameter which was identified as critically important in affecting BLM epidemics under protected cultivation (refer to chapter 5), was highly and significantly different in the two canopy levels in this experiment. One obvious factor for such a high gradient is canopy development during the growing season. This gradual increment has to be considered as playing a key role in influencing subsequent changes of microclimate especially during the later stages of growth whereby leaf wetness duration is prolonged and relative humidity is higher. This situation was detected in this research as relative humidity showed an increment as leaf area index in a greenhouse increased. A similar result has been

shown in *Cercospora*-sugar beet pathosystem (Wolf and Verreet, 2004). Other possible reasons for high *RH* around the lower canopy could be the prevailing lower temperature and proximity to evaporation from substrate (soil).

An intuitive observation of canopy morphology of FMTT260 in this research was found to be one of the reasons, in addition to all the above mentioned ones, in aggravating the confinement of the lower canopy. The AUC comparisons, only from the first four weeks when the canopy started hanging down, showed a reshuffling of substantial amount of leaf area from the middle canopy towards the lower canopy. This could implicate the confinement impact of canopy architecture as the growing season proceeds toward the harvesting time. Such an impact of canopy morphology has also been investigated in the pathosystems *Puccinia-Triticum* (Gasowski, 1990) and *Septoria*-winter wheat (Lovell *et al.*, 2004). Furthermore, this aspect of morphology and growth habit of host plant cultivars as affecting the microclimate and the severity of epidemics is well described in earlier literature (Schlösser *et al.*, 2000; Kranz, 2003). Royle (1994) also mentioned studies that have been addressing the roles of leaf development and canopy architecture in *Septoria tritici*, mainly because of the importance of vertical inoculum dispersal.

Pathosystems could respond differently to age of plants and leaves (Royle, 1994). As part and parcel of integrated approaches for management of BLM, it was possible to pinpoint the spatio-temporal dynamics of the disease across the plant canopy in this research. From one of the experiments dealing with time of BLM symptom appearance, it was found that symptom appearance, unless disease was carried in from the nursery, started at the leaf positions 5 to 10. Such information would help to know positions which could serve as a spring board for further disease dissemination. Protecting these leaf positions at early stages would not only serve to cull the pathogen but also to curb further dispersal of inoculum.

This has justified the application of mancozeb at 4 and 6 WAT, a time when the lower canopy contributes 75 – 80 % of total leaf area (Fig. 10). This goes also in synchrony with a high probability of controlling the source of inoculum at the early stage. Similarly, Madden *et al.* (1978) too underlined the major importance of determining the initial spray application for tomato early blight control which would eventually help to minimize the total number of applications required to manage the disease. Later the growing season, near the 10^{th} WAT for instance, this proportion of leaf area at the lower canopy was drastically reduced to a proportion range of only 10 – 25% depending on the season and other management practices.

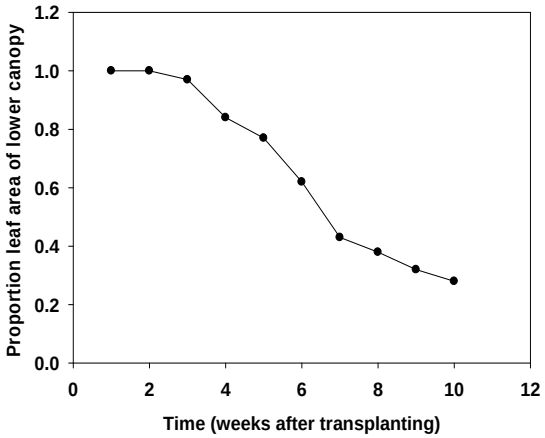

Fig. 10. Proportion leaf area of the lower canopy (up to 50 cm) as compared to the total leaf area of FMTT260 plants across time during destructive sampling in May to July 2006.

Starting the 1st harvesting, at about 10 WAT, the lower canopy (from leaf positions 9 to 12 or between 45 to 55 cm plant height from the substrate) burdens the plant photosynthetically than benefiting it. There could be two explanations for this assumption. First, the photosynthetically active radiation (PAR) rarely reaches this canopy level and even then 66% of the photosynthates are produced on the upper canopy (Acock *et al.*, 1978) and second, the greatest proportion of BLM severity (and for that matter other foliar diseases) was concentrated in this canopy. Thus, the customarily practiced pruning at this time would benefit the tomato by removing the stress of the diseased leaves without much compromise of the photosynthetic area. Further pruning of the next 5 to 10 leaves could also be considered depending on the variety and duration of the growing season. In cases of fungicide spray, there was a tendency of shift of the disease towards the middle canopy and by removing the next leaves during the last harvesting time would also benefit tomato production in a similar way.

In conclusion, BLM disease concentration at the lower canopy and assumption of associating this phenomenon to the age of the plant and the leaves was clarified from the outputs of this research. Accordingly, BLM disease, given equal chances at optimal conditions prefers younger tissues than older once. Regardless of the mechanism, the higher disease levels in the lower canopy underscore the importance of adequate application of fungicides to lower parts of the plants as also underscored by Ojiambo and Scherm (2005).

Chapter 5: Seasonal dynamics of black leaf mold (*Pseudocercospora fuligena*) on tomato (*Solanum lycopersicum*) grown under protected cultivation in the humid tropics

ABSTRACT

Epidemics of black leaf mold (BLM), caused by *P. fuligena*, were studied in 24 plantings of the tomato (*S. lycopersicum*) cv. FMTT260 under ambient greenhouse conditions at the Asian Institute of Technology, during the period of May 2005 to March 2007. Three periods with BLM peak-epidemics were identified, *i.e.* August and September in 2005 (1) and 2006 (2) and plantings in December 2005 to January 2006 (3). While maximum disease severity (*DS*) in proportion at the last assessment date on non-fungicide sprayed plants of these periods were 0.81, 0.50 and 0.56, the mean severities per plant across the growing time (*DS**) were 0.30, 0.14 and 0.20 for the 1st, 2nd and 3rd peak-epidemic periods, respectively.

Favorability indices of temperature (FI_T) and relative humidity (FI_{RH}) of the three respective periods were 0.90, 0.61 and 0.66 for FI_T and 0.51, 0.35 and 0.44 for FI_{RH}. Linear, multiple and stepwise regressions were performed to detect relationships between weather-disease-host. While FI_{RH} was highly correlated with *DS** (R^2 = 0.71), the multiple regression of both (including FI_T) in the model $DS^* = a + b \cdot FI_T + c \cdot FI_{RH} + d \cdot FI_T \cdot FI_{RH}$ improved R^2 value to 0.74 but with non-significant parameter estimates. Further regression analysis with the model, $DS^* = a + b \cdot FI_T + d \cdot FI_T \cdot FI_{RH}$ resulted in significant parameter estimates with nearly the same R^2. Maximum plant height (*MPH*) of all experimental plants (n = 252) was fairly correlated (r^2 = 0.32, $p < 0.0001$) with marketable yield (*MY*) in contrast to maximum leaf number (*MLN*) (r^2 = 0.13, $p < 0.0001$). Multiple regressions of both raised the R^2 to 0.33 with significance of both parameters at $p < 0.05$.

Further regressions excluding the combined term highly improved the significance of both linear terms. Despite the poor correlation between yield and *DS**, integration of the host parameter *MPH* in the model $MY = (a + b \cdot MPH) \cdot (1 - c \cdot DS^*)$ resulted in R^2 value of 0.35 and high significance of the parameters *b* and *c*. Accordingly, a 1% *DS** reduced *MY* by ≈ 1.2%. The actual yield loss recorded from comparison of sprayed and non-sprayed treatments of the 1st, 2nd and 3rd peak-epidemic periods was 34.1, 39.2 and 20.6%, respectively. Thus, the average yield loss of *MY* due to BLM under protected cultivation was 31.3%. Whereas the latest model explained the actual yield losses from the first and last peak-epidemic periods, the loss in 2nd period was higher than the model prediction.

Key words: ***BLM epidemics, seasonal dynamics, host growth, marketable yield, weather, favorability indices***

INTRODUCTION

From an epidemiological point of view, a priori knowledge of the quantitative relationship between the components of a disease pyramid, consisting of the interfaces host-pathogen-environment with pillar dimensions of time and space, is a prerequisite for designing novel strategies of disease management. Studies on temporal epidemics of a disease across seasons of a year and their effects on a host vis-à-vis prevailing weather parameters play a key role as a foundation to this end. Research studies that are undertaken with an aim of quantifying the amount of disease across seasons are essential (Cooke, 2006) and corner stones of epidemic analysis (Campbell and Neher, 1994) because without quantification of a disease, no studies in epidemiology, no assessment of crop loss and no plant disease surveys and their applications would be possible (Kranz, 1988). Outcomes from such studies obviously serve in pinpointing seasons of high and low epidemics on the basis of which models to predict a disease could be established. Based on these models, eventually feasible integrated disease and crop management strategies could be developed. Several scientists (Kranz, 2003; Strange, 2003; Cooke, 2006; Madden *et al.*, 2007) maintained that the measurement of plant disease and its effects on crop yield, quality and value are crucial for control priorities.

Despite the unparalleled merits of protected cultivation to quench the food demand of the alarming population growth through intensification, the consistent warmth and humidity of these confined systems create a fertile ground for perpetuation of many pests. Averting pest problems in these systems are entertained mainly through exclusion, a strategy which helps a lot for insect pests and vectors of viral infections, but not for foliar fungal pathogens and other microbes. The obvious reason for this is their microscopic size and hence the multitude of options to enter greenhouses (Paulitz and Bélanger, 2001). Consequently, fungal pathogens and others, which are highly favored by confined warmth and humidity, gained more and more economic importance in the past years and the use of fungicides remained the sole option to maintain optimal productivity. This tendency of heavy fungicide reliance for vegetable and fruit production is reflected mainly in Asian countries (Kuck and Gisi, 2007).

There are, however, increasing societal concerns about the environmental and health effects of fungicides. Searching for alternative human and environment friendly options of disease management in such protected systems is thus a timely demand, and epidemiological studies targeted on quantifications of seasonal dynamics of diseases are thus gaining momentum to alleviate such problems. The case in point in this research is the production of tomato

(*Solanum lycopersicum* L.), one of the highly cherished solanaceous vegetable crop world-wide, and its foliar disease black leaf mold (*Pseudocercospora fuligena*).

Tomato is one of the most popular vegetables world-wide (Rubatzky and Yamaguchi, 1997; Jones, 2008) and its production has been increasing globally and also in Thailand (FAO, 2007), where this research was conducted. Amongst many of the biotic constraints of tomato production (Blazquez, 1991; Anonymous, 2007; Jones, 2008), black leaf mold (BLM) was mentioned as widely distributed and economically important in many Asian countries (Roldan, 1938; Yamada, 1951; Chupp, 1954; Mohanty and Mohanty, 1955; Hsieh and Goh, 1990; Blazquez, 1991; Hartman *et al.*, 1991, Hartman and Wang, 1992). Recent reports indicated that the disease has spread further to Latin America, Brazil (Halfeld-Vieira *et al.*, 2006). Kandziora (personal communication) surveyed the disease as one of the most important foliar diseases in greenhouses in Thailand because of the prevailing highly conducive weather parameters like warm temperatures and high relative humidity (Hartman *et al.*, 1991). A report from artificial inoculation trials of Hartman and Wang (1992) in Taiwan indicated a loss of fruit weight ranging from 4 to 34% as compared to control plots treated with fungicide.

Even with the growing economic importance of plant diseases, the relationship between epidemic development and resulting yield was not studied rigorously for many diseases until recent decades (Madden *et al.*, 2007). The same held true for the foliar disease BLM. Despite its wide distribution and economic importance in tomato production under protected cultivation, studies related to seasonal dynamics of the disease as well as to the extent of damage it causes still remain scanty (Wang *et al.*, 1995) except few yearly reports (Anonymous, 2003, 2004a, 2004b) of the world vegetable centre (formerly known as Asian vegetable research and development centre – AVRDC).

P. fuligena is highly favored by high humidity, moderate to high temperatures and low night temperatures which result in extended periods of leaf wetness (Blazquez, 1991; Wang *et al.*, 1996). Initial symptoms of the disease appear as small, pale yellow lesions with no definite margin on either the upper or lower leaf surface. These lesions have white fungal growth that turns gray to black as the fungus sporulates. Later, black sooty fungal growth will occur on both the upper and lower leaf surfaces.

With a major objective of producing information on seasonal disease-weather-host relationship, this research attempted to show the progression and degression of BLM epidemics across different seasons in Thailand with the respective loss in yield. Data produced

from these plantings could also be applied to diseases of similar weather requirements and hosts grown under comparable production situations.

MATERIALS AND METHODS

Experimental overview and setup

Monthly and fortnightly plantings of the indeterminate type fresh market tropical tomato cultivar FMMT260 were carried out starting June 2005 in a greenhouse located at the campus of the Asian Institute of Technology (AIT). The experimental site is located 44 km north of Bangkok in Khlong Luang, Pathumthani province, central Thailand (14º 04′ N, 100º 37′ E, altitude 2.3 m above sea level). Climatic conditions of central Thailand can be broadly classified into two seasons: the rainy season, lasting from mid-May to October and the dry season from November to mid-May (Takahashi and Yasunari, 2006). Kleinhenz *et al.* (2006) indicated that the latter can further be divided in a cooler dry season (November to February) and a hot dry season (March to mid May) with respective mean temperatures of 28.3, 26.5 and 29.6 as well as average precipitation of 210, 27 and 97 mm month^{-1} for the three seasons. While there were 14 fortnightly (15 days interval) plantings in 2005, the rest 13 were planted on a monthly basis starting January 2006.

All plantings were carried out in a BioNet^{TM} greenhouse (east west oriented, 20 m long and 10 m in width) equipped with two exhaust fans for active ventilation with automatic threshold temperatures set to 30 and 33°C for the first and second, respectively. Automated fertigation was provided from nutrient solutions that were prepared from concentrated stock solutions of Kristallon™ 6+12+36+3+Micro (% N, P, K, Mg) and Calcinit™ 15.5+0+0+19 Ca (both Yara, Oslo, Norway) in a ratio of 70:30 using a Fertilizer Mixer (Micro 100, GVSystem, Odense, Denmark) and delivered to the plants through a drip irrigation system. Average composition of daytime nutrient solution was (in [mM]): N 7.4, P 0.8, K 5.9, Ca 3.1, Mg 0.7, S 1.7, Na 1.8 and (in [µM]): B 6.0, Fe 4.2, Cu 5.3, Mn 3.8, Mo 1.1, Zn 1.4 (J. Max, 2007, personal communication).

All the passive ventilation openings at the sidewalls, gables, and 0.8 m height vents underneath the roof ridge were covered with a 50-mesh UV absorbing insect proof net screen (BioNet^{TM}, Klayman Meteor Ltd. Petah Tikva, Israel). The whole floor was covered with white plastic mulch (Silo plus^{TM}, FVG, Dernbach, Germany). For staking, the plants were trained according to the high wire system (van de Vooren *et al.*, 1986) using Bato hangers (Bato Trading B.V., Zevenbergen, The Netherlands) attached to metal wires 4 m above the ground with 0.4 m distance between individual stems. On the middle pillar, a plastic curtain

was hanging dividing the greenhouse into two halves with some space left for walking at both ends.

Seeds of FMTT260 (AVRDC, Shanhua, Taiwan), an F1-hybrid which is considered to be heat tolerant, were sown in peat moss and raised in an evaporative cooled nursery (fan and pad equipped) for an average of two weeks and then transplanted to the 50-mesh sized greenhouse. Amongst the six rows within the greenhouse, the two exterior and two most interior rows were sprayed with fungicide every other week until harvesting and thus left as boarder rows and only the two inner rows (2nd and 5th) were used as experimental plots (Fig. 1). Within a row, 50 or 60 pots were spaced 35 and 30 cm apart resulting in a total population of 300 and 360 plants for double and single stemmed plantings respectively.

