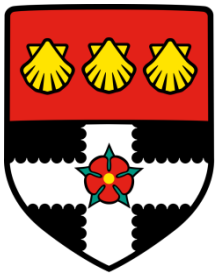

The University of Reading

‘Symptomless’ Infection by *Botrytis cinerea*

Thesis Submitted in Part Fulfilment of the

Degree of Doctor of Philosophy

School of Agriculture, Policy and Development

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Declaration

I confirm that this is my own work and the use of all material from other sources has been properly and fully acknowledged.

Emmanuel Christy Jeyaseelan

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Glossary

Conidium: An asexual spore produced by fungus. These microscopic propagules act as a source of inoculum for the infection. Some fungi, including *Botrytis cinerea*, produce two different sizes of conidia; macro-conidia and micro-conidia (Dewey & Grant-Downton, 2015).

Sclerotium (plural: sclerotia): A compact mass of hyphae, usually with a darkened rind, and capable of surviving under unfavourable environmental conditions (Holz *et al.*, 2007).

Micro-sclerotium: A microscopic knot of thick-walled vegetative hyphal cells produced as a resting structure by some fungi.

Infection: The invasion and multiplication of microorganisms that are not normally present in or on the plant body (Agrios, 2005).

Symptoms: The visible changes that occur in the host plant in response to infection by pathogens. *Botrytis cinerea* produces characteristic macroscopic necrotic lesions on host tissues (Agrios, 2005).

Necrotic infection: An infection that is associated with macroscopic necrosis: a form of cell injury which results in the premature death of cells in living tissue. The affected plant tissue usually turns brown to black in colour (Agrios, 2005).

Latent infection: A phase of infection in which the pathogen grows unapparent (not visible with naked eye). In order to complete the lifecycle the pathogen will express necrotic infection (Jarvis, 1994).

Symptomless infection: An infection is considered symptomless if a plant is a carrier for a pathogen but it fails to show the noticeable macroscopic symptoms with which it is usually

associated. In *B. cinerea* symptomless infection, the fungus grows with the plant and spreads to new developing plant parts.

Quiescent infection: An infection in which pathogen remains alive but not growing in the host tissue and remains localized until the host defences decline (Elad *et al.*, 2007).

Systemic infection: The fungus spread internally throughout the plant body in an infection (Agrios, 2005).

Endophyte: Micro-organism that lives within a plant for at least part of its life cycle without causing apparent disease (Agrios, 2005).

Epiphyte: Micro-organism that lives on the outer surface of plant for at least part of its life cycle without causing apparent disease (Agrios, 2005).

Stratification: The process of treating stored or collected seed prior to sowing to simulate natural winter conditions that a seed must endure before germination (Baskin & Baskin, 2004).

Abstract

The study was carried out to clarify the nature of symptomless infection by *Botrytis cinerea* and to what extent it differs from aggressive necrotic infection in *Lactuca sativa* (lettuce) and *Arabidopsis thaliana*. Symptomless plants were produced by dry spore inoculation in plants growing in controlled environmental conditions or in glasshouses. Plating out of surface-disinfected and non-surface-disinfected samples of inoculated, apparently healthy, plants on selective medium revealed that the fungus was spreading from the initial inoculation site to newly developing plant organs both internally and externally. Similar findings were obtained in microscope experiments in which host plants were inoculated with GFP labelled *B. cinerea* and symptomless spreading was monitored under confocal laser scanning microscope. Spore germination on leaf surface was followed by development of sub-cuticular vesicles and plant cell damage in the infected epidermal cell and a few nearby cells. Sparsely branched long hyphae arose from the vesicles and spread on the leaf surface; spread was mostly on the outer surface of the epidermal layer but occasionally below the cuticle or epidermal cells. In the late symptomless phase, mycelium arising from single vesicles formed several mycelial networks on leaves. Experiments were carried out to compare the extent of gene expression in symptomless and necrotic infections, using RT-qPCR. Expression of selected genes was quantified in tissue samples based on the amount of mRNA of the respective genes found. In both host species, the mRNA concentration of signalling genes *bcg1*, *bmp1* and calcineurin, and the pathogenicity genes *bcsod1* and *bcpg1* were similar to or slightly greater in symptomless samples than in necrotic samples. The mRNA of the signalling gene *ba*c and pathogenicity genes *bcbot1* and *bcnep1*, were not detected or detected in lower abundance than in necrosis. In lettuce, the leaves developing distant from the site of inoculation showed similar results to *A. thaliana*, but in healthy leaves close to the site of inoculation mRNA concentrations of *bac* and *bcnep1* were similar to necrotic samples. Thus, in both host species, the fungus grew along with the plant and moved to newly growing plant parts without

producing symptoms; during this growth some pathogenicity genes were less expressed than in necrotic infection.

Chapter 1 General Introduction

1.1 Plant–fungus interactions

In natural environments plants are continuously exposed to various kinds of microbial pathogens, such as fungi, bacteria, viruses and nematodes. These pathogens can exist in or on a plant body. Fungi are the predominant group of microorganism which causes disease on land plants; among the identified fungi about 10% of them are phyto-pathogens (Agrios, 2005).

Plant pathogenic fungi can be classified into several groups based on their mode of interaction with their respective host plant. Some fungal pathogens are grouped as obligate biotrophs; these fungi are intimately associated with host plants in their whole life cycle and strictly rely on living host tissues to complete their life cycle. Therefore, biotrophs have developed mechanisms to avoid host cell damage so that, they do not elicit host cell death and defence responses (Divon & Fluhr, 2006). Biotrophs exist at different locations and produce special structures such as haustoria in host plant tissues. Mendgen & Hahn (2002) reviewed biotrophic relations and pointed out the diversity present: *Venturia inaequalis* exists in the subcuticular region; *Claviceps purpurea*, *Ustilago maydis* and monokaryotic rust fungi are present in both inter- and intracellular regions; powdery mildews grow in extracellular spaces but produce haustoria within epidermal cells; dikaryotic rust fungi and downy mildews in intercellular spaces with haustoria within parenchyma cells (Mendgen & Hahn, 2002).

Necrotrophs, another group of plant pathogenic fungi, obtain their nutrition by triggering plant cell death by producing toxins and enzymes and then getting nutrients from dead tissues through saprotrophic growth. The fungi *Botrytis cinerea*, *Cochliobolus heterostrophus* and *Fusarium graminearum* are some common economically important necrotrophs. Certain fungal species, such as *Magnaporthe grisea*, *Phytophthora infestans* and some *Colletotrichum*

spp., lie between the above two extreme categories and are called hemi-biotrophs (Mendgen & Hahn, 2002). This group of fungi expresses a short biotrophic–like phase at the beginning of infection process and later switches to a necrotrophic lifestyle.

In order to complete their life cycle each group of fungi above have specialized adaptations and communicate with host plant through signalling molecules and physical contact (Tudzynski & Gronover, 2007). Fungus infection processes and fungal interactions with host plants are controlled by specific genes. Gene activation or inactivation alters the biochemical signalling pathways that control host behaviour so as to lead to the successful development of fungal disease (Tudzynski & Gronover, 2007).

In general, the life cycle of a fungus on a host surface starts with spore germination and germ tube development. In order to satisfy their energy needs, a developing fungus has to enter into the host tissues; this is achieved by either passive entry through natural openings (e.g. *Cladosporium fulvum*) or via wounds present on tissue surface or by active penetration of cuticle (e.g. *Ustilago maydis*) and spread inside the tissues using special structures (Ökmen & Doehlemann, 2014). Fungi such as *Magnaporthe grisea* and *Colletotrichum lindemuthianum* produce a specialized penetration structure called an appressorium; the melanised appressorium provides higher pressure to the infection peg to penetrate the cuticle (Deising *et al.*, 2000). The penetration process is also coupled with production of cutinases and cell wall degrading enzymes.

Production of cutinase enzymes have been detected in several pathosystems. In *Colletotrichum lindemuthianum*, expressing an endopolygalacturonase encoding gene, clpg2 was detected during conidial germination, in appressoria and in penetrating hyphae. The expression of genes coding for pectate lyase, pelA and pelD, are important for the penetration of plant tissue by the fungus *Fusarium solani* (Kahmann & Basse, 2001).

Following the penetration step there is a great difference in infection process between biotrophs and necrotrophs; biotrophs use several tactics to mute host responses and to maintain living host cells, but necrotrophs actively destroy the plant cells and protect themselves from host defence.

The gene-for-gene concept hypothesises that the plant immunity against pathogens is controlled by resistance (*R*) genes, which interact with avirulence (*Avr*) genes of pathogens. The R genes monitor the state of host components targeted by Avr molecules produced by the pathogen to establish disease, and these Avr molecules are also called 'effectors'(Dodds & Rathjen, 2010). The effector molecules act in apoplast or cytoplasm, where they function to modulate host physiology, suppress host defences or protect the pathogen from host defence responses, and through this they facilitate fungal growth (Jonge *et al.*, 2011). The *in planta* signals that trigger expression of effector genes presently remain largely unknown, but recent studies identified some transcriptional regulators involved in early infection process in some associations. In the fungus *Fusarium oxysporum*, the transcriptional regulator Sge1 is required for the expression of various effector molecules (Michielse *et al.*, 2009). In *Magnaporthe oryzae* the zinc finger transcription factor MoCRZ1 regulates various virulence factors (Kim *et al.*, 2010).

Jonge *et al.* (2011) have reviewed several kinds of effector molecules produced by fungi; cell wall degrading enzymes (CWDEs), necrosis and ethylene inducing protein (NEP 1) like proteins (NLPs) and cysteine rich secreted proteins. The fungi which grow in extracellular spaces secrete effectors mainly at hyphal tips, but fungi which can produce intracellular cellular structures, for example rust and mildew fungi, release their effectors through haustoria. Many of the effector molecules which are released in apoplast subsequently translocate into the host cytoplasm (Jonge *et al.*, 2011).

Biotrophic fungi have well established machinery to avoid or suppress host plant defence mechanisms. As the infection strategy of biotrophic fungi is different from necrotrophs, the expression of virulence genes greatly differs between biotrophic growth and necrotrophic growth; biotrophs produce more specialised effector proteins.

Microbe associated molecular patterns (MAMPs) is a general term denoting the signals for an ancient conserved system in plants to detect invading microbial pathogens. The immunity which develops from MAMPs restricts microbial growth and induces host cell death (Thomma *et al.*, 2011). Therefore, to develop a biotrophic infection the fungus has to avoid this immunity. The cell wall chitin of fungus acts as a MAMP in response to which plant triggers an immune response by producing chitinases. To avoid this plant response the biotrophic fungus *Cladosporium fulvum* produces two kinds of effector proteins: the effector Avr4 binds with fungal cell wall and protects from chitinase activity; Ecp6 binds to fragmented chitins and prevents further plant response (van den Burg *et al.*, 2006). Avr4 homologues have also been detected in *Mycosphaerella fijiensis* and *Dothistromo septosporum* (de Wit *et al.*, 2012). Proteins which function similarly to Ecp6 have also been detected in *Zymoseptoria tritici* as Mg3LysM protein and in *Magnoporthe oryzae* as Slp1 protein (Marshall *et al.*, 2011). The fungus *Fusarium verticillioides* produces metalloproteases to cleave chitinases (Naumann *et al.*, 2011).

Biotrophic fungi also have mechanisms to detoxify the antimicrobial secondary metabolites produced by host plants. For example, saponins produced by host plants have strong antifungal activity by causing pore formation in cell membranes (Okmen *et al.*, 2013). The fungus *Gaeumannomyces graminis*, causing take-all disease in oats, produces avenacinase enzyme to detoxify the saponin avenacin (Bowyer *et al.*, 1995). *Cladosporium fulvum* and *Septoria lycopersici* produces tomatinase against the saponin (Osbourn, 1996).

Host plants also produce reactive oxygen species (ROS) immediately after recognition of MAMPs. ROS react in two ways on pathogens; they directly act as antimicrobials and also they induce cellular signals that lead to accumulation of phytoalexin and hypersensitive response (Lamb & Dixon, 1997). *U. maydis* produces an effector molecule Pep1, which binds and inhibits peroxidases (Hemetsberger *et al.*, 2012).

Some plant proteases are involved in disease resistance; in an infected tissue proteases such as papain-like cysteine proteases (PLCPs), serine and aspartic proteases are detected in high amounts. Plant proteases inhibit pathogenic infection in two ways: by directly degrading pathogenic effectors and also by acting as plant defence inducers. The biotrophic fungus *C. fulvum* escapes from PLCPs by producing PLCP inhibitors (Shabab *et al.*, 2008).

Plant defences are also controlled by ubiquitination and the 26S proteasome system; they are responsible for oxidative burst, hormone signalling, induction of defence genes and programmed cell death. Biotrophic pathogens have effector molecules to target these sites. Avr3a effector of *P. infestans* binds to potato U-Box E3 ubiquitin ligase CMPG, and stabilizes it to suppress Inf1 (an extra cellular protein)-mediated cell death. The fungus *M. oryzae* suppresses chitin induced pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) by inhibiting the RIG E3 ubiquitin ligase Apip6 of rice (Bos *et al.*, 2010; Park *et al.*, 2012).

Biotrophic fungi also carry out metabolic reprogramming in host plants. Salicylic acid (SA) plays a key role against biotrophic pathogens; as a signalling hormone, SA is known to be involved in the induction of pathogen resistance (PR) genes as well as in the establishment of long-term immunity by way of systemic acquired resistance (SAR) pathways (Loake & Grant, 2007) . Therefore, there are some mechanisms used by fungi to prevent SA accumulation. *U. maydis* secretes a chorismate mutase (Cmu1), which re-channels the host chorismate metabolism to prevent SA synthesis (Hemetsberger *et al.*, 2012). The fungus

Hyaloperonospora arabidopsis produces a nuclear localized effector HaRxL44, which promotes shift from SA responsive defence to jasmonic acid or ethylene responsive defence mechanisms by degrading a protein called MED19. MED19 mediates the interaction between transcriptional regulators and RNA polymerase II (Shabab *et al.*, 2008).

In *Colletotrichum gloeosporioides*, a nitrogen starvation induced gene (*CgDN3*) is expressed at the early stage of infection. A fungal isolate having mutation of this gene was able to germinate and produce appressoria on the leaf, but failed to further infect, and also elicited a localized hypersensitive–like response in the host. These observations suggest that the product of the *CgDN3* gene mediates the suppression of hypersensitive-like responses in compatible hosts (Stephenson *et al.*, 2000).