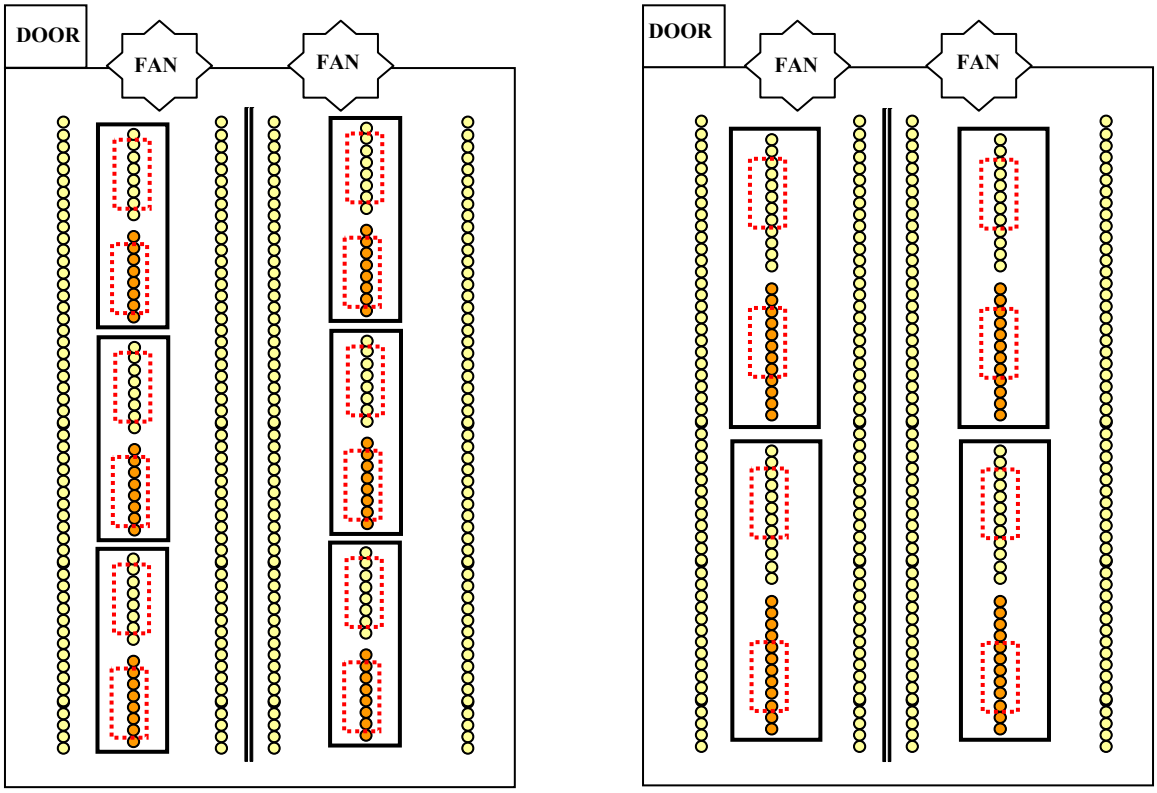

Fig. 1. Experimental layouts of seasonal epidemic study of BLM for the fortnightly (left) and monthly plantings (right) at AIT. NB: ○ = Fungicide sprayed ● = No fungicide sprayed, ▭ = main plot (planting), ⬚ = sub plot.

Following the single plant approach of Kranz and Jörg (1989), 24 plants were attended for the first three weeks (in case any wilting or establishment difficulties happened to the transplant, it would be replaced with these plants) out of which only 12 vigorous plants were selected for data assessment in each planting time (Fig. 1). These experimental plants were further divided

into two blocks, each with 6 plants, *i.e.* 6 plants sprayed with fungicide (F) at the age of 4 and 6 weeks after transplanting (WAT) and the rest 6 plants were left for natural epidemics without fungicide (NF) application. At any point in time, except at the inception and finish up of the study, up to 4 (monthly) or 6 (fortnightly) plantings were accommodated within the greenhouse. In addition to the regular spray of the fungicide (mancozeb) on the boarder plants and on the white plastic floor, any inadvertent spread of the pathogen inoculum within the greenhouse was mitigated by the curtain in the middle of the greenhouse. Moreover, the white floor was thoroughly disinfected with mixes of mancozeb and insecticides and exposed to sun for 2-3 days before each planting.

Data collection and analyses

Disease assessment

Plants were monitored on a weekly basis for prevalence of the foliar diseases BLM and early blight (EB), caused by *Altenaria solani*. At times when both diseases (BLM and EB) co-occurred, severity of both was estimated separately and then summed up on a plant basis. Eventually, however, since EB appeared only for a short time during the cool-dry seasons and even then, its effect was taken over by BLM during the growing season, disease assessment in this research was summarized into only BLM. BLM symptoms were assessed for an average of 14 to 18 WAT. Exception was the last planting which halted earlier (Table 1).

During each scouting, diseased leaves were counted and marked with multi-colored threads loosely wrapped on the petiole. Disease incidence (*DI*) was accordingly calculated in relation to the total number of leaves available on a plant at a particular time. At the same time, disease severity (*DS*) was estimated on a plant basis. Estimation of severity was practiced through a series of destructive sampling of non-experimental diseased plants from other greenhouses and routine quantification of diseased leaf area using the software AxioVision. Disease assessment at all times was done only by the researcher to minimize subjective errors. Procedurally, a walk through all the rows of the greenhouse was first made to have visual pictures, and systematically all blocks of plants with NF and F were estimated one after the other. Furthermore, disease intensities across plant canopy at harvesting were assessed destructively (refer the data presented in chapter 2). *DI* and *DS* as well as the later calculated standardized areas under their curves are given as proportion, not in percentage.

Host growth and yield parameters

In a similar way, host parameters like plant height (*PH*) and total leaf number (*TLN*) were monitored on a weekly basis for 14 to 18 WAT. *PH* was measured to the tip of the youngest tomato leaf and *TLN* was counted. For each planting, about 4 to 7 sequential harvestings were done starting after about 10 to 12 WAT. Harvesting was done on a plant basis whereby the ripe fruits were categorized as marketable and non-marketable following the customarily used standard, *i.e.* any of those fruits with < 50 g weight, with any form of crack, blossom end rot or with any other deformations not appealing for the consumers were classified as non-marketable.

Table 1. Calendar of events on the sequential plantings for the study of seasonal dynamics of the foliar disease black leaf mold.

Planting No.	Planting code	Starting and harvesting date	Julian calendar (weeks)	Experiment duration in weeks (days)
1	Jun.$_{I}$-05	16.06.05-30.09.05	24-39	15 (106)
2	Jun.$_{II}$-05	30.06.05-14.10.05	26-41	16 (105)
3	Jul.$_{I}$-05	15.07.05-11.11.05	28-45	18 (119)
4	Jul.$_{II}$-05	05.08.05-18.11.05	31-46	16 (112)
5	Aug.$_{I}$-05	19.08.05-02.12.05	33-48	16 (110)
6	Aug.$_{II}$-05	02.09.05-16.12.05	35-50	16 (112)
7	Sep.$_{I}$-05	16.09.05-30.12.05	37-52	16 (105)
8	Sep.$_{II}$-05	07.10.05-20.01.06	40-03	16 (112)
9	Oct.$_{I}$-05	21.10.05-20.01.06	42-03	14 (96)
10	Oct.$_{II}$-05	04.11.05-03.02.06	44-05	14 (96)
11	Nov.$_{I}$-05	18.11.05-24.02.06	46-08	15 (103)
12	Nov.$_{II}$-05	02.12.05-03.03.06	48-09	14 (95)
13	Dec.$_{I}$-05	23.12.05-01.04.06	51-13	15 (100)
14	Dec.$_{II}$-05	06.01.06-14.04.06	01-15	15 (106)
15	Jan.$_{I}$-06	20.01.06-05.05.06	03-18	16 (110)
16	Feb.$_{I}$-06	17.02.06-02.06.06	07-22	16 (107)
17	Mar.$_{I}$-06	17.03.06		
18	Apr.$_{I}$-06	21.04.06		
19	May$_{I}$-06	19.05.06		
20	Jun.$_{I}$-06	23.06.06-29.09.06	24-39	16 (107)
21	Jul.$_{I}$-06	21.07.06-20.10.06	29-42	14 (98)
22	Aug.$_{I}$-06	18.08.06-17.11.06	33-46	14 (95)
23	Sept..$_{I}$-06	15.09.06-14.12.06	37-50	14 (91)
24	Oct.$_{I}$-06	20.10.06-01.02.07	42-05	16 (110)
25	Nov.$_{I}$-06	16.11.06-08.03.07	46-10	16 (115)
26	Dec.$_{I}$-06	07.12.06-29.03.07	49-13	16 (112)
27	Jan.$_{I}$-07	12.01.07-30.03.07	02-13	12 (84)

Weather parameters

Weather parameters were acquired both from the computerized data logging system of the project and from the weather station of AIT. Ambient weather data outside the greenhouse among others included temperature (°C), relative humidity (%), solar radiation (W/m^2) and rainfall which was recorded as yes (1) or no (0) from the project data logger and in mm from the rain gauge of AIT weather station. More importantly, temperature, *T*, and relative humidity, *RH*, were measured using two sensors that were hung in each greenhouse. Until 5th July 2005, the data logger was set to every 10 minutes, followed by adjustment to 5 minutes until 30th October 2006 after which the interval was reduced only to a minute. Due to some technical errors of having some extreme values for both *T* and *RH*, day and night mean as well as maximum and minimum values were calculated manually and offset values were excluded.

Statistical analyses

Areas under the curves of *DS* and *DI* values were calculated using a trapezoidal method (Campbell and Madden, 1990) and standardized considering duration of each planting (Fry, 1977). Standardized *DS* and *DI*, symbolized as *DS** and *DI** respectively, were then analyzed using a PROC MIXED statement of SAS (2003). Progress curves of host parameters like *PH* and *TLN* were similarly depicted graphically using Microsoft Excel 2003 and fitted to linear and non-linear regression model using SigmaPlot (2006).

Eventually, favorability indices (*FI*) based on prevalence of optimal *RH* (> 84.5%) and *T* (26-33.4°C) for the first 56 days (after transplanting) of each planting were computed. The ranges were delineated based on earlier reports from Hartman *et al.* (1991) and Wang *et al.* (1996). For each day *i*, the favorability index of each of the weather parameter during the day (FI_{TDi} or FI_{RHDi}) and night (FI_{TNi} or FI_{RHNi}) was set to 1 when the condition was favorable else a value of 0 was assigned:

$$FI_{TDi} \text{ or } FI_{TNi} = \begin{cases} 1 \text{ if the mean day } (TDi) \text{ or night } (TNi) \text{ time temperature of day } i \text{ is in the interval } 26°C \leq T < 33.5°C \\ 0 \text{ if } TDi \text{ or } TNi \text{ of day } i \text{ is } < 26°C \text{ or } \geq 33.5°C \end{cases}$$

$$FI_{RHDi} \text{ or } FI_{RHNi} = \begin{cases} 1 \text{ if the mean day } (RHDi) \text{ or night } (RHNi) \text{ time relative humidity of day } i \text{ is } > 84.5\% \\ 0 \text{ if } RHDi \text{ or } RHNi \text{ of day } i \text{ is } \leq 84.5\% \end{cases}$$

FI of temperature of the day (FI_{TD}) and night (FI_{NT}) for the first 56 days was then computed using equations 1 and 2, respectively.

$$FI_{TD} = \sum_{i=1}^{56} (FI_{TDi}) / 56 \quad (1) \qquad FI_{TN} = \sum_{i=1}^{56} (FI_{TNi}) / 56 \quad (2)$$

The same procedure was followed to compute *FI* of relative humidity of the day (FI_{RHD}) and night (FI_{RHN}) as shown in equations 3 and 4.

$$FI_{RHD} = \sum_{i=1}^{56} (FI_{RHDi}) / 56 \quad (3) \qquad FI_{RHN} = \sum_{i=1}^{56} (FI_{TNi}) / 56 \quad (4)$$

For each planting, favorability values for temperature (FI_T) and relative humidity (FI_{RH}) were formed by calculating the mean value of the day and night favorability indices, i.e. $FI_T = 0.5 \cdot (FI_{TD} + FI_{TN})$ and $FI_{RH} = 0.5 \cdot (FI_{RHD} + FI_{RHN})$, respectively.

Weather–disease relationships were first analyzed using linear relationships of each favorability index (FI_T and FI_{RH}) separately and then using multiple regression analyses including the product of both favorability indices (eq. 5).

$$DS^* = a + b \cdot FI_T + c \cdot FI_{RH} + d \cdot FI_T \cdot FI_{RH} \quad (5)$$

In a similar way, maximum host growth parameters like maximum plant height (*MPH*) and maximum leaf number (*MLN*) were first regressed to marketable yield (*MY*) using linear as well as multiple regression (eq. 6) in SigmaPlot (2006). After fitting of *MPH* and *MLN*, the statistical significance of the coefficients was tested using SAS (2003).

$$MY = a + b \cdot MPH + c \cdot MLN + d \cdot MPH \cdot MLN \quad (6)$$

Finally, relationship of marketable yield (*MY*) of FMTT260 to that of host growth in terms of maximum plant height (*MPH*) and to the disease severity (*DS**) was tested using equation 7.

$$MY = (a + b \cdot MPH) \cdot (1 - c \cdot DS^*) \quad (7)$$

RESULTS

Disease intensities: progress curves and integral variables

Due to high heat stress and fertigation problems, three of the monthly plantings in 2006, *i.e.* March to May 2006, were discarded, thus making the number of plantings considered for the experimental analyses to be 24. Besides, although early blight (*Alternaria solani*) occurred during the cool season plantings in October and November of each year, its appearance was only sporadic for the first 3 to 6 WAT and taken over by BLM thereafter. For the reason that both diseases shared an overlapping niche and considering the minor importance of early blight in the BioNet^{TM} greenhouse where the seasonal study was undertaken, only the impact of BLM was analyzed in this research.

Standardized areas under disease progress curves, representing mean values of disease intensities over the experimental period, in terms of incidence of leaves (*DI**) and severity per plant (*DS**), are presented in Fig. 1. Statistical analyses of disease intensities from the first 14 fortnightly plantings in 2005 using a PROC GLM procedure in SAS indicated non-significant difference for the repetitive plantings within a month for both *DI** (p = 0.823) and *DS** (p = 0.087). All the plantings as of January 2006 were carried out on a monthly basis.

*DI** from the non-fungicide (NF) treatments was high in all the fortnightly plantings of 2005 except those two plantings at the beginning in June 2005 (Fig. 1a). In a similar way, weekly progress curves indicated that disease incidence of leaves (*DI*) at the last assessment date on those plantings as of July 2005 ranged between 0.7 to 1.0 (graphs not presented). Obviously, there were more diseased leaves and thus a higher *DI* on non-protected plants than on those sprayed with fungicide (Fig. 1a). In some cases, however, like for instance at times of heavy disease epidemics in August 2005, both fungicide sprayed (F) and non-sprayed (NF) treatments showed high *DI* values (0.73 and 0.95, respectively) at the last assessment date (16 WAT). *DI** was comparatively lower after of February 2006 than those plantings before the interruption (Fig. 1a). The high heat stress forced us to discard the plantings from March to May 2006. Consequently, the inoculum carryover was interrupted and thus there was generally less *DI** from June 2006 to January 2007 (Fig 1a).

The *DS** values were clearly lower as compared to *DI** (Fig. 1a and b). Despite prevalence of BLM throughout all plantings, its severity was highly variable. Four fortnightly plantings in August and September 2005, two plantings in December 2005 and January 2006 as well as two more plantings in August to September 2006 could roughly be categorized as three peak

periods among the plantings (Fig. 1b) with *DS** values ranging between 0.20-0.30, 0.14-0.20 and 0.09-0.14 for the first, second and third periods, respectively (Fig. 1b). Similarly, the maximum *DS* at the last assessment date of each of these respective peaks were 0.65-0.81, 0.48-0.56 and 0.30-0.45 (data not shown).

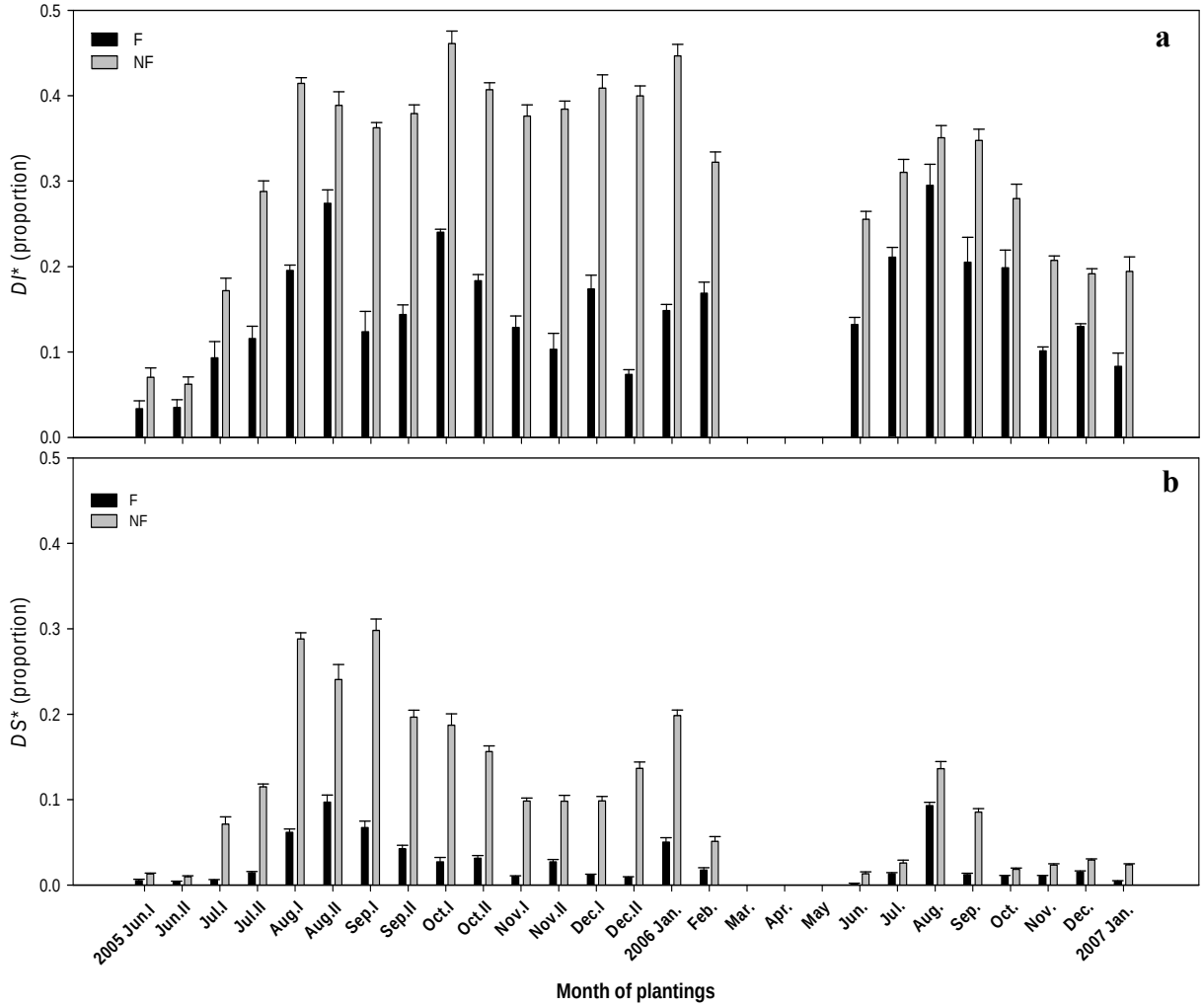

Fig. 1. BLM epidemics in terms of the standardized a) disease incidence ***DI**** and b) severity ***DS**** of the 24 sequential plantings from June 2005 to January 2007 with (**F**) and without (**NF**) fungicide treatments. NB: Fortnightly plantings in 2005 are differentiated with **I** and **II** planted at the 15th and 30th day of each month.

The difference between fungicide treated and non-treated plants in terms of BLM severity was even more conspicuous than that of incidence as was observed from the progress curves (graphs not presented) and the integral variables (Fig. 1). Mancozeb generally curbed the disease very well and at all times during the plantings. Even at the last assessment of the high

disease severity in August-II 2005 (0.81), the corresponding *DS* for fungicide protected plants (F) was only 0.29 (data not shown). A similar observation was made from the comparison of the *DS** value of 0.24 and 0.097 for NF and F treatments of the same planting (Fig. 1b).

Weather parameters: favorability indices of temperature and relative humidity

Favorability indices of temperature (FI_T) and relative humidity (FI_{RH}) for the first 56 days of each planting based on optimal weather parameter requirements (equations 1 and 2) of BLM were depicted on Fig. 2a. Except the cool-dry seasons which extended from October to December, FI_T was high enough to favor BLM epidemics. This could be detected from the comparison of the lowest FI_T values of 0.43 (November 2005) and 0.40 (December 2006) with that of the hot-wet seasons of June to September 2005 which always ranged above 0.82 (Fig. 2a). FI_T during the hot months of March to May 2006 was not at optimum as it could be expected surpassing the optimal range of 26 to 33.4°C.

FI_{RH}, on the other hand, highly corroborated to the seasonal classification but was also partly affected by other factors, like the host leaf area accommodated at a point in time, which in turn influenced the climatic conditions within the greenhouse. For instance, FI_{RH} was low during the first two plantings in June 2005 (Fig. 2a) which could partly be attributed to a new start of the experiment after renovation of all greenhouses despite the fact that they fall within the hot-wet season. There were fewer plants in the greenhouse during these plantings as compared to the others. The steady progress of FI_{RH} in the months July to September 2005 and 2006, too, was a clear indicator of high favorability for BLM (Fig. 2a). The fact that there were few plants in the greenhouse during the hottest months in 2006 (March to May), as plants with extreme stress were consistently discarded, has partly affected the greenhouse microclimate and contributed to the low FI_{RH} (Fig. 2a).

Detailed analyses of day-night comparisons of the favorability indices of temperature (FI_{TD} and FI_{TN}) and relative humidity (FI_{RHD} and FI_{RHN}) revealed two scenarios as shown in figures 2b and c. On one hand, FI_{TD} was higher than 0.8 nearly in all plantings whereas FI_{NT} values during the cool season went as low as 0.04 (Fig. 2b). FI_{RHN} was exclusively different from that of FI_{DRH} whereby the former was with a significantly higher favorability index as compared to the latter (Fig. 2c). Average values of all the 27 plantings in terms of favorability indices of temperature were 0.88 during the daytime (FI_{TD}) to 0.61 at nighttime (FI_{TN}). The most significance of night relative humidity could be traced when these mean values were compared, *i.e.* 0.07 to 0.64 for day and nighttime respectively.

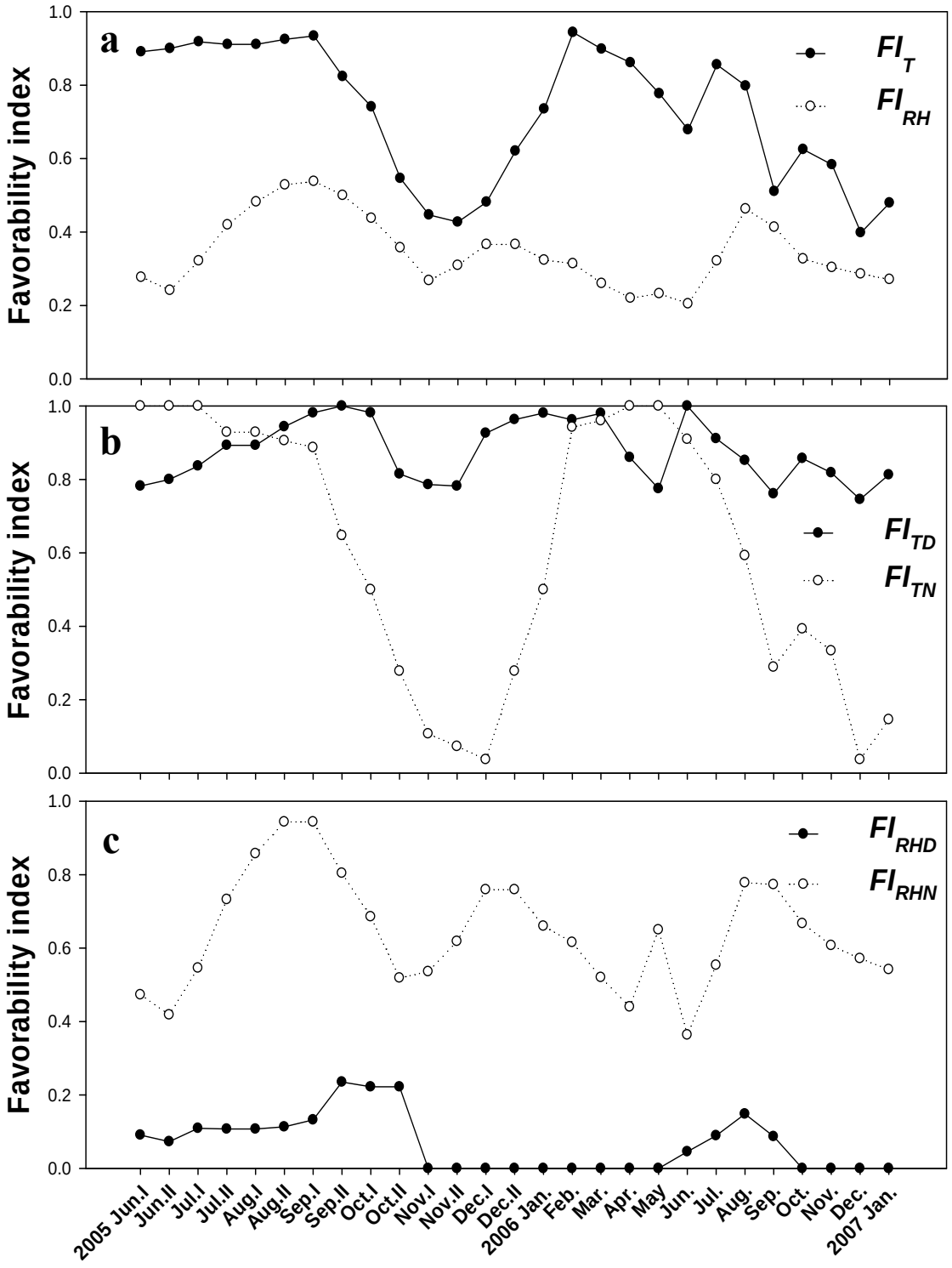

Fig. 2. Favorability indices of a) daily temperature (FI_T) and relative humidity (FI_{RH}) computed for the first 56 days of each planting, b) contribution of day- and nighttime favorability indices of temperature (FI_{TD} and FI_{TN}) according to equations 1 and 2 and c) relative humidity (FI_{RHD} and FI_{RHN}) according to equations 3 and 4.

Weather – disease relationships

In contrary to the observed trend of favorability of daily temperature (FI_T) in following the pattern of disease epidemics (*DS**), the relationship was not explained with the linear line (Fig. 3a). Both the intercept (p = 0.9591) and slope (p = 0.1177) terms were non-significant and r^2 value was only 0.10. This could partly be attributed to the wide range of optimal mean temperature (from 26 up to 33.4ºC) which is mostly prevalent except a few cases of cooler or extremely hot months. Besides, the diluting effect of low *DS** values at the beginning of the experiment in 2005 and during the plantings that were carried out after the interruption in 2006 contributed to the poor correlation. This low *DS** is most likely arising from the few initial inoculum sources of *P. fuligena* during four plantings at inception of the experiment in 2005 and all the plantings after the interruption in 2006 (refer to Fig. 1a and 2a).

Linear regression of the favorability index of relative humidity (FI_{RH}), on the other hand, proved to be highly correlated with *DS**. This was witnessed from the high significance of both the intercept and slope terms ($p < 0.0001$) as well as high value of r^2 (0.71) (Fig. 3b). Multiple regressions of both favorability indices gave a slightly better fit than the unilateral linear fits as the coefficient of determination was further improved to 0.74. Nonetheless, all the parameters estimates *a*, *b*, *c* and d (eq. 5) were non-significant. In further attempts, the model was simplified by removing parameters finally resulting in the modified function $DS^* = a + b \cdot FI_T + d \cdot FI_T \cdot FI_{RH}$ that fitted well (R^2 = 0.74) with significance of the three parameters, *i.e. a* (p = 0.0360), *b* (p = 0.0006) and *d* ($p < 0.0001$). The final output with the parameter estimates of *a* (0.09 ± 0.04), *b* (-0.34 ± 0.08) and *d* (1.00 ± 0.14) is depicted in Fig. 3c.

Host parameters: progress curves, integral variables and marketable yield

The overall assessment of host growth parameters indicated that after the first three weeks of adjustment after transplanting (relatively slow growth) FMTT260 plants grew nearly linearly until about a height of 175 cm (Fig. 4a). Although the cultivar was an indeterminate type and thus able to grow indefinitely, it grew up to more than 3 m of height until harvesting during the cool season (Fig. 4a). High temperatures during the last weeks of growth of the plant as well as fertigation problems in some of the plants as of January 2006 visibly affected the plant height across the plantings (Fig. 4a). In a similar way, except for the differences due to single and double stem plantings, total leaf number on a plant basis followed the same trend of nearly a linear growth to the time when 30 and 40 leaves were borne on single and double

stemmed plants respectively (Fig. 4b). Because of external variations amongst plantings with respect to fertigation and heat stress, however, contrasting seasonal variations of host growth among the plantings became difficult. High R^2 values as well as the significance of parameters of logistic growth functions fitted to plant height and total leaf number of the selected four plantings in 2005 (refer to chapter 3) could implicate better fit to represent host growth of FMTT260 plants than using linear fits.

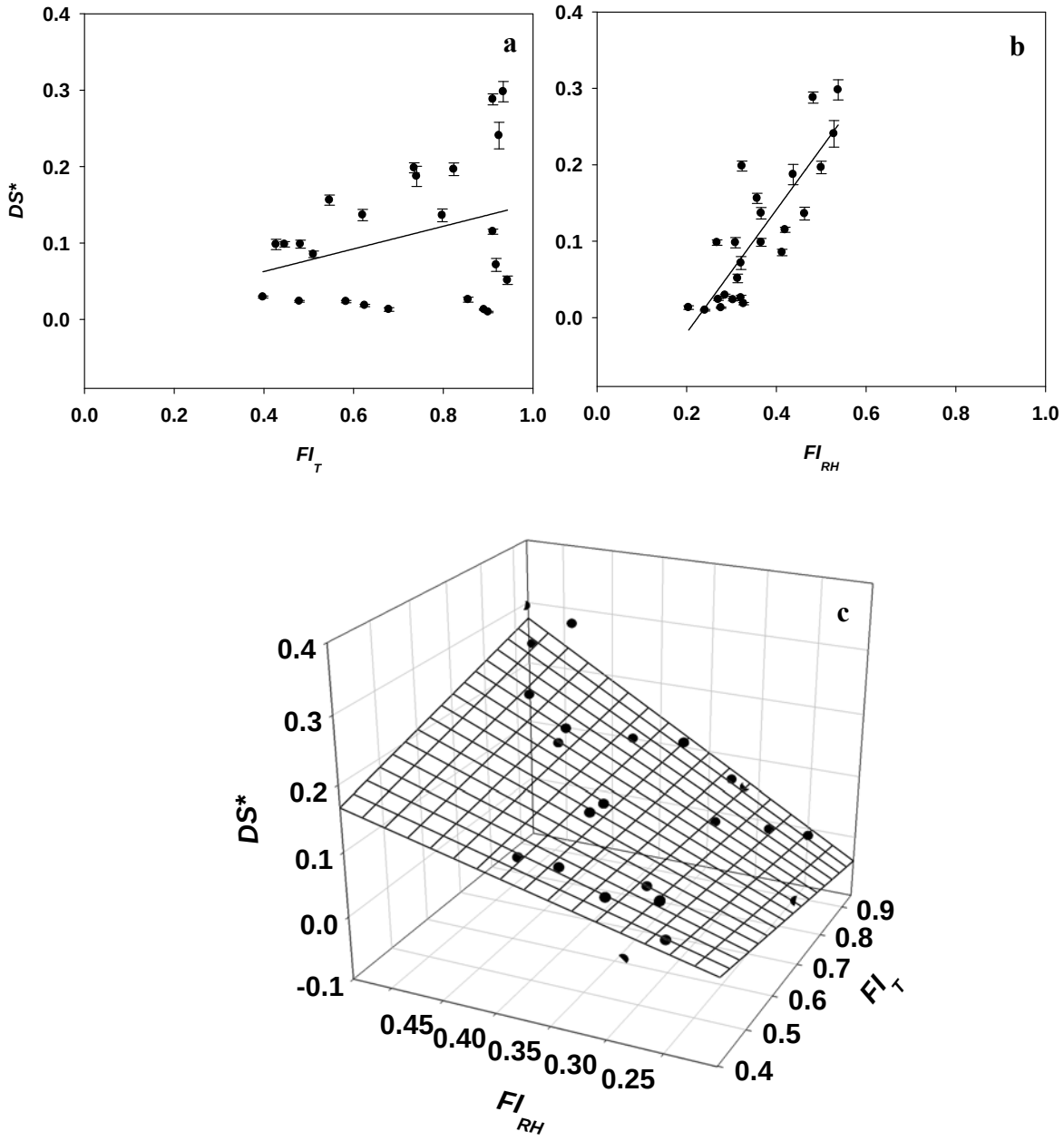

Fig. 3. a) **Linear regression** of favorability indices of temperature ($\boldsymbol{FI_T}$) and b) relative humidity ($\boldsymbol{FI_{RH}}$) alone and **multiple regression** of both indices (c) to standardized proportion of BLM severity (*DS**) under natural epidemics at AIT, Thailand.