On the other hand necrotrophic fungi secrete a range of toxins and enzymes to kill the plant tissues and degrade plant tissues. In *Colletotrichum nicotiana*, terpenoid compounds called colletotrichins function as non-host-specific phytotoxins. External sprays of this toxin produce symptoms similar to tobacco anthracnose. The toxin reacts in two ways: it causes rapid loss of membrane integrity and inhibits the electron flow in the mitochondrial respiration chain (Munch *et al.*, 2008). Necrosis and ethylene inducing protein (NEP 1) like proteins (NLPs), present in many pathogenic microorganisms, generally induce cell death in dicotyledonous plants through membrane permeabilization. The gene *MgNLP* of *Mycosphaerella graminicola* induce cell death in *Arabidopsis thaliana* leaves –a non-host - but did not induce cell death or elicit immune responses in leaves of the host plant wheat (Motteram *et al.*, 2009). Cell wall degrading enzymes produced by fungal pathogens are relatively well characterized apoplastic effectors. However, these enzymes are also produced by saprophytic fungi and do not determine host range or host specificity. In several fungi the sucrose non-fermenting 1 protein (SNF1) regulates CWDE expression (Jonge *et al.*, 2011). Depolymerases such as endo-polygalacturonases, pectin lyases and proteases are secreted to destroy plant tissues. The resulting sugars are an excellent nutrient supply to the pathogens.

1.2 Fungal endophytes

A group of fungi that can infect and remain alive within healthy plants without producing any visible host symptoms in part of their life cycle or their whole life cycle is called endophytic fungi (Zabalgogeazcoa, 2008). The diversity of endophytes inhabiting a single plant varies with the age of the plant. Within a plant, mature plant parts have more endophytes than younger plant parts (Arnold *et al.*, 2003). Most endophytes cause localized infection; they are restricted to plant parts such as root, stem, or leaf. But some endophytes spread systemically throughout the plant body: endophytes such as *Neotyphodium* and *Epichloë* species systemically infect the intercellular spaces of leaves, reproductive stems, and seeds of their hosts (Stone *et al.*, 2004). Geo-climatic conditions also determine the diversity and quantity of endophytes in host plant samples. The climatic conditions in the tropics favours endophytes; enhanced temperatures and humidity values together with longer annual vegetation periods are conditions which facilitate the infection of plants (Garcia-Guzman & Heil, 2014). The host ranges of endophyte vary with fungus species; endophytes like *Neotyphodium* have a narrow host range, other endophytes such as *Alternaria*, *Penicillium* or *Piriformosfora* have a wide host range (Stone *et al.*, 2004).

The interaction between the fungal endophytes and host plants varies with the type of fungus. Several endophytes are beneficial to host plants, in many ways. Endophytes such as *Neotyphodium* and *Epichloë* protect the host plants from herbivores and drought, and also provide nutrients to their host plants (Schardl *et al.*, 2004). *Piriformospora indica*, *Acremonium strictum* and some *Stagonospora* species enhance the growth of their hosts (Zabalgogeazcoa, 2008). On the other hand, some endophytes are latent pathogens or latent – non-growing - saprophytes. Some plant pathogens cause latent infection and remain as endophytes for certain period in their lifecycle until conditions become favourable for their pathogenic mode of life (Photita *et al.*, 2004). Likewise, some saprophytes remain as

endophytes in plant tissues until the tissues senesce and die, and then act as saprophytes (Promputtha *et al.*, 2007).

Based on phylogeny and life history traits, the endophytes can be classified into two major groups, clavicipitaceous and non-claviciptaceous endophytes. These two groups are further sub-divided according to their host range, mode of transmission, rate of colonization and tissues on which they colonize (Rodriguez, 2009).

Clavicipitaceous endophytes, also named class I endophytes, mostly infect grasses and they have limited phylogenetic diversity compared to non-clavicipitaceous endophytes. Clay & Schardl (2002) reported that the host-pathogen relationship of clavicipitaceous endophytes ranges from pathogenic species with symptomatic infection through mixed interactions to asymptomatic infections; they are named as Type I, Type II, and Type III endophytes respectively. These three categories also differ in the mode of their transmission; type I endophytes are mostly transmitted horizontally, type III endophytes mostly show vertical transmission by infecting seeds of a host plant; both vertical and horizontal transmission can be seen in type II endophytes (Rodriguez, 2009).

One of the well-known example for the clavicipitaceous endophytes is *Epichloë* sp. (anamorphs: *Neotyphodium*), an endophytic symbiont of cool-season grasses. The mycelium occurs in the intercellular spaces of leaf sheaths, culms, and rhizomes. Some species of *Epichloë* have lost the capacity for development of the sexual stage; these are classified under type III endophytes as species of *Neotyphodium* (Rodriguez, 2009).

In grasses the two genera, *Epichloë*, with anamorphs in *Neotyphodium*, and *Balansia*, with *Ephelis* anamorphs show systemic endophytic growth. The fungus infects the host plant at a site and then spread throughout the plant body. The same kind of infection is expressed by *Acremonium* sp. in rice plant (Jeffrey *et al.*, 2000).

Non-clavicipitaceous endophytes are a highly phylogenetically diverse group of endophytes having broad range of host plants, ranging from non-vascular plants to conifers and angiosperms (Arnold & Lutzoni, 2007). These fungi can be classified into three classes based on several characters such as host colonization pattern and mechanism of transmission between host generations. Class II endophytes may grow both above and below ground tissues, and some are capable of extensive tissue colonization. This group of fungi may be vertically transmitted through seed coat or via rhizomes. Members of the group spread through the plant body mainly through intercellular spaces, with little to no impact on host cells. In healthy plants, this group of fungi may produce low level of sporulation, but in senescing host plant the fungus emerges and sporulate rapidly (Rodriguez, 2009).

Class III endophytes occur in a plant mainly in the above ground tissues. They are transmitted to other plants by horizontal transmission, and in the plant body they produce localized but invisible infections. Some members of ascomycota and basidiomycota are showing characters of this class. Class IV members are mainly restricted to roots of the host plant. These members having characteristic melanised septa, and they spread in plant tissues through inter and intracellular hyphae (Rodriguez, 2009).

Endophytes can be detected through several experimental methods. Plating out strongly disinfected tissues on selective medium is commonly used and the easiest way to disclose endophytic growth (Bacon & White, 2000). However, the type of disinfectants used, their concentration and the duration for which the tissues are exposed to the chemical are important factors influencing the results. In addition to that, this method has some limitations: some obligate biotrophs cannot grow on nutrient medium, and it is not possible to determine the actual quantity of endophytic fungus in the tissue samples. Microscopic visualization of infected plant tissues can provide more certain evidence for endophytic growth. There are several staining techniques available to visualize systemic pathogens; confocal microscopic observation with fungi with fluorescently labelled proteins will give the least ambiguous

results (Sesma & Osbourn, 2004). Nowadays polymerase chain (PCR) based methods are widely used to detect and identify endophytes in plant tissues. Fungus DNA is amplified with specific primers from the mixture of plant and fungus DNA (Arnold & Lutzoni, 2007). The real time PCR technique can be used to quantify the amount of the endophyte in the tissue samples but, besides the higher cost for the PCR experiments, it is difficult to distinguish living and dead fungus. Recently, next generation sequencing techniques provided more insight into the endophytic fungal diversity and ecology (U'Ren *et al.*, 2014), in terms of resolution and magnitude (Lindahl *et al.*, 2013).

Mycorrhizae are a special kind of endophyte involving association of fungus and feeder roots of flowering plants; both plants and fungi get benefit from this symbiotic association in several ways. Based on the arrangement of fungal hyphae in the cortex tissues of the root there are two kinds of mycorrhiza: ecto-mycorrhiza and endo-mycorrhiza. Ecto-mycorrhizal fungus produces a tightly interwoven fungus mantle around the outside of the root. It also grows inside the root, around the cortical cells. An endo-mycorrhizal fungus produces hyphae inside the cortical cells either by forming special feeding hyphae called "arbuscules", or by forming food storage hyphal swellings called vesicles. They also grow on the outside of the feeder roots as loosely arranged hyphae (Agrios, 2005).