Since there were no conspicuous effects of BLM on plant height and total leaf number, as it was observed throughout the plantings, no further statistical analyses was made for the weekly progress curves. This was obviously visible from the nearly the same growth of fungicide treated (green dotted line) and non-treated (red line) plants (Fig. 4). Considering the same non-significant observations of *PH* and *TLN* from the four selected plantings in 2005 (refer to chapter 3), further analyses in this study were proceeded to include aspects of yield.

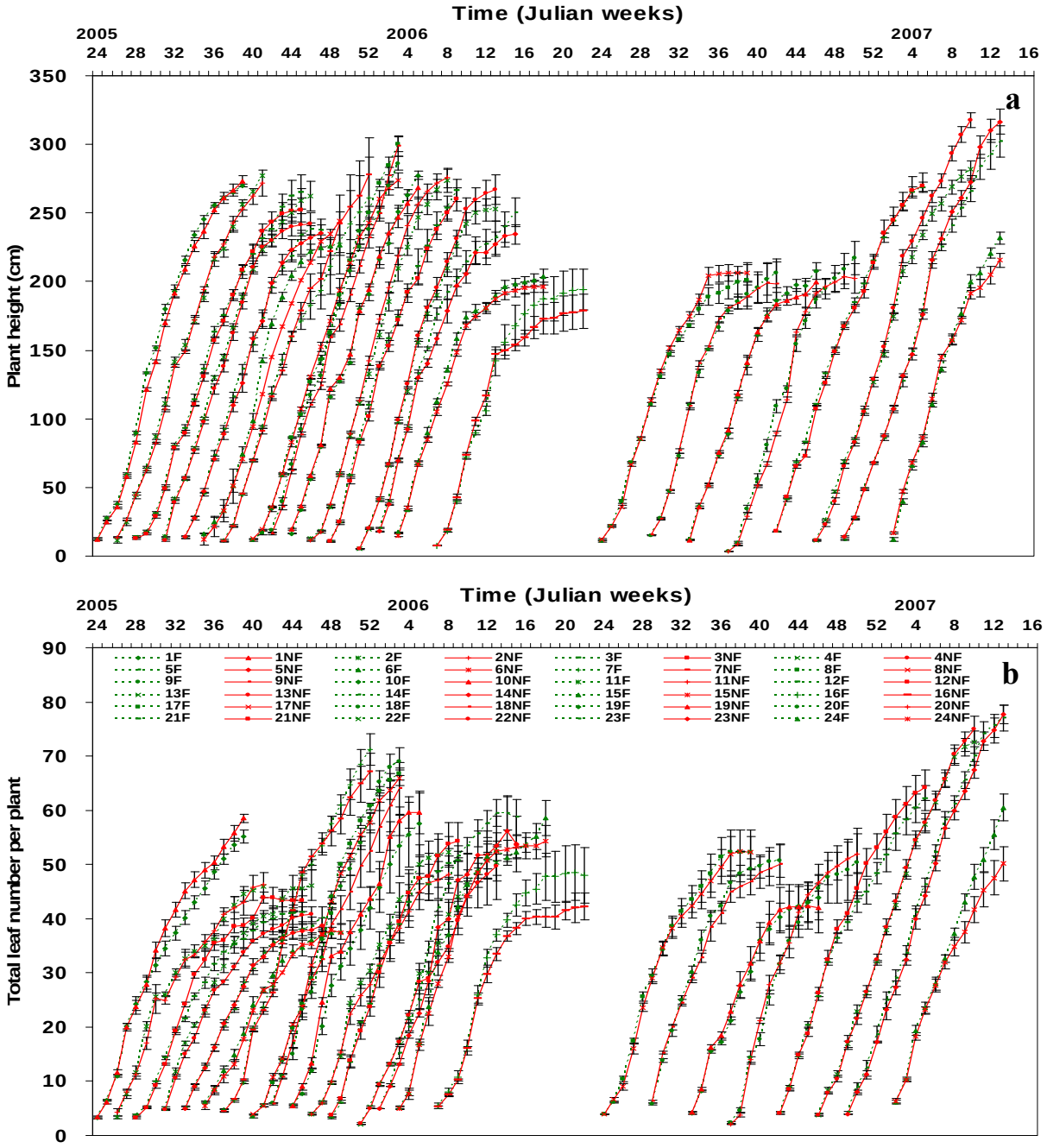

Fig. 4. Progress curves of weekly **host growth** assessments of FMTT260 plants during the 24 plantings in terms of a) **plant height** and b) **total leaf number**. NB: Each couple of red line and green dotted line represented a planting and the same legend is used for both graphs.

In the same way as host growth, comparison of yield across seasons and plantings became erroneous as FMTT260 responded to external stimuli like variations in temperature and fertigation very highly. There was a clear tendency of higher yield during the cool-dry season as compared to the hot-wet season. This was observed from the relatively higher yield from plantings during the months of October to December in both years, *i.e.* plantings 9 to 14 in 2005 and 24 to 26 in 2006 (Fig. 5a). In 2005, for instance, the yield per plant was more than a double in October to December (plantings 9 to 14) compared to June to August (plantings 1 to 6). This scenario was repeated in the plantings of 2006 (Fig. 5a).

Although yield loss attributable to BLM was lower at plantings when there was a harvest of relatively higher yield, the highest peaks of BLM severity still caused a marketable yield loss ranging from 75 to 150 kg per greenhouse when the fungicide sprayed treatment F of each planting was compared with that of the non-sprayed treatment NF (Fig. 5b). Accordingly, four of the fortnightly plantings in August to September 2005 (plantings 5 to 8) had a relative yield loss of 30.5 to 37.5% with an average of 34.1% and the two plantings in December 2005 and January 2006 (plantings 14 to 16) resulted in a percent yield loss of 17.6 to 41.4 (an average of 31.9%). The last peak-epidemic plantings in July to September 2006 (plantings 21 to 23) resulted in an average yield loss ranging from 4.3 to 30.5% (an average of only 20.6%) (Fig. 5b). In overall, these three heavy epidemic plantings gave rise to an average yield loss of 28.9%.

In 21 out of the 24 plantings from which tomato fruits were harvested, marketable yield loss (*MYL*) in percent was positive (ranging between 2.9 and 41.1%) but the reverse was too observed in three (plantings 12, 25 and 26) when marketable yield of fungicide protected plants (MY_F) were compared with that of non-sprayed plants (MY_{NF}) (Fig. 5b).

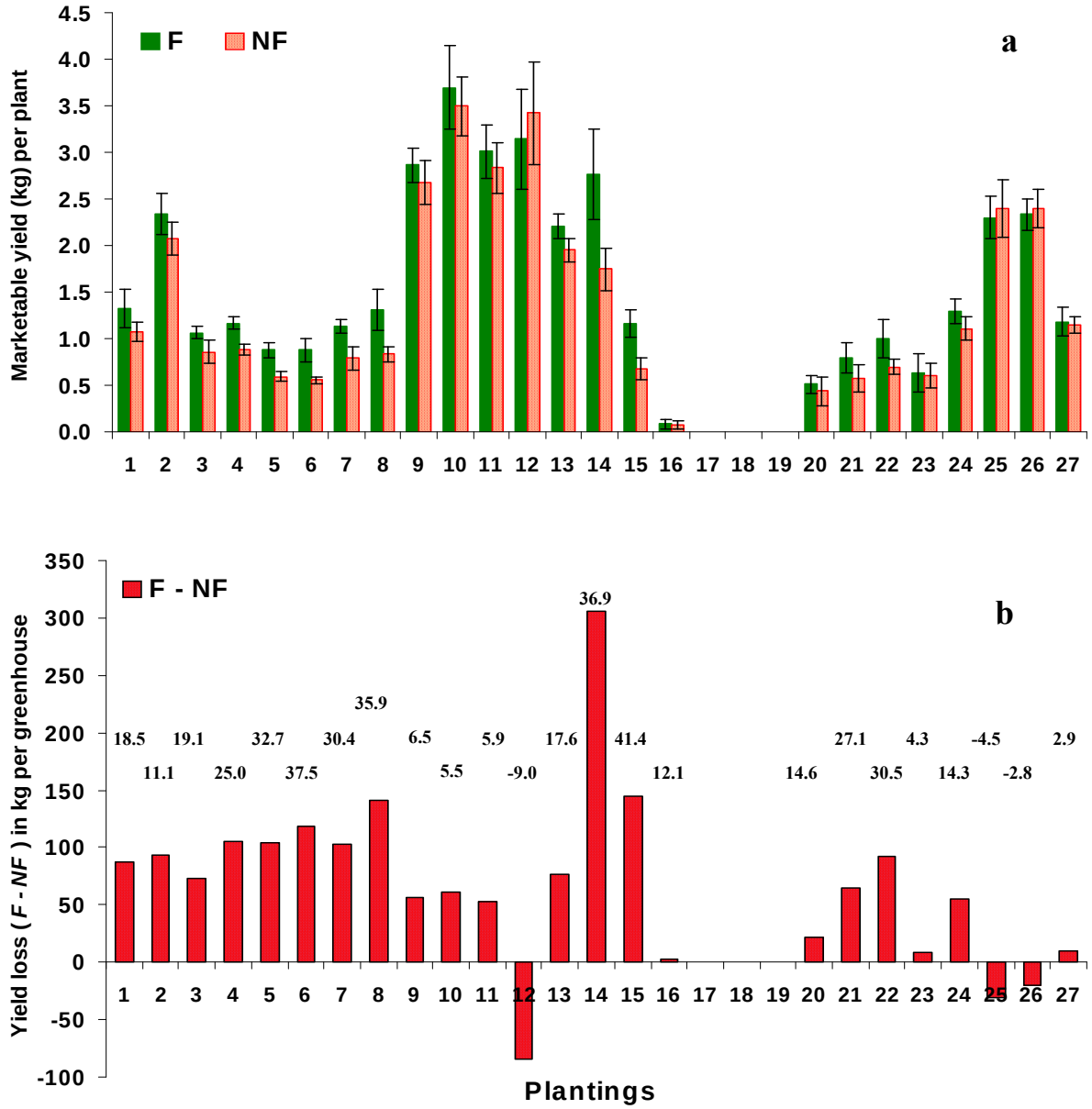

Fig. 5. Marketable yield (kg fruit harvest/plant) from fungicide (*F*) and non-fungicide (*NF*) treated FMTT260 plants across 24 plantings (a) and associated differences (*F-NF*) in yield due to BLM expressed as kg yield loss per greenhouse (b). NB: numbers on top of each bar in b indicate marketable yield loss (*MYL*) in percent, *i.e.* $MYL\ (\%) = 100 - (100 \cdot MY_{NF}/MY_F)$

In fact, the marketable yield on a plant basis was positively correlated with the host growth parameters maximum plant height (*MPH*) in cm and with the count of maximum leaf number (*MLN*) as could be seen from the linear and multiple regressions (Fig. 6). *MPH* fitted better to the linear line (Fig. 6a), with both the intercept (-2.27 ± 0.35) and the slope (0.015 ± 0.001) terms highly significant ($p < 0.0001$) but relatively low r^2 value (0.32), compared to *MLN*.

Only the slope term (0.03 ± 0.005) of *MLN* was significant with a very low r^2 value (0.13). This could partly be because of mixed plantings of single and double stem at different seasons. Multiple regressions of both host growth parameters (eq. 6) has shown a better fit with improved r^2 value (0.33) and significance of the intercept ($p = 0.0026$) and parameter estimate of the linear term *MPH* ($p = 0.0002$). Because of non-significance of the other two parameters, *i.e.* c ($p = 0.0652$) and d ($p = 0.1075$), however, equation 6 was modified excluding the final product term. Accordingly, the parameters *a* and *b* in the modified equation ($MY = a + b \cdot MPH + c \cdot MLN$) remained significant ($p < 0.0001$) but the parameter *c* was non-significant ($p = 0.1307$) (Fig. 6c).

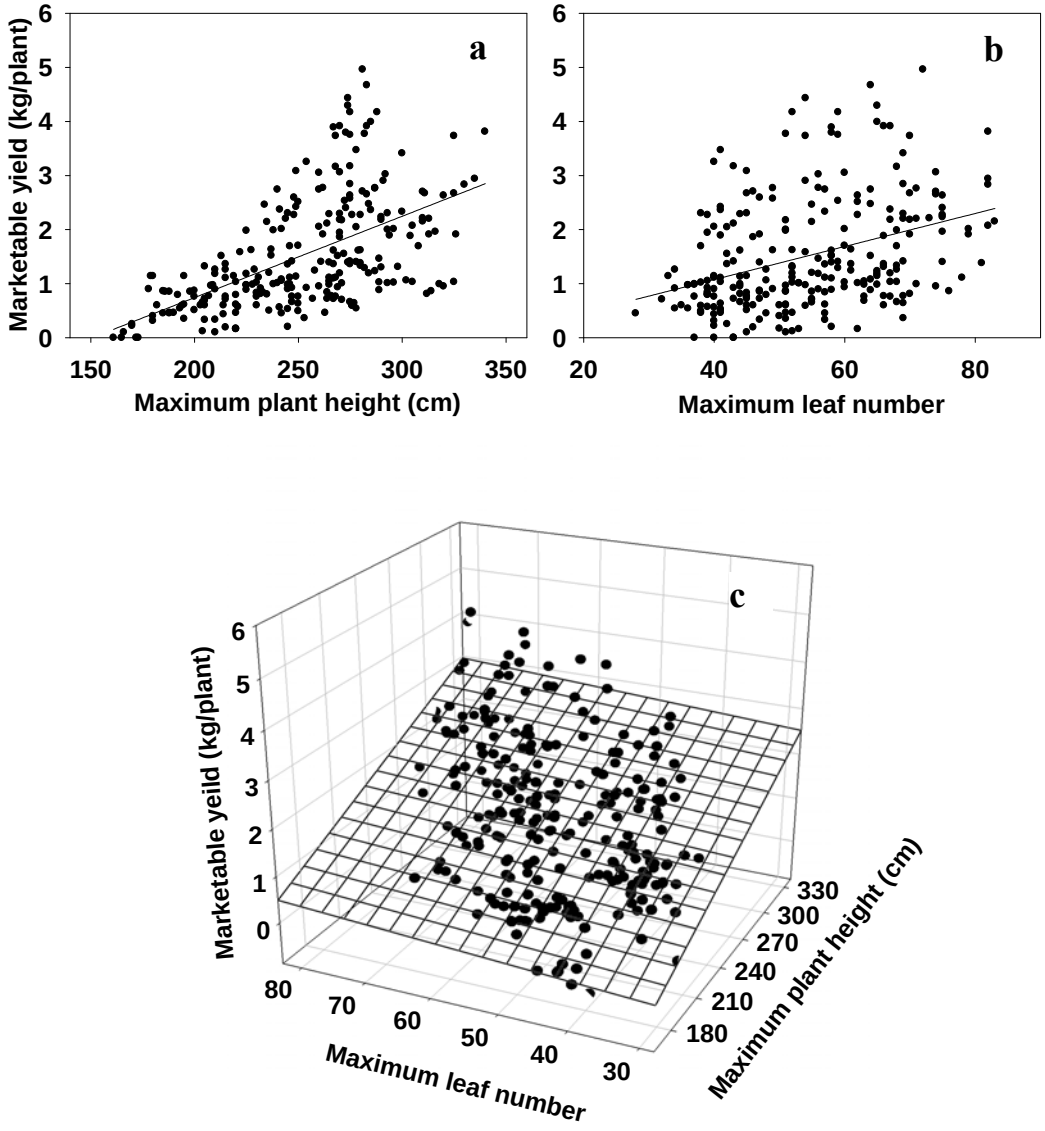

Fig. 6. Linear regression of the host growth parameters maximum plant height (a) and total leaf number (b) alone and multiple regression of both (c) with marketable yield of FMTT260 from all experimental plants (*n =255*).