Beneficial microbes such as rhizobial and mycorrhizal symbioses are initially recognized by the host as potential invaders. But later, mutualists are able to short circuit plant defence responses to enable successful colonization (Zamioudis & Pieterse, 2012). Endogenous plant derived molecules that arise from enzymatic degradation of cell wall are called damage associated molecular patterns. Pathogenic fungi produce several cell wall degrading enzymes during their infection. The genome of the ectomycorrhizal fungus *Laccaria bicolor* lacks several gene families that encode for enzymes involved in the degradation of plant cell wall; However, the fungus prevents plant defence activation (Zamioudis & Pieterse, 2012).

Several cysteine-rich mycorrhiza-induced small secreted proteins (MiSSP) show significant similarity to cysteine-rich apoplastic effectors of pathogenic fungi(Martin *et al.*, 2008). The genome of *Laccaria bicolor* encodes a number of small secreted proteins (SSP), many of which are induced during the symbiotic interaction. Effector MiSSP7 of this fungus promotes auxin related gene expression in host plant (Plett *et al.*, 2011). Up-regulation of auxin signalling may make plant defences weaker, as demonstrated for pathogenic interactions (Kazan & Manners, 2009). Likewise, the SP7 effector molecule of the arbuscular mycorrhizal fungi *Glomus intraradices* possesses host defence-suppressive activity (Kloppholz *et al.*, 2011). SP7 expression is induced upon contact with the host root and the secreted protein is translocated into the nucleus, where it interacts with the defence related ethylene responsive factor ERF19 to block the ERF19- mediated transcriptional program (Kloppholz *et al.*, 2011). In plants, ethylene signalling regulates plant immunity by regulating receptors that are required for the oxidative burst (Mersmann *et al.*, 2010).

1.3 The fungal pathogen *Botrytis cinerea*

Botrytis cinerea (teleomorph *Botryotinia fuckeliana*) is a plant pathogenic fungus, causing grey mould disease in more than 200 crops ranging from ornamentals to vegetables and field crops. It also causes postharvest losses in stored and transported agricultural products, especially fruit and flowers (van Kan, 2005). The life cycle of *B. cinerea* is provided in Figure 1-1. Asexual spores of *Botrytis*, conidia germinate rapidly on host tissue. Infection, mycelium growth and conidiation on susceptible host tissues under a wide range of microclimate conditions create severe disease management problems all around the world. *Botrytis* species are present in places wherever their hosts are grown. Therefore, their geographical distribution ranges from tropical and sub-tropical to cold areas (Elad *et al.*, 2007). Because of the world wide importance of this fungus and the availability of molecular genetics tools, the fungus has been extensively studied; the journal *Molecular Plant Pathology* has recognized

this fungus as the second most important plant pathogenic fungus in the world (Dean *et al.*, 2012).

The fungus can attack different plant organs, including flowers, fruits, leaves, shoots and underground plant parts. The most severely affected plant groups are field crops, vegetables such as cabbage, lettuce, broccoli and beans, and small fruit crops such as grape, strawberry (*Fragaria* × *ananassa*), raspberry (*Rubus idaeus*) and blackberry (*Rubus fruticosus*). The fungus can survive and grow at just above the freezing point, therefore, the fungus also causes postharvest loss during storage in fruits and cut flowers (Williamson *et al.*, 2007).

B. cinerea is a necrotrophic pathogen. After the death of host tissue the fungus behaves as a saprophyte on the necrotic tissues, and sporulates or produces long term survival structures. The fungus exists in different habitats and hosts as mycelia, micro-conidia, macro-conidia, chlamydospores, sclerotia, apothecia and ascospores (Holz *et al.*, 2007).

Sclerotia are the most important survival structures produced by *B. cinerea* to protect itself from adverse environmental conditions. The size and shape of the sclerotia differ depending on the type of isolates and growing conditions. Sclerotia are capable of producing successive crops of conidia or may produce apothecia after a sexual process (Holz *et al.*, 2007). Chlamydospores are hyaline cells of extremely variable form and size, they can develop from terminal or intercalary cells. Chlamydospores can serve as short term survival structures which may help the fungus to overcome short unfavourable periods. Depending on the available nutrients, chlamydospores develop in different ways; under moist conditions without added nutrients chlamydospores germinate on the leaf by micro-conidia, but in the presence of fresh nutrient supply germinate with hyphae or produce a new crop of macro-conidia (Holz *et al.*, 2007). Macro-conidia are short lived propagules produced by *B. cinerea*; their survival depends on avoiding temperature extremes, dessication, microbial activity and sunlight exposure (Holz *et al.*, 2007). Micro-conidia are generally present in old cultures or

cultures contaminated by other organisms, and on sclerotia. Micro-conidia develop from germ tubes of macro-conidia, more mature hyphae, inside empty hyphal cells, appressorium and sclerotia (Jarvis, 1980).

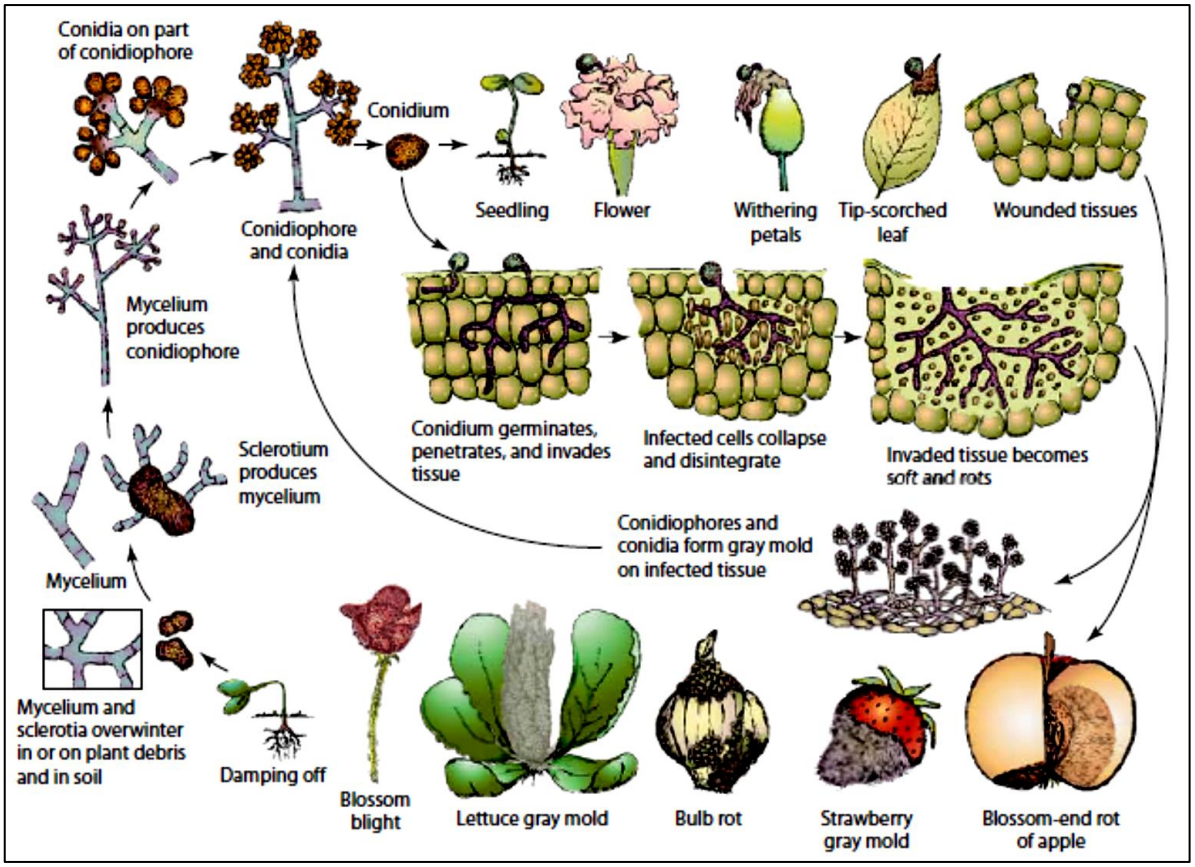


Figure 1-1: Life cycle of *Botrytis cinerea* (Agrios, 2005)

1.3.1 Symptoms produced by *B. cinerea*

Symptoms produced by *B. cinerea* range from restricted lesions to dry or spreading soft rots which often produce conspicuous sporulating colonies. The soft rot symptom develops accompanied by collapse and water soaking of parenchyma tissues, followed usually by rapid appearance of grey masses of conidia on the lesion (Williamson *et al.*, 2007). *B. cinerea* causes blossom end rot in many fruits and vegetables, where the infection commonly begins on attached senescent flowers and then as soft rot spreads to affect the adjacent developing fruits. Depending on environmental conditions symptom development varies on flower petals;

from rapidly appearing minute marks indicating host reactions to full scale soft rotting (Williamson *et al.*, 2007).

1.3.2 Epidemiology of *B. cinerea*

Sclerotia develop in dead host tissues and on the surface of soil and are important survival mechanisms in winter, also serving as a primary source of inoculum in early spring. Mycelium also survives within debris of dead host tissues and inside some seeds (Elmer & Michailides, 2007; Williamson *et al.*, 2007). *B. cinere*a causes seed borne infection in more than fifty host plant species, including flax, sunflower and lettuce (Elmer & Michailides, 2007). As *B. cinerea* has a wide host range, several alternative hosts exist in the field. Herbicide treated or senescing weeds can also act as source of inoculum in field crops (Elmer & Michailides, 2007). Hallett *et al.* (1990) found that groundsel (*Senecio vulgaris*) which was already infected by rust (*Puccinia lagenophorae*) was more vulnerable to *B. cinerea* infection than healthy or wounded plants; mortality was more rapid if plants were inoculated with *B. cinerea* as rust colonies first erupted through the host's epidermis.

Air-borne conidia are the major source of inoculum of *B. cinerea*. Conidia are typically present in highest concentration in the atmosphere during day time, especially shortly after mid-day (Fitt *et al.*, 1985). Rain also has been associated with large concentration of air borne conidia. Rain drops hitting the leaves dislodge dry conidia from infected leaves (Fitt *et al.*, 1985). In general, conidia are very hydrophobic. Laboratory based experiments have shown that raindrops can carry conidia within or on the outside of splash droplets (Hislop, 1969). However, very few of the *B. cinerea* conidia dispersed by raindrops become wet enough to enter the droplets, and the majority are carried on this droplet surface as a dry coating (Jarvis, 1962a).

The surviving mycelium and sclerotia produce conidiophores and multinucleate conidia. Conidia formation is stimulated by specific wavelengths of light, especially near-UV light.

The production and dissemination of conidiospores is regulated by fluctuation in temperature and humidity; a rapid decline in humidity with rise in temperature in early morning causes twisting and drying of conidiophores to eject conidia into air currents (Jarvis, 1962b). The conidia can move on air currents from neighbouring crops, yet most conidia are probably generated from primary sources within the crop (Williamson *et al.*, 2007). Insect vectors also have considerable amount of contributes in *B. cinerea* dispersal; in grapes, for example, insects disperse viable conidia either on their external appendages or even inside the gut, depositing on the surface of fruits (Elmer & Michailides, 2007).

The successful germination of a dry inoculated spore depends greatly on relative humidity (RH). Williamson *et al.* (1995) found that for the germination and infection of conidia on rose petals RH values need to be more than 93%; free water is not needed. The germinated conidia produce germ tubes; based on the available nutrients number and length of the germ tubes varies (Williamson *et al.*, 2007). *Botrytis* appressoria differ from the classic type present in *Colletotrichum* or *Magnaporthe* species; there is a septum between appressorium and germ tube in the classic type, but not in *Botrytis*. Therefore, the pressure given by the appressorium to the penetration peg, the fungal structure that penetrates cuticle, is lower in *B. cinerea* (Doss *et al.*, 1995). *B. cinerea* appressoria breach the plant surface mainly by secreting several enzymes, such as cutinase and lipase (van Kan *et al.*, 1997; Reis *et al.*, 2005). The H_2O_2 produced by the tip of the penetration pegs may contribute by causing an oxidative burst in the cells below and therefore destruction of cuticle (Tenberge *et al.*, 2002). After successful penetration of the cuticle layer, a penetration peg often grows into the anticlinal wall of underlying epidermal cells and produces significant amount of pectinases to degrade the pectin in the cell wall (van Kan, 2006). In order to have a successful infection process *B. cinerea* needs to kill the plant cells; this is accomplished by the continuous oxidative burst and production of variety of phytotoxic metabolites such as botrydial and botcinolides (van Kan, 2005).

In most temperate regions, by early spring there is ample inoculum in the environment for infection (Elmer & Michailides, 2007). In strawberry (Sutton & Peng, 1993) and grapes (Holz *et al.*, 2003) newly emerged and partially expanded leaves are infected by *B. cinerea*, but the infection remains at a quiescent stage. These early quiescent infections provide little inoculum for flower infection unless the leaves undergo premature senescence. As the leaves senesce and die, the fungus colonizes the tissues and sporulates; conidia formed on dying and necrotic leaves are the principal source of inoculum for *B. cinerea* fruit rot epidemics (Holz *et al.*, 2003). A similar story was observed in kiwifruit sepals: in the early season sepals had quiescent infection, but they did not sporulate and therefore did not contribute to flower infection (Elmer & Michailides, 2007).

In plant *Phaseolus vulgaris*, infected wind-blown petals can act as dispersal propagules in some cases, or they could act as sites of secondary inoculum production (Johnson & Powelson, 1983). In small fruits such as grapes, raspberry and strawberry (*Fragaria* × *ananassa*) floral parts are important sites for primary infection. After primary infection the pathogen remains inactive for a considerable period, until fruit maturity, and then rapidly destroys the tissues (Williamson *et al.*, 2007).

B. cinerea is known to have many cryptic species, possibly with partial ecological separation. Giraud *et al.* (1997) suggested that *B. cinerea* populations were structured as two genetically isolated subgroups characterized by the presence or absence of the transposable elements *Boty* and *Flipper*. *B. cinerea* var *transposa* strains possess both *Boty* and *Flipper*, whereas *B. cinerea* var *vacuma* strains lack both. There are isolates which have either *Boty* or *Flipper* (Rajaguru and Shaw, 2010). Walker *et al.* (2011) identified a new cryptic species, *B. pseudocinerea* in French vineyards. None of the morphological criteria tested was able to discriminate, but population genetics analysis revealed a high level of diversity within each species.