Obviously, marketable yield on a plant basis was negatively but poorly ($r^2 = 0.021$) correlated with that of standardized BLM severity (*DS**) as seen from the linear regression of all experimental plants of NF treatments (Fig. 7a) even though both the intercept and slope terms were significant. However, considering the better linear fit of *MPH* ($r^2 = 0.32$) as shown in Fig. 7b, multiple regression of both, *i.e. MPH* and *DS** (eq. 7), improved the fit ($R^2 = 0.35$) with high significance all the three parameters a = -2.4822 ($p < 0.0001$), b = 0.0164 ($p < 0.0001$) and c = 1.1630 ($p = 0.0007$) as shown in Fig. 7c. The final parameter estimates of c suggested that a 1% *DS** reduced the marketable yield by ≈ 1.2%.

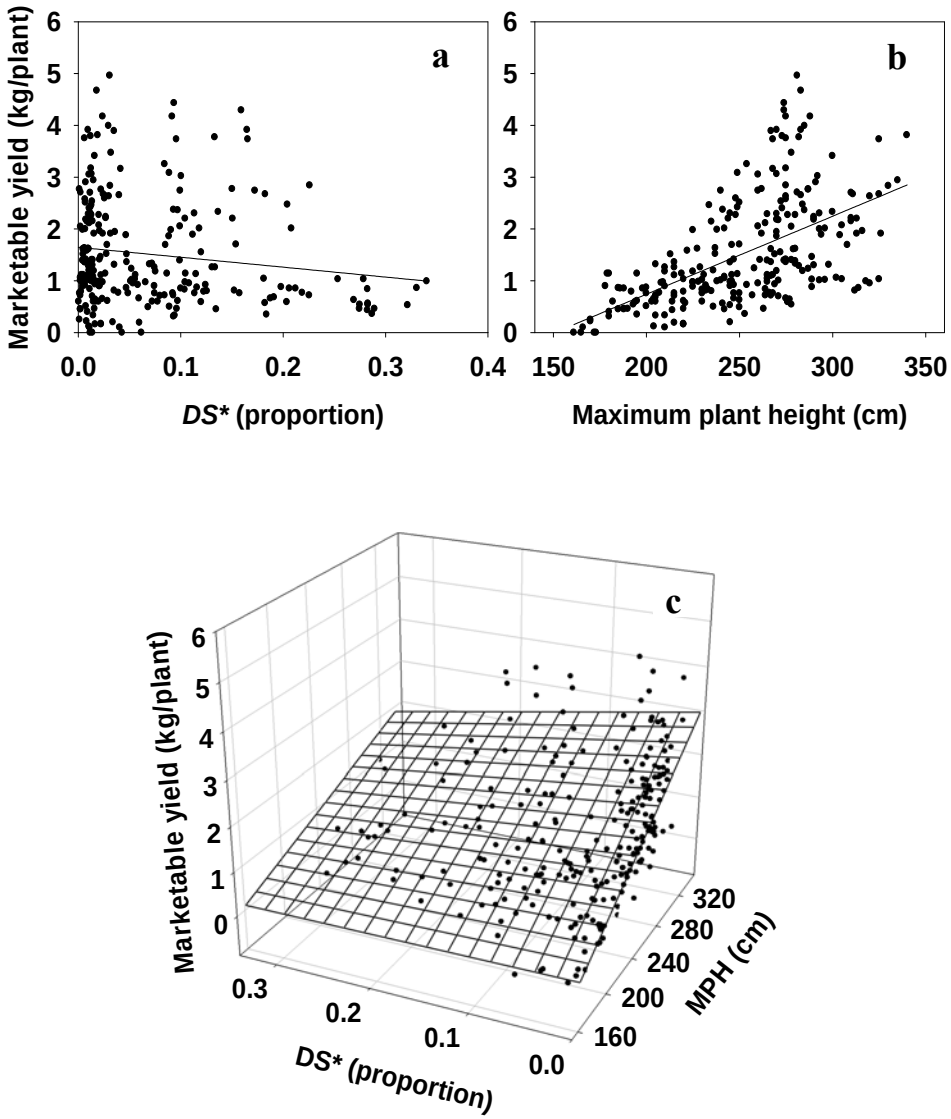

Fig. 7. Linear regression of the a) standardized BLM **severity (*DS**)** and b) **maximum plant height** in cm alone and c) multiple regression of both with **marketable yield** of FMTT260 plants.

DISCUSSION

BLM occurred throughout the year when the susceptible cultivar FMTT260 was planted in greenhouses at AIT, Thailand, but only two peak-epidemic periods of BLM within a year could be identified. The most obvious one was the hot-wet season, particularly the plantings from August to September. This goes in agreement with earlier results of Hartman and Wang (1992) and Wang *et al.* (1996) at AVRDC where maximum area under disease progress curve (*AUDPC*) was recorded during these months. In Thailand, however, plantings during June to July could be of high risk potentially considering the observed favorability indices. Thus the actual recorded low epidemics (in the first four plantings in 2005) despite high favorability could most likely be attributed to the low level of inoculum as it was a time of a new start of research after renovation of all the greenhouses. Indeed, level of inoculum and plant density within the greenhouse during any of the plantings may have influenced the level of BLM epidemics. This was also noticed from those plantings that were carried out in 2006 right after interruption due to heat stress. Since conidia of BLM are airborne (Hartman *et al.*, 1991; Wang *et al.*, 1996), low initial inoculum at the beginning of this study right after renovation of all the greenhouses of the project in June 2005 may have masked the prevailing disease favorability factors. With the same analogy, the interrupted gap of three plantings during the hot months from March to May 2006 may have partly contributed to the low disease level in 2006.

In 2005, mean disease severity (*DS**) and incidence of leaves (*DI**) per plant was up to 0.3 and 0.4 respectively, from plantings during the hot-wet season. Maximum disease severity and incidence at the last assessment date (about 16 WAT) reached up to a proportion of 0.81 and nearly 1.0 respectively. Hartman and Wang (1992) found an average severity of 53 and 60% at two assessments during fruit onset on 10 tomato entries with a record of 53% for the fresh market tomato varieties FMTT274 and FMTT305. The same peak was observed in 2006 from plantings of August and September with lower BLM disease level as compared to the same plantings in 2005. This was most probably due to the three month interruptions (March to May 2006) that led to a break-up of inoculum build-up withinthe greenhouse. In addition, it could also presumably be the hottest temperature that could be detrimental to the survival of fungal propagules.

The second peak BLM epidemic was during plantings of December and January which fall in the seasonal climatic category of cool-dry season. As it was noticed in both years, despite no access of rain into the greenhouse, rainfall has influenced the relative humidity inside the

greenhouse. Rainy to wet seasons during all the plantings highly favored the disease through increased relative humidity inside the greenhouse. More importantly, the range of temperature was found to be conducive not only to black leaf mold but also to early blight. Although a separate assessment of the later was attempted, at the later growth stages of tomato, BLM was always dominant and it was decided to sum up severities of both until a certain point in time and account the later epidemics only to the major disease, *i.e.* BLM.

Whereas disease severity as well as its integral variable (*DS**) gave the essential clue about the extent of disease progression and degression in time, disease incidence of leaves did not show any clear pattern amongst the plantings. As the conidia of *P. fuligena* are mainly disseminated by air (Wang *et al.*, 1996), the distribution of the symptoms withinthe plant canopy should be random. However, leaves at the lower canopy stratum mostly showed higher BLM severity and 100% incidence under natural undisturbed epidemics. In addition, there was a gradual shift of the disease upwards in cases of fungicide protection. This aspect of spatio-temporal distributions of the disease within the plant canopy was discussed in chapter 4.

BLM is favored by high humidity, moderate to warm as well as cool night temperatures which result in extended periods of leaf wetness (Yamada 1951; Blazquez, 1991; Wang *et al.*, 1995). During their detailed analysis of BLM's pathogenecity, Hartman *et al.* (1991) found out 26-28°C to be optimum for mycelial growth and observed culmination of growth at a maximal limit of 34°C. Besides, they stated that conidia of BLM did not germinate at or below 84.5% relative humidity. Despite some irregularities, these limits were followed in this research too. The most highly correlated favorability index with that of *DS** in this research was found when sums of the first 56 days of favorability indices of temperature (FI_T) and relative humidity (FI_{RH}) were accounted. Although multiple regression of both parameters gave better fit, 75% of the variations in *DS** were accounted solely from relative humidity. This could partly be attributed to the wide range of optimal temperature that coincided with the existing situation of the research area.

Detailed scenario of weather favorability in the greenhouse was explicitly explained by depicting hourly mean temperature (*T*) and relative humidity (*RH*) values of three days within each month (1st, 8th and 15th) as shown in Fig. 8. Since data for the month of May lacked in both years, it was only possible to represent the hot-dry season using June 2005. Accordingly, within a day, an average of 19 and 8 hours were within the above mentioned *T* and *RH* ranges (Fig. 8a). If we consider the month of September, for instance, 24 and 15 hours of favorability existed for BLM (Fig. 8c). The final category in cool-dry season in December on the other hand was with 9 and 0 hours favorability. Considering the mild weather requirements of early

blight, however, temperature favorability may increase up to 24 hours (Fig. 8e). Even for BLM epidemics, transplantings in December and January will extend to the beginning of the favorable *T* and *RH* in March and April when an average of 16 weeks growing period was considered. Thus a certain level of peak epidemics could be expected. Summarized data of maximum and minimum as well as mean temperature and rainfall from AIT weather station (data not presented) corroborated with earlier seasonal descriptions by Matsumoto (1997), Zhang *et al.* (2002b), Kleinhenz *et al.* (2006) and Takahashi and Yasunari (2006).

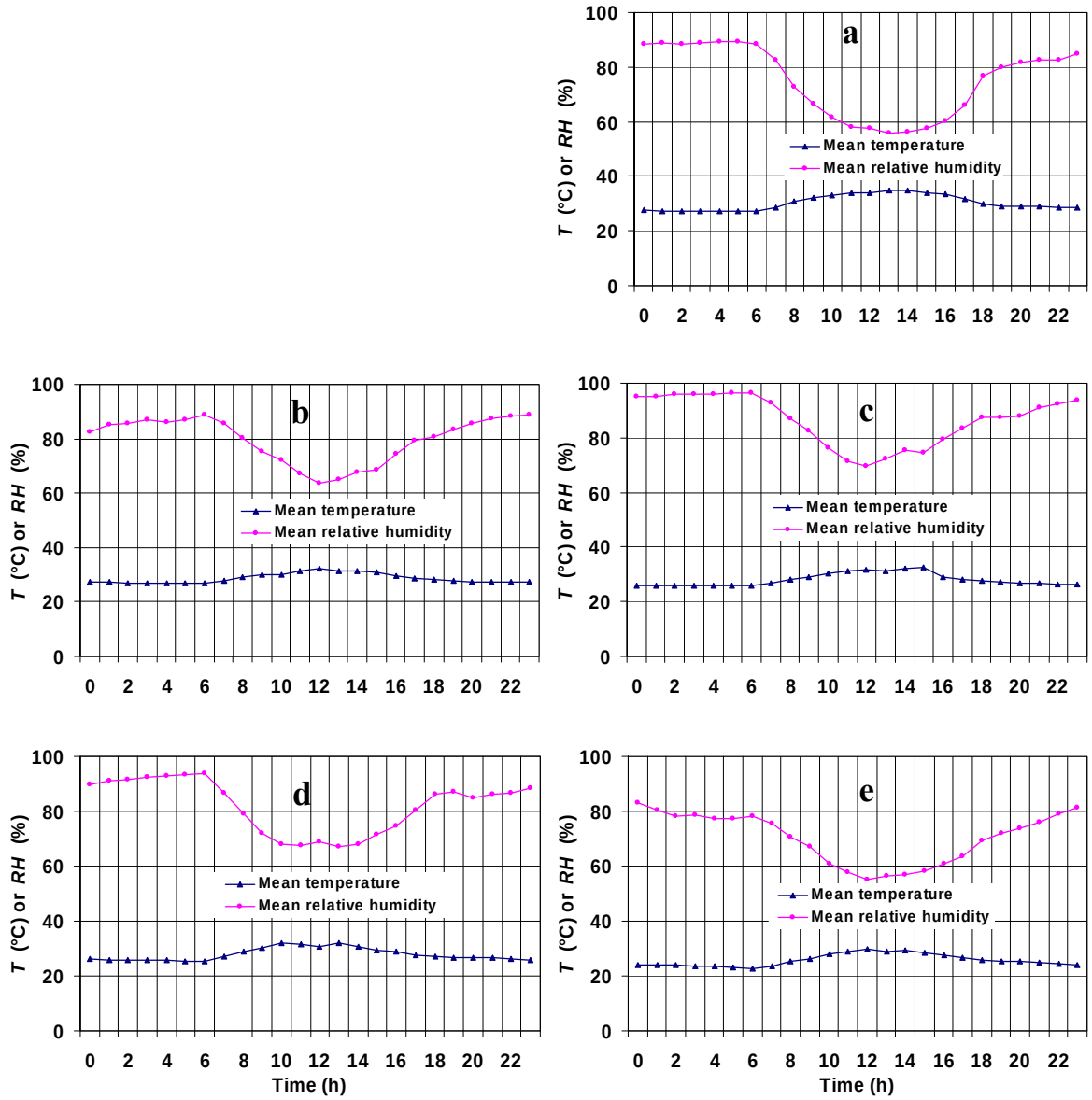

Fig. 8. Mean hourly temperature (*T* in °C) and relative humidity (*RH* in %) in the 50-mesh BioNet^{TM} greenhouse during the hot-wet season in June (a), August (b) and September (c) 2005 and cool dry season of November (d) and December (e) 2005. NB: Mean of the 1st, 8th and 15th day of each month was considered and there was no data for May 2005 as the greenhouse was on renovation.

Although previous researches from Charles-Edwards (1982) and Boote *et al.* (1983) broadly classified effects of diseases on hosts, Burdon (1993) simplistically stated that disease may cause damage to plants as castrators, killers and debilitators. Debilitators certainly interfere negatively with the source-sink relationship of the host plants that cause yield loss as accumulated photosynthates in crops. Although the major impact of BLM belongs to this category, its effects on the host parameters plant height and total leaf number was not conspicuous. This aspect was briefly discussed on selected plantings in chapter 3.

In this research, however, impact of BLM on marketable yield was assessed from all the plantings. Since yield constituted from the integral of all biotic and abiotic stresses, verifying losses solely due to effect of BLM across plantings under ambient conditions was difficult. Marketable yield comparison of each planting was thus made with reference to its own control. Accordingly, a yield loss 34.1, 39.2 and 20.6% was observed for the 1st, 2nd and 3rd BLM peak-epidemic periods, respectively. This resulted in an average yield loss of 31.1% which is similar to the report of Hartman and Wang (1992) which mentioned a yield loss of up to 32% when control treatments were compared to those sprayed with fungicide. James (1983) and Cooke (2006) stated many of the confounding factors that weaken the statistical relationship between disease levels and yield. This phenomenon prevailed in our research and resulted in a poor correlation of marketable yield and *DS**. Nonetheless, integrating host parameters like maximum plant height in the model $MY = (a + b \cdot MPH) \cdot (1 - c \cdot DS^*)$ improved the fit and suggested a yield reduction of ≈1.2% from a 1% *DS**. Interestingly, this model further explained the actual yield loss of the 1st and 3rd BLM peak-epidemic seasons.