1.3.3 Control measures against *B. cinerea* infection

B. cinerea is controlled by various methods such as spraying chemicals, cultural practices and application of biological control agents. Among these, fungicides often very effectively control the fungus and therefore have been widely used in different cropping systems. Five different types of fungicides are used to control *B. cinerea*; affecting fungal respiration, sterol biosynthesis, microtubule assembly, osmoregulation or unspecified processes whose toxicity is reversed by amino acids (Rosslenbroich & Stuebler, 2000). Multisite fungicides such as thiram, mancozeb and maneb have also been used to control *B. cinerea* for a long time (Leroux, 2007). There are some excellent fungicides that have been used to control *B. cinerea*; anilino pyrimidines, phenylpyrrol fludioxonil and hydroxyl anilide fenhexamid (Rosslenbroich & Stuebler, 2000). All these fungicides strongly inhibit germ tube elongation and mycelial growth. However, development of fungicide resistance is a serious issue in *B. cinerea* disease control; several major botrycides such as benzimidazoles, phenylcarbamates and dicarboximides (Leroux, 2007) are useless in most settings. Recent studies have revealed that *B. cinerea* isolates can show resistance against fungicide fenhexamid (Esterio *et al.*, 2007).

Conditions such as high humidity, reduced light and moderate temperature are highly favourable for the grey mould disease development. Therefore, management of these conditions with some cultural practices can reduce losses in crop fields. In general, there is a range of cultural practices employed in grey mould disease management, but they vary with plant species and cropping system. Conditions such as creating an open canopy to provide adequate air movement and good light interception can help to dry the water droplets on plant tissues from rain or irrigation (Williamson *et al.*, 2007). Gubler *et al.* (1987) found that pruning of excessive vegetative plant parts can greatly reduce disease incidence in perennial woody plants such as grapevine. In soft fruits such as strawberries, rainfall during blossom period causes significant loss in the crop production. Spore inoculation to wounded stems of

tomato resulted in stem rotting but no symptoms developed following inoculation of unwounded stems (O'Neill *et al.*, 1997). A similar observation was made by Sharabani *et al.* (1999) in sweet basil; cut ends of stems were highly susceptible soon after harvest. Xiao *et al.* (2001) reported that growing plants in shelters or plastic tunnels can reduce the disease incidence by 90%.

There are some microbial antagonists available to control *Botrytis* infection on crops. An isolate of *Paenibacillus polymixa* has shown an antagonistic potential against *B. cinerea* in strawberries (Helbig, 2001). Mari *et al.* (1996) has shown that grey mould was reduced in fresh market tomatoes treated with *Bacillus anyloliquefaciens*. The combined effect of biological control agent and chemical spray has been proved in several experiments; Elad *et al.* (1993) discovered that in tomato, rotation of *Trichoderma harzianum* with chemicals resulted in more consistent reduction of grey mould than exclusive use of chemicals. One of the yeast isolates, *Saccharomyces cerevisiae* BSc68, was able to inhibit mycelial growth of *B. cinerea in vivo* on grape berries at both 2 °C and 25 °C (Nally *et al.*, 2012). Sylla *et al.* (2015) reported that combined application of *Bacillus amyloliquefaciens* FZB42, *Aureobasidium pullulans* DSM 14940 and DSM 14941 and *Beauveria bassiana* ATCC 74040 effectively reduced the *B. cinerea* infection in field growing strawberry (*Fragaria* × *ananassa*) fruit. Treatments with any single one of these biocontrol agents were not effective.

1.3.4 Mode of infection of *B. cinerea*

1.3.4.1 Necrotic infection

In general, *B. cinerea* is considered to be a necrotrophic pathogen which mainly produces localized lesions. The air borne conidium attaches to the host surface in two stages: in the first stage, weak adhesion results from hydrophobic interaction between host and conidium

surface. The second, stronger, stage of attachment happens after the germination of a spore. Extracellular matrix material which covers the germ tube provides the adhesive surface (Doss *et al.*, 1995). As an opportunist pathogen invasion can be achieved by active penetration or passive entrance. Free surface water or high relative humidity (>93% RH) is needed for germination and penetration of the host epidermis (Williamson *et al.*, 1995). Fungal hyphae produce diffusible molecules that trigger programmed cell death in a ring of cells around the hyphae (Govrin & Levine, 2000). Oxalic acid, reactive oxygen species and toxins like botcinolide and botrydial may all act as these diffusible molecules (van Kan, 2005). Lignification, biosynthesis of phytoalexins and accumulation of pathogenesis related proteins were detected in *B. cinerea* infection, as in the hypersensitive reaction to a biotrophic pathogen (van Kan, 2005). Depending on the host tissue and physiological state of the host, the pathogen may then enter a quiescent stage or become an aggressive form with expanding lesions. During aggressive growth the fungus shows vigorous outgrowth, resulting in rapid maceration of plant tissues. In order to actively degrade the plant cells, *B. cinerea* possesses a set of cell wall degrading enzymes including pectin lyase, pectin methylesterase, exo- and endo-polygalacturonase and cellulase. In the final stage of the cycle, the fungus produces a large number of spores on the macerated plant tissues. The whole cycle may be completed within 3 to 4 days if the conditions favour the fungus (van Kan, 2005).

1.3.4.2 Latent or quiescent or symptomless infection

As explained above, the grey mould pathogen *B. cinerea* is generally known as a necrotroph and most experiments looking at infection focus only on symptomatic, aggressive, infection. However, *B. cinerea* can also cause latent or quiescent or symptomless infection in host plants.

The definition for latent infection is broad. For an epidemiologist view, latency is the period from one infection to produce inoculum for another infection (Vanderplank, 1963). Verhoeff (1974) describe pathogen quiescence as a latent infection. Jarvis (1994) regards latent

infection as a spectrum running from un-germinated spore, through quiescent and symptomless internal infections, to visible but non-expanding lesion.

During quiescent infection the fungus remains alive inside the plant body without causing any visible symptoms or growing. If the conditions become favourable the fungus may show aggressive growth and starts to produce spores (Elad *et al.*, 2007).

Quiescent infection of *B. cinerea* is well known in small berry crops, grapes, strawberries and raspberries; the fungus establishes quiescent infection in the floral parts and then aggressive infection is noticed in ripe fruit (Jarvis, 1994). Grape berry infection takes place during bloom and fungus remains quiescent in immature berries. Nair & Parker (1985) found that *B. cinerea* invades the stigma and then becomes quiescent in necrotic style tissue at the stylar-end of the berry. Grape clusters remain symptomless between the flowering period and the beginning of ripening. Coertze and Holz (2002) found that spore dust inoculated *B. cinerea* may retain on grape berries in three different dormant stages: dormant conidia may adhere to the skin; there may be germlings that had colonised but not penetrated the skin; or there may be germlings that had penetrated the skin, but were restricted to a small zone by host defences. Coertze & Holz (2002) also mentioned that that infection on grape berry skins does not contribute to a gradual build-up of secondary inoculum in the vineyard. Braun & Sutton (1988) noticed quiescent infection of *B. cinerea* strawberry (*Fragaria* × *ananassa*) leaf discs; the spore dust-inoculated leaves remained green until senescent, but microscopy revealed quiescent *B. cinerea* in the epidermal cells.

Three major stages are often recognized in quiescent infection: establishment; containment/arrested phase; and resumption of active growth (Wade & Cruickshank, 1992; Latunde-Dada, 2001; Elmer & Michailides, 2007). There are several host-pathogen factors which could account for quiescent infection. Grape berries exude a variety of compounds, such as phenolic compounds and malic acid, through the cuticle layer, onto the berry surface.