Two applications of Mancozeb at the 4th and 6th week were found to significantly reduce the level of BLM in most of the plantings. During plantings of high epidemics, however, fungicide treatments shall start earlier and three applications including the 2nd week could be considered. Despite the significant reduction of BLM, a complete control was not achieved as there were latent infections during those fungicide-free weeks. Hartman and Wang (1992), however, reported that no disease occurred on two lines that were inoculated but fungicide protected. Merits of these early applications of mancozeb as part and parcel of integrated disease management of BLM could also be explained by their impact of reducing the inoculum dispersal from the lower confined strata of the plant. Similar results were obtained when early blight of potato (*A. solani*) was controlled more efficient by early applications than was late blight (*P. infestans*), particularly when the latter was severe (Fry and Shtienberg, 1990).

In summary, despite the sporadic threat of the disease during the above mentioned three peak-epidemic periods, FMTT260 as indeterminate variety showed a tendency of more growth of flushed that at times dilute the impact of the disease. Besides, impact of the disease seemed to be not in proportion to observed disease intensities as the disease becomes more aggressive later in the growing period. A similar comments were mentioned in earlier studies of Wang *et al.* (1996). Hartman and Wang (1992) also pointed out that yield loss attributable to black leaf mold may not be as severe as other tomato diseases. However, without control of the disease, yield losses of earlier mentioned quantities could happen under protected cultivation. Thus, stringent follow-up of weather parameters and two sprays of mancozeb, at times of high epidemics, coupled with late pruning of lower older leaves could be the best options of managing the disease. The economic aspects of disease management versus demand-supply and price of tomato under local market dynamics should, however, be studied for future affirmative recommendations. Moreover, future technological advancements of the greenhouse industry shall consider holistic approaches that would incorporate aspects of managing foliar fungal pathogens besides the mainly focused area of insect-vector exclusion strategy.

Chapter 6: Comparative epidemics of the foliar diseases black leaf mold (*Pseudocercospora fuligena*) and early blight (*Alternaria solani*) on tomato (*Solanum lycopersicum*) cultivated in four different greenhouse setups in the humid tropics

ABSTRACT

Epidemics of the two major foliar fungal diseases under protected cultivation, namely black leaf mold (*P. fuligena*) and early blight (*A. solani*), were compared in four greenhouses with different cooling methods located at the campus of the Asian Institute of Technology, Thailand. The cooling methods were use of fan and pad (FAD), 50-mesh (BioNet^{TM}) and 78-mesh (Econet-T^{TM}) natural ventilation (NV), and NV plus shading a nearly infrared pigment on the roof of a 78-mesh greenhouse (Econir). Severity of both diseases (DS_{TOT}), *i.e.* black leaf mold (DS_{BLM}) and early blight (DS_{EB}), were positively correlated to that of increased cooling and increased relative humidity (*RH*).

Cooling of the FAD greenhouse was accompanied by high *RH* which unabatedly created a fertile ground for epidemics of both diseases. Accordingly, there was 50 to 94% and 54 to 61% more disease observed in the FAD greenhouse during the hot-wet and cool-dry seasons respectively, as compared to the other three. Next to FAD were Econir and BioNet^{TM} greenhouses in which average seasonal temperature was 1 to 2ºC higher and relative humidity (*RH*) 10 to 20% less than in FAD. The lowest disease was observed in Econet-T^{TM} where temperature was highest at noontime. During the cool-dry season, DS_{EB} was dominant, with its 68.9% share of the *AUDPC*, in the FAD greenhouse. An early application (2 to 3 times) of the fungicide mancozeb during season 3 in FAD, Econet-T^{TM} and Econir greenhouses, on the other hand, resulted in reduction of these foliar diseases up to 36%.

Key words: ***Black leaf mold, early blight, epidemics, greenhouse cooling methods, favorability indices***

INTRODUCTION

Averting challenges of agricultural production from the dynamic fluxes of ambient weather and biotic constraints in the humid tropics heavily rely on use of different cladding materials to protect the crops and thereby create a secluded environment for their growth. Such protected systems, while providing intensification opportunities, also present challenges like high temperature (due to high ambient solar radiation and the confinement structures) and high relative humidity. In addition, once pests entered these protected systems, their perpetuation is highly triggered by the monoculture stand practiced in most greenhouses as well as by the prevailing warmth and humid situations. Use of different cooling methods (Ajwang, 2002; Klose and Tantau, 2004; Salokhe *et al.*, 2005; Harmanto, 2006; Harmanto *et al.*, 2006; Baille *et al.*, 2006; Mutwiwa *et al.*, 2006; Mutwiwa, 2007) and exclusion of insect pests using different mesh sizes (screens) or altering the visual behaviour of insects through modification of the spectral radiation (Michelle and Baker, 2000; Ajwang *et al.*, 2002; Mutwiwa *et al.*, 2005; Kumar and Poehling, 2006) are amongst the highly focused strategies to alleviate these problems.

In some instances, however, efforts of protected cultivation systems may worsen the situation and aggravate problems of fungal diseases which are not only favored by moderate warmth and high humidity but also non-excludible from greenhouses owing to their microscopic nature (Javis, 1992; Paulitz and Bélanger, 2001). High risk of fungal infection due to increased relative humidity, for instance, is mentioned as one among other demerits of using a fan and pad cooling system (Arbel *et al.*, 2003; Kittas *et al.*, 2003). Comparison of epidemics under such circumstances is thus vital to develop integrated strategies for a more efficient, economic and sustainable disease management (Kranz, 2003) in greenhouses.

Despite the rigorous efforts of greenhouse technology improvements towards reducing temperature by active cooling methods like ventilation through the use of mechanical fans and/or evaporative cooling or passively using natural ventilation and/or shading (Willits, 2003), the effects on disease epidemics are barely studied. In this research, an attempt is made to bridge this gap by monitoring and analyzing epidemics of two foliar fungal diseases, namely black leaf mold (BLM) and early blight (EB) on the fresh market tropical tomato (*Solanum lycopersicum* L.) cultivar FMTT260 in four greenhouses at central Thailand with different cooling methods.

Black leaf mold disease caused by *Pseudocercospora fuligena* (Roldan) Deighton (syn. *Cercospora fuligena* Roldan) is one of those fungal diseases reported to cause substantial yield loss on tomato in countries like Taiwan (Hartman and Wang, 1992), India (Mohanty and Mohanty, 1955), Japan (Yamada, 1951) and Thailand (Mersha and Hau, unpublished). The disease is highly favored by the macro- and microclimate of greenhouses in the humid tropics which are characterized by high relative humidity, moderate to high temperatures and extended periods of leaf wetness (Hartman *et al.*, 1991; Blazquez, 1991). Initial symptoms of the disease appear as small, pale yellow lesions with no definite margin on either the upper or lower leaf surface. These lesions have white fungal growth that turns gray to black as the fungus sporulates. Later, black sooty fungal growth will occur on both the upper and lower leaf surfaces of tomato.

EB caused by the necrotrophic fungus *Alternaria solani* (Ellis & Martin) Jones & Grout, on the other hand, is a common foliar disease of tomatoes over a wide range of climatic conditions, but is most prominent in areas with heavy dew, rainfall and high relative humidity. Disease development is favored by mild temperature, and the presence of heavy dew leads to a profuse sporulation (Sherf and MacNab, 1986; Blazquez, 1991). On tomato, it causes damping-off, collar rot, leaf spots, stem lesions and fruit rot. The most noticeable symptoms are leaf spots (Sherf and MacNab, 1986). This research focused on the aspect of leaf spot which could result in a complete loss of the crop at times of heavy epidemics as yields are reduced by destruction of foliage and the fruits are damaged directly by the pathogen and by sun blotch on defoliated plants (Rotem, 1994).

MATERIALS AND METHODS

Experimental overview

This experiment was conducted in four greenhouses of the "Protected cultivation project" located in the campus of the Asian Institute of Technology (AIT), 44 km north of Bangkok, Pathumthani province, central Thailand (14º 04′ N, 100º 37′ E, altitude 2.3 m above sea level). Each greenhouse was on an area of 200 m^2 (20 m long by 10 m width, a height of 6.4 m at ridge and 3.8 m at gutter) with the gutters oriented east-west and covered with a roof made of a UV absorbing polyethylene (PE) plastic film (Wepelen™, FVG, Dernbach, Germany). This PE film was 200 μm thick, anti-dust and anti-fog UV-absorbing. All the four greenhouses were equipped with a two-door entrance system with a sunken footbath between the two doors which was filled with a disinfectant to help maintain high phytosanitary standards inside the greenhouses. Whereas the floor of all the greenhouses was covered with the black and white PE mulch film (FVG, Dernbach, Germany) with the white surface facing the topside, fertigation in all of them was through automatically controlled drip irrigation system.

The four cooling methods compared, each representing one greenhouse, were a) use of fan and pad (FAD), b) natural ventilation using 50-mesh sized walls (BioNet™), c) natural ventilation using 78-mesh sized walls (Econet-T™) and d) natural ventilation coupled with coating a nearly infrared reflecting pigment on the roof of the 78-mesh greenhouse (Econir). BioNet™ (Klayman Meteor Ltd, Petah-Tikva, Israel) had an additive to reduce transmission but Econet-T™ (Ab Ludvig Svensson, Kinna, Sweden) was UV transmitting. On the fourth greenhouse, Redunet (Mardenkro BV, Baarle Nasau, The Netherlands), a newly developed shading paint with a near infrared reflecting pigment was sprayed using a high pressure system (Harmanto, 2006; Mutwiwa, 2007). These four greenhouses hereafter would be labeled as FAD, Bnet, Enet and Enir for purposes of clarity. Further details of greenhouse specifications could be referred in Mutwiwa *et al.* (2006) and Mutwiwa (2007). Weather parameters including temperature (*T*), relative humidity (*RH*), solar radiation, and rainfall were recorded every 5 minutes using a central computerized data logging system developed by the Institute of Horticultural and Biosystems Engineering (Leibniz Universität Hannover, Germany) connected to sensors inside and outside each greenhouse.

FMTT260 seeds were sown in peat moss and raised in FAD equipped nursery for about two weeks. A single seedling was then transplanted into 10 L capacity perforated white plastic pot containing a local commercial potting mix substrate (Textural classes: 30% sand, 31% clay,

39% silt, 28% organic matter and a pH of 5.3; Dinwandeekankasat, Ayutthaya, Thailand). In each greenhouse, six rows spaced 1.3 m apart and 300 plants were accommodated forming a plant density of 1.5 m^{-2}. Samples of nine plants, selected from rows with the same management practices of each greenhouse, were marked and assessed for the intensity of the two foliar diseases, *i.e.* black leaf mold (BLM) and early blight (EB).

Assessments in terms of severity (*DS*) and incidence (*DI*), both as proportions, were made up to four times in each greenhouse in three consecutive seasons (Table 1). For *DI*, the total number of leaves (*TLN*) on the plants was counted and symptomatic leaves were marked by wrapping colored threads on the petiole. *DI* of leaves was accordingly calculated considering the proportion of symptomatic leaves to *TLN*. *DS* was visually estimated on a plant basis as a percentage of diseased leaf area compared to total leaf area. *DS* was estimated for BLM (DS_{BLM}) and EB (DS_{EB}) separately and final disease effect was summed as total (DS_{TOT}). Proportion *DI* too was categorized with respect to BLM (DI_{BLM}), EB (DI_{EB}) and both diseases (DI_{TOT}).

Transplanting dates were 8th June 2005, 8th November 2005 and 8th May 2006 for seasons 1, 2 and 3 respectively. Whereas comparisons were made only at harvesting time in season 1 (3rd October 2005), assessments in the other two seasons were done 4 times at each (Table 1). Since the greenhouses were used for multidisciplinary studies, three of the greenhouses (except Bnet) were sprayed with mancozeb during season 3. To control insect pests, blue and yellow sticky traps were hung inside each greenhouse and around the vicinity of the experimental station. Besides, alternating insecticide sprays of Cypermethrin™ (2 ml L^{-1}), Abamectin™ (1.5 ml L^{-1}) or Spinosad™ (1.5 ml L^{-1}) were done on demand.

Table 1. Summary of experimental schedule (dd/mm/yy) and data assessment dates.

Season	Date of disease assessment			
	Assessment 1	Assessment 2	Assessment 3	Assessment 4
1	-	-	03/10/05	17/10/05
2	26/12/05	11/01/06	18/02/06	03/03/06
3	08/06/06	07/07/06	11/08/06	29/08/06

Data collection and analyses

Analysis of variance for both diseases (DS_{TOT} and DI_{TOT}) at time of harvesting was computed using SAS (2003). Besides, disease progress curves in terms of *DS* during seasons 2 and 3 were fitted to the three parametric logistic function (eq. 1) using SigmaPlot (2006). The parameters y_0 and $y_{\max}$ are the respective initial and maximum disease severity (*DS*) while r_y refers to the rate of infection (1/day) of the logistic function.

$$y(t) = y_{\max} / (1 + (y_{\max} / y_0 - 1) \cdot e^{-r_y \cdot t}) \qquad (1)$$

In addition, for the assessments during seasons 2 and 3, areas under disease progress curves for both diseases ($AUDPC_{TOT}$) were calculated following the trapezoidal method (Campbell and Madden, 1990).

Favorability indices based on temperature of the day (06:00 a.m. to 17:59 p.m.) (FI_{TD}) and night (18:00 p.m. to 05:59 a.m.) (FI_{TN}) were calculated over a period of 112 days for BLM ($FI_{TD\text{-}BLM}$ and $FI_{TN\text{-}BLM}$) and EB ($FI_{TD\text{-}EB}$ and $FI_{TN\text{-}EB}$) in each greenhouse. The favorability indices of temperature for BLM during day, $FI_{TD\text{-}BLM}$ (eq. 2) or night $FI_{TN\text{-}BLM}$ (eq. 3) time considered a particular day or night temperature (DT_i or NT_i) as favorable if the temperature (*T*) fell in the range of 26 and 33.4°C. This optimal range was delineated based on earlier reports from Hartman *et al.* (1991) and Wang *et al.* (1996).

$$FI_{TD-BLM} = \sum_{i=1}^{112} (FI_{TDi}) / 112 \qquad (2) \qquad FI_{TN-BLM} = \sum_{i=1}^{112} (FI_{TNi}) / 112 \qquad (3)$$

Where:

FI_{TDi} or FI_{TNi} = 1 if the mean day (TD_i) or night (TN_i) time temperature of day *i* is in the interval 26°C ≤ *T* < 33.5°C

0 if TD_i or TN_i of day *i* is < 26°C or ≥ 33.5°C

For EB too, $FI_{TD\text{-}EB}$ (eq. 4) and $FI_{TN\text{-}EB}$ (eq. 5) were determined based on a lower range of optimal temperature, *i.e.* 22 to 25.9°C. Despite wide ranges of recommendations about optimal temperature for germination and penetration of conidia of *A. solani*, the above mentioned range was chosen by modifying the 22.5°C report of Bashi and Rotem (1975) for production of conidia. Chaerani *et al.* (2007) have also used an optimal temperature range of 21 to 22°C for their *in vitro* experiments of *A. solani*. Bashi and Rotem (1974) have similarly indicated a nearly complete spore germination of *A. solani* at 20°C temperature and a minimum of 2 h wetting period.

$$FI_{TDi} \text{ or } FI_{TNi} = \begin{cases} 1 \text{ if the mean day } (TD_i) \text{ or night } (TN_i) \text{ time temperature of day } i \text{ is in the interval } 22°C \leq T < 25.9°C \\ 0 \text{ if } TD_i \text{ or } TN_i \text{ of day } i \text{ is } < 22°C \text{ or } \geq 25.9°C \end{cases}$$

The same procedure was followed to compute *FI* of relative humidity of the day (FI_{RHD}) and night (FI_{RHN}) as shown in equations 3 and 4.

$$FI_{TD-EB} = \sum_{i=1}^{112} (FI_{TDi})/112 \qquad (4) \qquad FI_{TN-BLM} = \sum_{i=1}^{112} (FI_{TNi})/112 \qquad (5)$$

Similarly, favorability indices based on relative humidity of the day (FI_{RHD}) and night (FI_{RHN}) were calculated over a period of 112 days for BLM ($FI_{TD\text{-}BLM}$ and $FI_{TN\text{-}BLM}$) and EB ($FI_{TD\text{-}EB}$ and $FI_{TN\text{-}EB}$). Since high relative humidity, *i.e.* > 84.5% (Hartman *et al.*, 1991; Wang *et al.*, 1996) was considered as optimal for BLM and as high as 12 h wetness was reported as a necessity for successful (100%) EB infection (Waggoner and Horsfall, 1969), a common minimal requirement of *RH* like that of BLM was considered for both diseases. Waggoner and Horsfall (1969) have accounted *RH* values > 90% for five day durations in their famous EPIDEM model. Accordingly, favorability indices for both diseases were computed using equations 6 and 7.

$$FI_{RHD} = \sum_{i=1}^{112} (FI_{RHDi})/112 \qquad (6) \qquad FI_{RHN} = \sum_{i=1}^{112} (FI_{TNi})/112 \qquad (7)$$

Where:

$$FI_{RHDi} \text{ or } FI_{RHNi} = \begin{cases} 1 \text{ if the mean day } (RHD_i) \text{ or night } (RHN_i) \text{ time relative humidity of day } i \text{ is } > 84.5\% \\ 0 \text{ if } RHD_i \text{ or } RHN_i \text{ of day } i \text{ is } \leq 84.5\% \end{cases}$$

In each greenhouse, favorability index values for temperature (FI_T) and relative humidity (FI_{RH}) were formed by calculating the mean value of the day and night favorability indices, i.e. $FI_T = 0.5 \cdot (FI_{TD} + FI_{TN})$ and $FI_{RH} = 0.5 \cdot (FI_{RHD} + FI_{RHN})$, respectively.

RESULTS

Early blight (EB) was dominantly observed only during season 2 and only in the FAD greenhouse. Therefore, EB was separately analyzed in this season. In other greenhouses, EB appeared only for a short time duration (for about 3 to 6 weeks) during the cool-dry season and was masked by BLM during the last assessments and thus excluded from analysis. All variables with the subscript TOT thus refer to BLM during seasons 1 and 3 but as summation of both diseases during season 2.

There was a distinct and statistically significant difference between the four greenhouses with respect to BLM severity (DS_{TOT}) with values of 0.82, 0.42, 0.14 and 0.05 during the first sampling of season 1 (October 2005) in FAD, Enir, Enet and Bnet (Fig. 1a) greenhouses respectively. Except Bnet, however, DI_{TOT} of the three greenhouses did not show any statistical difference (Fig. 1a).

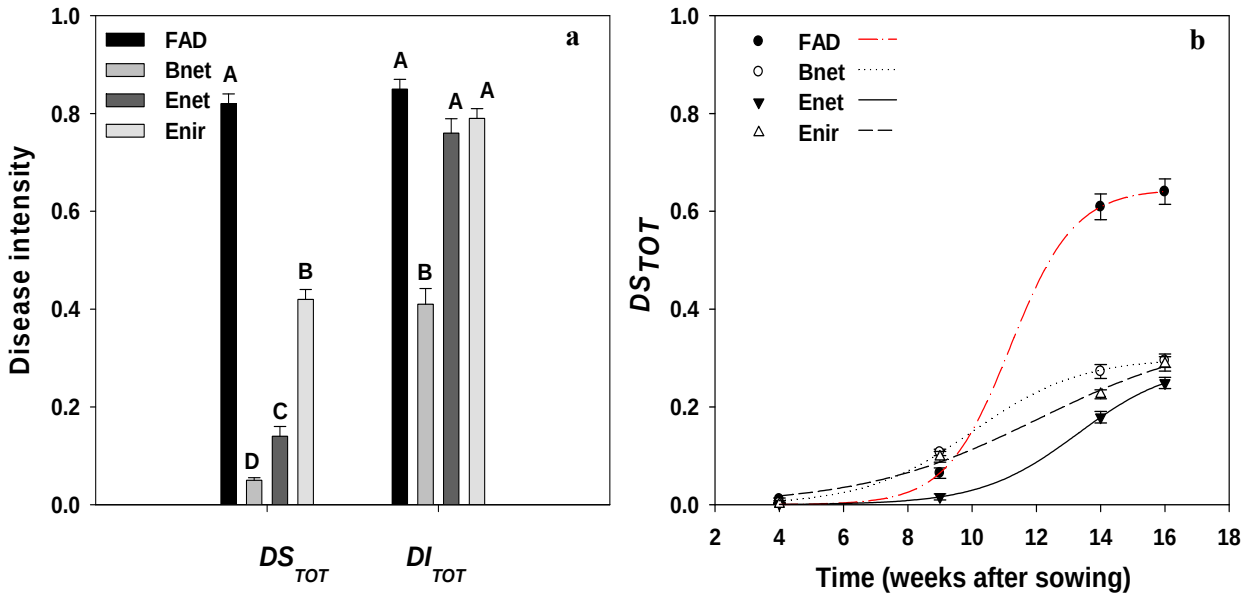

Fig. 1. a) Disease intensities expressed as severity (DS_{TOT}) and incidence (DI_{TOT}) at harvesting in season 1 and b) temporal disease progress of both diseases fitted to a logistic function during season 2 in four greenhouses with different cooling methods: fan and pad (FAD), 50-mesh (Bnet) and 78-mesh (Enet) naturally ventilated greenhouses, and Enet with near infra-red pigment on its roof (Enir). NB: DS_{TOT} and DI_{TOT} in season 1 represent only black leaf mold epidemics.

Temporal progress of disease epidemics during season 2 were fitted to logistic functions with $R^2 > 0.98$ (Fig. 1b). Again the highest epidemic level in this season was recorded in the FAD greenhouse followed by Bnet, Enir and Enet. Disease in Enet was the lowest in most of the assessments except during the first season in which the disease level in the Bnet greenhouse was lower.

Unlike season 1, however, disease epidemics in Bnet and Enir during season 2 showed statistically non-significant difference when *AUDPC* is considered (Table 2). In this cool-dry season, high relative humidity coupled with mild temperature in the FAD greenhouse caused a high prevalence of EB and hence a significant share in disease level, *i.e.* 68.9% of $AUDPC_{TOT}$ (Table 2).

Due to intervention of fungicide sprays in all except the Bnet greenhouse during season 3, however, disease progress was distinctly higher in this greenhouse as was shown by the actual $AUDPC_{TOT}$ (Table 2). Further comparison of epidemics in the four greenhouses was carried out assuming an equal reduction of disease level in all by 35.7% (considering the actual reduction of $AUDPC_{TOT}$ in Bnet from 1.79 to 0.64). Accordingly, $AUDPC_{TOT}$ was extrapolated to be 1.12, 0.57 and 0.35 in FAD, Enir and Enet respectively (Table 2). With this assumption, total disease epidemics in FAD were seen unparalleled as compared to other greenhouses in all the seasons.

Table 2. Summary of $AUDPC_{TOT}$ in seasons 2 and 3 including projections in season 3.

Greenhouse	Season 2			Season 3	
	$AUDPC_{TOT}$	$AUDPC_{BLM}$	$AUDPC_{EB}$	$AUDPC_{TOT}$ (Actual)	$AUDPC_{TOT}$ (Projected[1])
FAD	3.12 ± 0.14a	0.97	2.15	0.25 ± 0.020b	1.12 ± 0.05a
Bnet	1.79 ± 0.08b	1.79	0	0.64 ± 0.050a	0.64 ± 0.05b[2]
Enet	0.96 ± 0.07c	0.96	0	0.05 ± 0.002c	0.35 ± 0.03c
Enir	1.57 ± 0.08b	1.57	0	0.23 ± 0.019b	0.57 ± 0.03b

[1]Projected $AUDPC_{TOT}$ values in FAD, Enet and Enir Bnet greenhouses.

[2] Actual $AUDPC_{TOT}$ value in the Bnet greenhouse.

Comparison of mean hourly *T* and *RH* in the four greenhouses depicted lower *T* and incomparably higher *RH* in FAD as compared to the others (Fig. 2). Day *T* in Enet was highest during noontime whereas day *RH* was substantially higher in FAD as compared to the others (Fig. 2). This explained the significant difference of day time relative humidity (FI_{RHD}) in FAD as compared to others (Fig. 3).

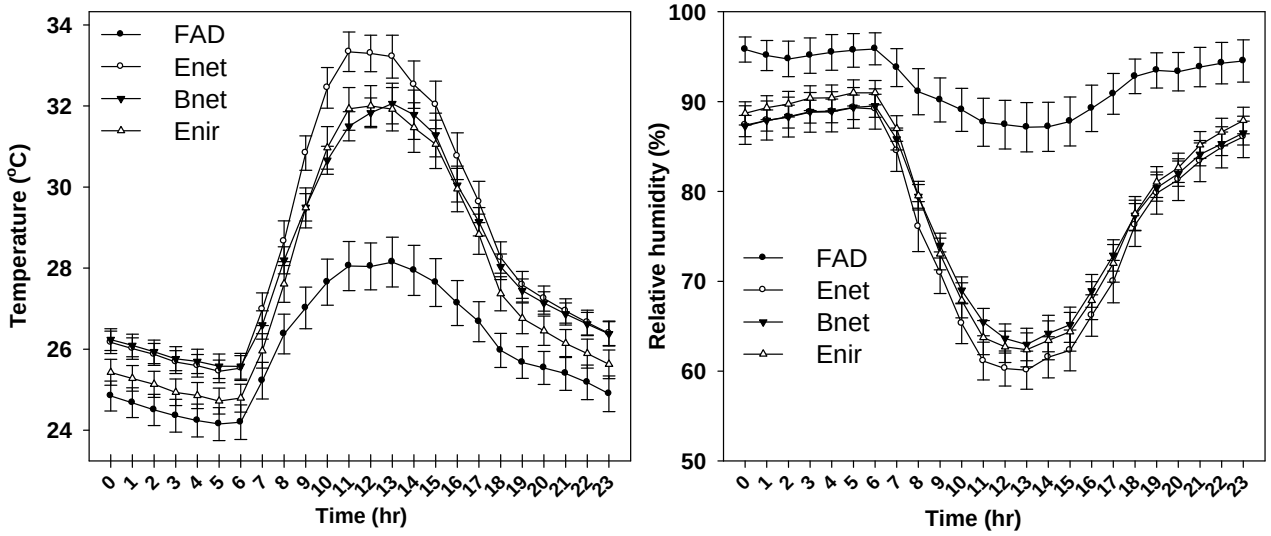

Fig. 2. Mean hourly temperature (left) and relative humidity (right) of the four greenhouses with different cooling methods. NB: mean values were calculated from the 1st, 8th and 15th day of each month from July 2005 to May 2006.

Whereas the significant interaction between season and FI_T from the output of ANOVA using a PROC GLM in SAS (2003) led for a separate analyses of each season, FI_{RH} was not statistically affected by season and thus data were pooled together (Fig. 3). FI_{RH} was unambiguously high during the night time (nearly > 0.6 for all) but the contrast to the day time was drastically different whereby the FAD greenhouse showed incomparably the highest value (Fig. 3). Considering the pattern of epidemics in the four greenhouses and based on earlier results from comparison of seasonal dynamics of BLM, level of epidemics in this study was highly explained by the prevailing relative humidity withina greenhouse.

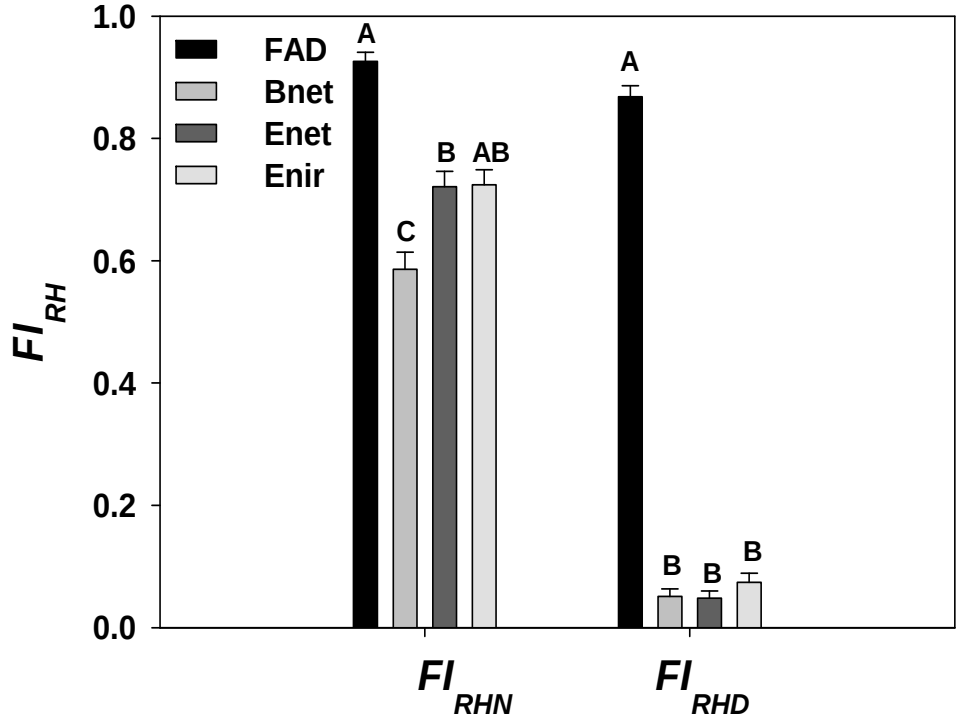

Fig. 3. Mean favorability indices of the night (FI_{NRH}) and day (FI_{DRH}) relative humidity for pooled data of all the seasons in the four greenhouses.

During seasons 1 and 3, $FI_{T\text{-}BLM}$ was much higher than $FI_{T\text{-}EB}$ in all greenhouses. While comparing within greenhouses, however, it was slightly higher in Bnet, Enet and Enir (≥ 0.86) as compared to FAD (Table 3). EB was favored in the FAD greenhouse during season 2 whereby $FI_{T\text{-}EB}$ was the highest recorded as compared to others (Table 3). In all but two cases in season 2 (in Enir and FAD), $FI_{T\text{-}BLM}$ was higher than $FI_{T\text{-}EB}$ (Table 3). This heavy EB epidemics in this season could also be explained by the high $FI_{T\text{-}EB}$ (0.60) in FAD greenhouse compared to $FI_{T\text{-}BLM}$ (0.03) (Table 3).

Coupled with the high FI_{RHN}, BLM epidemics were generally higher in all greenhouses. In FAD and Enir greenhouses in season 2, however, besides the ambient cool temperature of the season, the reduced temperature due to the cooling methods has contributed to the lower $FI_{T\text{-}BLM}$. Since consistently high relative humidity prevailed in FAD, epidemics of EB were dominantly expressed with nearly 80 percent share of the total disease amount at the last assessment. In Enir, however, this upper hand in $FI_{T\text{-}EB}$ could not bring about a major shift in epidemics of the two diseases as FI_{RHD} was so low. As relative humidity correlated higher to disease severity than temperature in this research (refer to chapter 5), this was expected.

Table 3. Favorability indices of temperature for BLM ($\boldsymbol{FI_{T\text{-}BLM}}$) and EB ($\boldsymbol{FI_{T\text{-}EB}}$) in the four greenhouses during the three seasons.