There is a negative correlation between the concentrations of these exudates and transition from latency to aggressive growth (Padgett & Morrison, 1990). If the cuticle is breached, uninfected adjacent cells start suberinisation to isolate the invaded mycelium. The suberinization process will reduce diffusion of nutrients into the isolated cells, causing the fungus to be trapped in the cells and preventing growth. Alternatively, the triggering of defence responses leading to the accumulation of phytoalexins and synthesis of PR proteins may lead the fungus to transfer to a quiescent stage of growth (Elmer & Michailides, 2007).

Quiescent infections may eventually become aggressive symptomatic infections in the field or post-harvest products. Therefore, detailed understanding of the factors involved in quiescent development and the transition to aggressive growth are needed to control disease (Jarvis, 1994). The development of quiescence or symptomless infection and its maintenance always depends on an equilibrium among host plant, pathogen and environment. Physiological and physical changes in the host, its environment, or both, trigger changes in that equilibrium to permit the pathogen to resume aggression (Jarvis, 1994).

Quiescence can occur at any stage between spore germination and sub-cuticular hyphal development (Prusky, 1996). In soft fruits such as strawberry, raspberry and grape, *B. cinerea* mainly infects the host flowers and then remains quiescent in the developing fruit tissue for several weeks. High levels of fungitoxic or fungistatic compounds (phytoalexins) and proteinaceous inhibitors of fungal cell wall degrading enzymes (Polygalacturonase Inhibiting Proteins – PGIPs) in the tissues cause the fungus to become quiescent (van Kan, 2005). When the fruit becomes ready to ripen, fungal growth resumes and starts to produce symptoms.

In symptomless infection *B. cinerea* grows with plant and spread to new developing plant parts without showing macroscopic symptoms. Symptomless infection of *B. cinerea* in blackcurrant flowers was reported by McNicol *et al.* (1989); pollinated flowers were inoculated with dry conidia of *B. cinerea.* The observation of pistils under UV fluorescence

revealed that conidia germinated in the stigmatic fluid and hyphae spread symptomlessly throughout the style to infect the pericarp and ovules. Pezet & Pont (1986) showed in their histological studies of laboratory-inoculated bunches that *B. cinerea* colonises the stamens and invades their bases on the receptacle. From there it grows systemically to the pedicel and vascular tissue in berries. Horst (1985) reported infection of *B. cinerea* in symptomless rose flowers; infection in the petals remained symptomless, but it became apparent under humid condition during transport.

Some recent studies revealed that *B. cinerea* also can cause symptomless systemic infection in host plants including *Primula vulgaris* (primrose) and *Lactuca sativa* (lettuce) (Barnes, 2002; Sowley, 2006; Rajaguru, 2008). In this type of infection fungus grows in parallel with plant growth inside, or closely associated with the surface of, the plant without producing any symptoms until the plant becomes physiologically susceptible to necrotrophic infection, typically at flowering.

In some of these studies, symptomless *B. cinerea* was isolated from different tissues of plants. In symptomless *Primula vulgaris* Barnes (2002) isolated the same *B. cinerea* isolate from all tissues of plants, including roots, but different isolates were found in different plants. Based on this result it was concluded that *B. cinerea* spreads systemically throughout the plant. In 2006, Sowley carried out an experiment to reveal symptomless systemic infection of *B. cinerea* in lettuce. Root, stem, leaves and flowers of inoculated lettuce plants were sampled on selective medium and stained with different stains including a *Botrytis* specific immunolabelling technique. The results of plating and qPCR were inconsistent with non-systemic infection, but the visual evidence of distributed infection was not completely convincing. Internal mycelium was detected mainly in roots, but background auto-fluorescence and thickness of plant tissues were major drawbacks for precise visualization. Rajaguru (2008) tried to visualise symptomless systemic *B. cinerea* infection in *Primula vulgaris* inoculated with GFP labelled *B. cinerea* isolates, but failed. The reason suggested for

this result was auto-fluorescence from plant tissues and the limited penetration of the confocal microscope, which could not observe below the epidermis of the thick *Primula vulgaris* leaves. Finally, Shafia (2009) found that *B. cinerea* can cause symptomless infection throughout wild *A. thaliana* plants: sampling of surface disinfected stem, leaves, root and flowers of *A. thaliana* plants produced *B. cinerea* colonies on selective medium.

With this background I focused in the research described in this thesis on symptomless infection of *B. cinerea* in host plants *Arabidopsis thaliana* (a well-known model plant, which appears to host symptomless *B. cinerea*) and lettuce (a well-known host of *B. cinerea*). My aims were to clarify the nature of the interaction and where exactly the pathogen was within plants with symptomless infection. In order to check whether there was any difference in the pattern of expression of pathogenicity or signalling genes of *B. cinerea* between symptomless infection and aggressive necrotic infection, amounts of mRNA of a sample of genes related to pathogenicity or the control pathways associated with infection were quantified and compared at different time points. In order to reveal the symptomless growth of *B. cinerea* in or on host tissues, I tried to follow the disease progress of GFP-labelled *B. cinerea* in these two host plants with the aid of confocal laser scanning microscope, from spore landing, to symptomless infection and then to necrosis.

1.4 Objectives of this study

To find out suitable growth and inoculation conditions that would facilitate the development of non-symptomatic infection of *B. cinerea* in *Arabidopsis thaliana* and *Lactuca sativa*.

To find out the extent of non-symptomatic infection of *B. cinerea* in wild *A. thaliana*.

To compare gene expression of *B. cinerea* during symptomatic and non-symptomatic infections in *A. thaliana* and *L. sativa*.

To visualize *B. cinerea* in *A. thaliana* and lettuce during symptomless infection.

Chapter 2 General Methodology

2.1 Surface disinfection

A. thaliana (wild type, Columbia ecotype) seeds were surface disinfected in 70% ethanol for 1 min and then 20% bleach (JANGRO Bleach, Jangro Limited, Bolton) for 2-3 min and finally thoroughly rinsed in 5 to 6 changes of sterile water.

Where required, before plating on Botrytis selective medium, plant tissue samples were surface disinfected. They were rinsed in 20% bleach for 2-3 min and then washed in three changes of sterilized distilled water.

2.2 Growth media

2.2.1 Botrytis selective medium (BSM)

Botrytis selective medium was used to recover *B. cinerea* from infected plant tissues by suppressing the growth of other microbial contaminants. BSM was prepared as described by Edwards & Seddon (2001). One litre of medium contained glucose 2g; $NaNO_3$ 0.1g (Sigma, USA); $MgSO_4.7H_2O$ 0.2g (Fisher scientific, UK); K_2HPO_4 0.1g (Sigma, USA); KCl 0.1g (Fisher scientific, UK); Chloramphenicol 0.2g (Duchefa biochemie, The Netherlands); Pentachloronitrobenzene 0.02g (Sigma, USA); Maneb 80 (80% Manganese Ethylene Bis dithiocarbamate) 0.02g (Sigma, USA); Rose Bengal 0.05g (Fisher scientific, UK); Tannic acid 5g (Sigma, USA); Agar 20g (Oxoid, UK). The pH was adjusted to 4.5 with 1M NaOH (Sigma, USA) prior to the addition of agar. The mixture was kept in a boiling water bath for 15 min and poured whilst still hot.

2.2.2 Malt extract agar (Oxoid, UK)

Malt extract agar (MEA) was used for the routine *B. cinerea* sub-culturing and spore production. One litre of medium contained malt extracts 30 g; mycological peptone 5g; agar 15 g. The mix was autoclaved at 115 °C for 15 min.