Season	Bnet		Enet		Enir		FAD	
	$\boldsymbol{FI_{T\text{-}BLM}}$	$\boldsymbol{FI_{T\text{-}EB}}$	$\boldsymbol{FI_{T\text{-}BLM}}$	$\boldsymbol{FI_{T\text{-}EB}}$	$\boldsymbol{FI_{T\text{-}BLM}}$	$\boldsymbol{FI_{T\text{-}EB}}$	$\boldsymbol{FI_{T\text{-}BLM}}$	$\boldsymbol{FI_{T\text{-}EB}}$
1	0.89	0.02	0.97	0.01	0.86	0.11	0.77	0.20
2	0.56	0.38	0.55	0.39	0.38	0.49	0.03	0.60
3	0.92	0.05	0.96	0.04	0.88	0.11	0.78	0.22

DISCUSSION

Using screens of different mesh sizes and altering the visual behaviour of insects through modification of the spectral radiation (Michelle and Baker, 2000; Ajwang *et al.*, 2002; Mutwiwa *et al.*, 2005; Kumar and Poehling, 2006; Harmanto *et al.*, 2006; Mutwiwa *et al.*, 2006) to manage insect pests and vectors of viral disease were elaborated in depth. Besides, the effects of different cooling methods on physiological responses of tomato plants (Mutwiwa *et al.*, 2006; J. Max, personal communication) were studied and briefly reported in earlier scientific works. In a sharp contrast, nonetheless, research of similar type on foliar fungal pathogens is barely found in literature. Van Lenteren and Woets (1987) have forwarded an in-depth review of some earlier works regarding biological control and integrated pest management in greenhouses. On tomato, besides insect and other arthropod pests (including those vectors of viral diseases), there were many reviews on viral diseases principally on tomato mosaic virus (TMV) and soil-borne diseases caused by *Fusarium* and *Verticillium*. The main strategy mentioned as a remedy to foliar diseases like leaf mold caused by *Cladosporium fulvum* was the use of resistant varieties.

The distinct and significant variation of DS_{TOT} (representing only BLM severity) across the four greenhouses during season 1 goes along with the observed favorability index of night time relative humidity (FI_{RHN}) than favorability indices of temperature (FI_T). Similar observations were made in other components studies too (Chapters 3 and 5). Interestingly, the day relative humidity is incomparably high in FAD in contrast to the three other greenhouses which showed nearly a non-existent favorability for both diseases during the daytime. In this season, FI_T was nearly 1.0 in all greenhouses but FI_{NRH} was significantly higher in the FAD followed by Enir, Enet and Bnet in descending order. The 2nd epidemic level in Enir could also be expected due to optimal temperature favorability (0.86) and FI_{RH} which is fairly high enough as compared to Enet and Bnet. Though disease level in this greenhouse was consistently high as compared to Enet, the fact that the greenhouse was used for multidisciplinary purposes including screening of varieties for heat stress tolerance may have affected the disease epidemics.

It could obviously be seen that the evaporative cooling strategy in the FAD greenhouse was accompanied with high relative humidity. This observation, which corroborated the recommendations of Arbel *et al.* (2003) and Kittas *et al.* (2003), has consequently resulted in 50 to nearly 100% more disease in the FAD greenhouse during season 1 as compared to the

other greenhouses. Low relative humidity (particularly during the daytime) in Bnet was responsible for the lowest epidemics of both diseases. In Enir, on the other hand, beside the favorable weather parameters, traffic of more people working in the greenhouse during season 1 may apparently have affected the favorability factors like relative humidity and inoculum dispersal thereby contributed to the high level of BLM epidemics.

Results from the second season were undisturbed and gave a clear picture of level of BLM epidemics across the four greenhouses. The epidemic in FAD was still unparalleled after the second assessment time as it started a steep progress about 10 weeks after transplanting. Bnet and Enir produced similar level of BLM epidemics whereas disease level in the Enet greenhouse was at lowest. As compared to the other two seasons, weather parameters were highly favorable for early blight during this season. The FAD greenhouse was, nevertheless, exceptionally conducive for EB as it was seen from comparison of the favorability indices.

Disease severity of both diseases (DS_{TOT}) was inversely correlated to cooling (reduced temperature) but positively to that of increased relative humidity. For instance, slope values of -1.27 ($R^2 = 0.95$, $p = 0.0270$) for FI_T and 0.64 ($R^2 = 0.97$, $p = 0.0168$) for FI_{RH} were obtained from linear regression of weather-disease during season 2. 50 - 94% (hot-wet) and 54 - 61% (cool-dry) more disease was observed in FAD greenhouse than the other three. Temperature in Bnet and Enet greenhouses was 1 to 2 ºC higher than FAD and Enir. Temperature was at its highest during noontime in Enet implicating the need for studies related to physiological stresses. The high solar radiation and its impact on temperature could also hamper the growth of fungal propagules. This high noontime temperature could possibly have played a role for the low epidemics in Enet. This goes in agreement with maximum temperature limit reports for *P. fuligena* ranging between 34 (Hartman *et al.*, 1991) to 37.7°C (Chupp and Sherf, 1960).

Cooling of the fan and pad greenhouse was accompanied by high relative humidity and hence interventions like restricted use of the pad system (in conjunction with the season and daily ambient solar radiation) and minimal protective fungicide applications could be considered as strategies to combat the foliar diseases. The drastic reduction of disease epidemics in the last season due to 2 to 3 applications of the fungicide mancozeb were indicating the need for augmenting disease management strategies at times of high disease risk. Besides, DS_{TOT} in Bnet during seasons 2 and 3 could be assumed to be higher due to inoculum build-up in the greenhouse since it was used for purposes of epidemic studies without interruption. This highlighted that disease epidemics were certainly influenced by cooling methods but also

other external factors like amount of inoculum and phytosanitary measures within each greenhouse.

Management of pest problems, which so far are mainly entertained through a strategy of exclusion, could not solve the problems of fungal pathogens and other microbes. The obvious reason for this is their microscopic nature and hence multitude of options for their entrance into greenhouses (Paulitz and Bélanger, 2001). Consequently, fungal pathogens and others, which are highly favored by confined warmth and humidity, are gaining more and more economic importance in the past years and the use of fungicides remains the sole option of maintaining optimal productivity. This tendency of heavy fungicide reliance for production of vegetables and fruit is given mainly in Asian countries preceded by fungicide sales in Western Europe because of the dominant position of the cereal crop production (Kuck and Gisi, 2007). Hence, results from this study underscore that future greenhouse improvement and integrated crop management strategies shall take into account aspects of fungal disease management in order to cub such heavy reliance on pesticide use.

GENERAL DISCUSSION

Data presented in the past six chapters of this dissertation principally aimed at finding scientific explanations for three categories of research problems that were formulated as part of a multidisciplinary research project on sustainable vegetable production in the humid tropics. The first aspect was investigating the biology and infection mechanisms of the fungal pathogen *Pseudocercospora fuligena* (Roldan) Deighton (syn. *Cercospora fuligena* Roldan) including its monocyclic components while causing black leaf mold (BLM) or syn. *Cercospora* leaf mold disease on tomato. The second target problem area was mainly related to effects of BLM on overall growth parameters of the host and to the spatio-temporal distribution of the disease across the canopy. The final inquisition was to acquire a response to the fundamental question of weather-disease-yield relationship under protected cultivation system and impact of greenhouse cooling systems on BLM epidemics.

Black leaf mold (BLM) disease on tomato (*S. lycopersicum* L.) under protected cultivation in Thailand was confirmed to be caused by *P. fuligena*. Identification proceeded after following Koch's postulate from axenic cultures of the pathogen (in collaboration with fungal biodiversity centre - Centraal Bureau voor Schimmelcultures – CBS), macroscopic observations of signs and symptoms of the disease in greenhouses and through molecular methods. Appearance of indistinct effuse patches on both sides of tomato leaves; amphigenous fructification and prolific fuliginous sporulation predominantly on the abaxial side but also on the adaxial were some of the peculiar features of the pathogen. This was further confirmed by comparison of the observed basic fungal structures with that of earlier reports (Chupp, 1954; Blazquez and Alfieri 1974; Hsieh and Goh, 1990; Hartman *et al.*, 1991; Blazquez, 1991; Halfeld-Vieira *et al.*, 2006). This study experimentally complimented many of the earlier assumptions of stomatal mode of penetration of *P. fuligena* (Sherf and MacNab, 1986; Hartman *et al.*, 1991) and proved the stomatal mode of egress from observations made using light and scanning electron microscopy. Though primary and secondary infection hyphae were observed in this research, there was no cross-sectional study conducted to observe the third type of hyphae, internal hyphae. Despite improvement of sporulation after using combinations of mycelial wounding, incubation of cultures on black light using tomato oatmeal agar and carrot leaf decoction agar, such a profuse sporulation to enable harvesting ample conidia, as reported from Hartman *et al.*, (1991) were not achieved in this research.

Incubation and latent period of BLM were found to be varying according to temperature regimes, leaf wetness duration and leaf ages. Onset of BLM symptoms appeared highly favored by 28°C, with wetness duration of 14 to 16 hours on young leaves. Considering the ambient environments in protected cultivation systems in Thailand, however, the optimal range of temperature for causing epidemics was assumed to extend up to 30°C. The shortest onset of BLM symptomatic units in this research was 8 days after artificial inoculations in the greenhouse at AIT. Interestingly, Wang *et al.* (1996) even reported initial lesion formations as early as only 6 days in controlled conditions at 28°C. During assessments of the natural epidemics, however, BLM symptom appearance were extended from 11 to 25 days. This finding went in agreement with earlier reports from AVRDC.

With reference to effects of BLM on its host, a clear effect of reduced host growth particularly in terms of healthy leaf area (*HLA*) was discernible from a comparison of treatments with (F) and without (NF) fungicide application at times of heavy epidemics as elaborated in chapter 3. The observed standardized disease severity proportion (*DS**) of 0.3 from the undisturbed epidemics of the two plantings with high epidemics resulted in a 68% loss of *HLA* when treatments with (*F*) and without (*NF*) fungicide treatments were compared. Furthermore, a comparison of healthy leaf area index of healthy plants ($HLAI_{HP}$) with that of plants with F ($HLAI_F$) and NF ($HLAI_{NF}$) treatments showed a respective loss of 11% and 50%. Interesting results were obtained with regard to spatio-temporal dynamics of canopy distribution of BLM (Chapter 4). In contrary to attributing high epidemics of BLM on the lower strata to the age of tomato leaves, it was possible to pinpoint the main factors causing this assumption. Instead of leaf age, proximity to substrate evaporation coupled with down-hanging nature of tomato leaves created a confound microclimate which led to higher relative humidity within the range of plant height of about 50-70 cm. Under artificial inoculation, given equal chance of the fungal conidia to land and infect all the plant leaves at a time, however, the lower leaves were not particularly preferred. Considering the age of leaves across plant strata, *P. fuligena* preferred fully expanded younger leaves for incubation as compared to the old leaves. This was further supported by the results of experiments on monocyclic components (Chapter 2). In artificially inoculated 6-week-old plants in a BioNet^{TM} greenhouse, disease was more prevalent in the middle part of the plant canopy as compared to the lower and top portion of the plant. Despite the cumulative trend of concentration of disease symptoms of black leaf mold on the lower plant canopy, spore landing and infection started randomly at about an average of leaf positions 5 to 10. This research, coupled with other components studies in this

dissertation came up with further implications of disease management as it would be discussed at the end of this discussion.

Studies related to weather-disease-yield relationships were carried out after 24 sequential monthly and bi-monthly plantings that were made from June 2005 until January 2007 in a naturally ventilated greenhouse at AIT. Three periods with peak BLM epidemics were traced from these plantings. These were plantings of August to September in both years, namely 2005 (1) and 2006 (2) and that of December to January plantings in 2005 (3). *DS* at the last assessment date on non-fungicide sprayed plantings were 46, 56 and 81% for the 1st, 2nd and 3rd period respectively, the maximum being in August 2005. The respective *DS** for each period was 30, 14 and 20%. Whereas linear regression of favourability index of relative humidity (FI_{RH}) showed high correlation to the disease epidemics ($r^2 = 0.71$), the multiple regression of both improved r^2 value to only 0.74. Stepwise regression of day and night values of both FI_T and FI_{RH} on the other hand showed much relevance of night humidity alone ($r^2 = 0.62$, $p < 0.0001$). Accordingly a yield loss ranging between 30-36, 17-41 and 4-30% was recorded for the three BLM peak periods, respectively. This is in agreement with the report of Hartman *et al.* (1992) which mentioned a yield loss of up to 32% when treatments are compared to controls which were treated with fungicides.

The final chapter (Chapter 6) on comparative epidemics of BLM across four different types of cooling methods illustrated the differences which resulted from the cooling methods. The cooling methods which included use of fan and pad (FAD), natural ventilation (NV) using two mesh sizes (50 and 78, BioNet™ and Econet T™, respectively) and NV plus shading a nearly infrared pigment on the roof of a 78 -mesh greenhouse (Econir) showed variation in terms of severity and incidence of BLM during the three season assessments. There was 50 - 94% (hot-wet) and 54 - 61% (cool-dry) more disease in the FAD greenhouse as compared to the other three. Next to FAD were Econir and BioNet™ greenhouses in which average seasonal temperature (*T*) was 1 to 2ºC higher and relative humidity (*RH*) 10 to 20% less than in FAD. Severity of both diseases (DS_{TOT}), *i.e.* black leaf mold (DS_{BLM}) and early blight (DS_{EB}) was thus inversely correlated to cooling (reduced *T*) but positively to that of effect on *RH*.

In view of concepts of integrated crop management, major achievements of the study and future outlooks for improvement of vegetable (tomato) production in the humid tropics are elaborated as follows.

Macroscopic as well as microscopic characterizations as well as other *in vitro* cultural and molecular methodologies could be used for further studies of *P. fuligena* or similar pathogens.

Of particular interest would be to use these methodologies on extensive surveys to make the geographical distribution of BLM in different provinces of Thailand and neighbouring countries, where the disease has not been reported. Studies on alternate hosts of the pathogen could also proceed in a similar way. Stomatal modes of penetration and egress of *P. fuligena* could shade light on designing appropriate hypotheses for future studies of managing the disease. One potential could be a strategy of strengthening components of induced resistance of the host instead of structural barriers.

Results from the monocyclic studies were informative on the potential of BLM epidemics to impact tomato production under the ambient environment of greenhouses in Thailand. This implicates a need for scrutinized weekly assessment of foliar diseases, especially for the first ten weeks time after transplanting. If the production falls in the hot-wet season, the frequency of assessment shall be more often. As BLM epidemics usually gets severe late in the growing season, any prophylactic measures including phytosanitation and 2 to 3 fungicide sprays (only if heavy epidemics are expected) would drastically reduce the damage by the disease. The timely pruning of the lower canopy after the time of the 1st harvest would then augment the disease management by removing the hot spot of inoculum. The results from the spatio-temporal dynamics of BLM epidemics had the practical implications with this respect.

Studies from the seasonal epidemics have witnessed the same situations. BLM epidemics were correlated to the favorability indices of relative humidity (particularly night relative humidity) of each planting time. The mere fact that FMTT260 transplanted in March, April and May (for instance in 2005) ended with a high physiological stress and thus without yield indicated that more efforts in producing heat tolerant varieties are in demand. From the point of view of BLM management, very high temperatures are detrimental for the germination and growth of its conidia and thus epidemics are expected to be lower in very hot situations. Cooling methods as an option to reduce the heat stress could be of great help as far as the effect of reduced temperature is not overwhelmed by the accompanying relative humidity.

Outputs from this study and from that of AVRDC unambiguously traced seasons of high and low BLM epidemics and more importantly its aggressiveness in a short period of time provided optimum favorability factors. While implementing integrated approaches by using resistant cultivars along with phytosanitation, tomato producers shall be vigilant of updated weather forecasts as well as disease progresses. Successful production of vegetables under protected systems in Thailand needs a concerted effort of solving the multifaceted problems by integrating the already available knowledge from the different disciplines. For a given

cultivar of vegetable, models integrating such aspects as physiological and nutritional requirements, optimal greenhouse cooling methods, thresholds of insect pests and epidemics of diseases would play a bigger role towards sustainable solutions.

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Declaration

I, the undersigned, declare that this dissertation, to the best of my knowledge does not incorporate without acknowledgement any material previously published or written by another person except where reference is made. It is an original piece of work conducted by myself and has never been submitted elsewhere.

Hannover, May 2008

Zelalem Mersha