2.3 *B. cinerea* incubation conditions

For routine sub-culturing and spore collection, MEA plates with fungus inoculum were incubated in darkness at 20 °C for 3-5 days to facilitate mycelial growth, then moved to near-UV incubators (combination of white light and UV light (SYLVANIA, Black light-Blue, F8W/BLB-T5)) with 16 hours day length to enhance spore production.

For isolation, BSM plates with plant tissue samples were incubated at 20 °C in UV incubators with 16 hours day length. *B. cinerea* was confirmed based on colour change of medium from pink to brown, and characteristic conidia bearing structures. The hyaline conidia are borne on grey, branching tree-like conidiophores.

2.4 Plant growth conditions

2.4.1 *A. thaliana*

2.4.1.1 Isolation propagator and seed sowing

Seeds were stratified at 4 °C for 4 days before planting. In order to isolate individual plants growing in the same growth chamber, a special experimental setup was made using covered pots (Figure 2-1). The pots and plastic transparent lids used to cover the plants were surface disinfected in 20 % bleach (1 % NaOCl) solution for 10 min then rinsed with sterilized water three times. Surface disinfected pots were half filled with sterilized compost (seed and modular compost, Clover, www.cloverpeat.co.uk) and placed on individual surface disinfected trays. The trays were filled with sterilized water and kept for a few hours until the compost become completely moist. When the surface of the compost became fully moist one

seed was planted per pot. The pots were covered with transparent covers (14 cm in height) and air tightly sealed to the base. Finally, all the pots were connected to the air circulation system.

Aeration was provided into each pot through closed robust tubes from an air pump via a hole above soil level. To ensure air circulation a small hole had already been made on top of the transparent lid and loosely plugged with cotton wool. An air filter was made with cotton wool and funnels and fixed in the airline after the pump and before branches to individual pots. The absence of *B. cinerea* contamination in the controlled environment chamber, air released from the pump and air in the closed circulation unit were tested by exposing Botrytis selective medium in the pots prior to seeding.

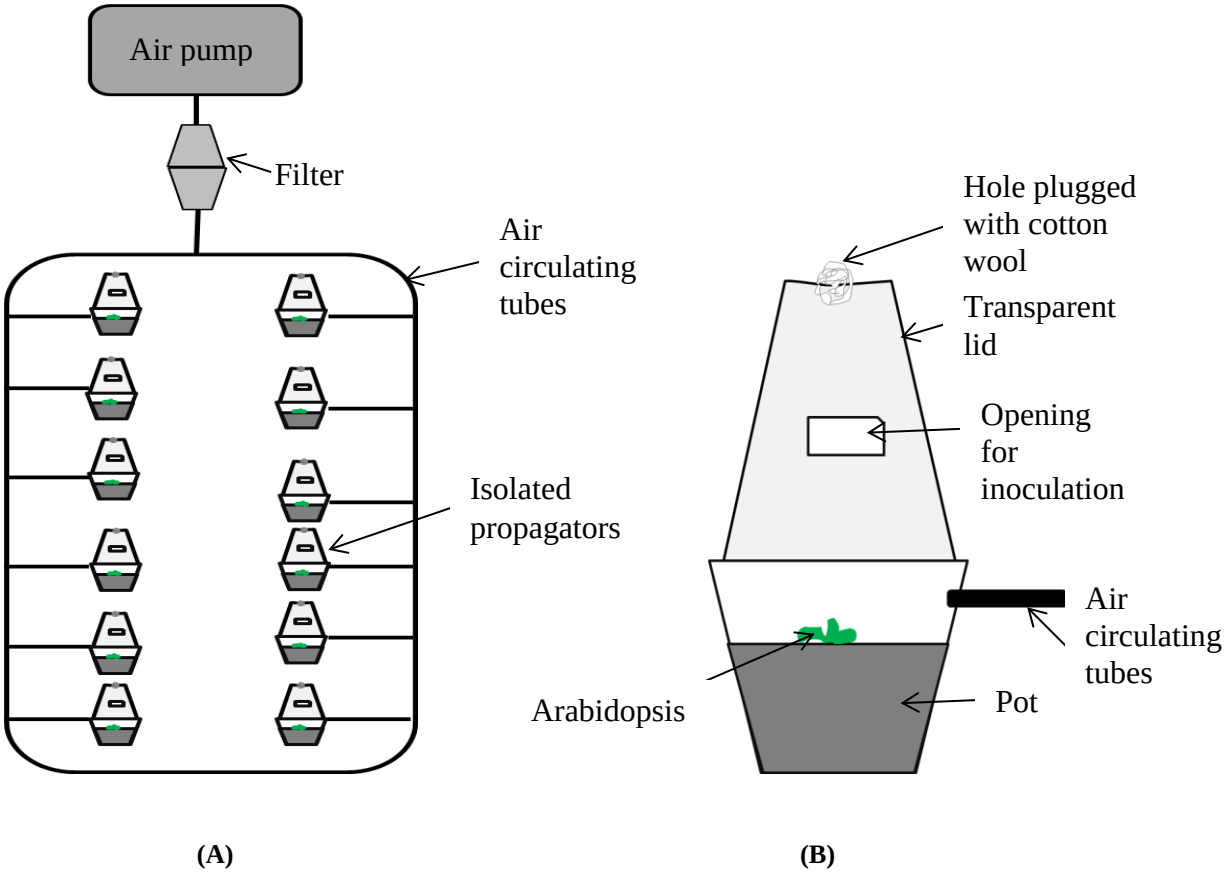


Figure 2-1: Isolation propagator. (A) The air released from the pump is passed through the filter before entering the circulation tubes, (B) individual propagators connected to common air circulation tube.

2.4.1.2 Growing conditions in controlled environment chamber

The plants were grown in a controlled environment chamber (Fitotron, Weiss Gallenkamp, UK) set to 22 °C day and 18 °C night temperature, 65% relative humidity, 16 h light and 8 h dark period, 200-250 µmol/m^2/s light intensity. Light intensity was measured using light sensor (Skye Instrument Ltd, UK). The plants were watered every day sufficiently to keep the compost just moist up to two weeks from sowing, then at two-day intervals. Some physical parameters inside the isolation propagator were monitored by inserting probes from the top and side of the transparent cover. Conditions inside the isolation propagator was measured using environmental meter connected with probe (Instrotech, UK), average day time temperature was 26.5 °C and night 18.5 °C; relative humidity in day and night ranged between 80% to 85%. The dew point temperature in the day was about 22 °C and at night about 16 °C. Light intensity was between 220 and 170 µmol/m^2/s.

2.4.2 *Lactuca sativa* (Lettuce)

Seedling trays were filled with compost (Seed and modular compost, Levington Horticulture Ltd, Suffolk, UK). Seeds were sown at about 0.5 cm depth and covered with compost. Seed germination and initial seedling growth was in controlled environment chambers: 20° C in 12 h day and 18°C in night, RH 65%. The compost was kept wet all the time by daily watering.

Seedling transplantation was done seven days after inoculation; seedlings were transplanted into 1L pots filled with compost mix of 1 L potting compost (Vitax Grower, Leicester, UK) with 4 g Osmacote (Scotts Australia Pty Ltd, Bella Vista). The pots were moved to a glasshouse, where the temperature ranged between 18° C at night and about 30° C in day time. The plants were watered every day.

2.5 Inoculation procedures

2.5.1 *A. thaliana*

Dry spores were collected from 15-20 days old *B. cinerea* cultures by using a cyclone spore collector (Long Ashton research station, Bristol) or by tapping the culture plate over aluminium foil. 10 mg spores were thoroughly mixed with 90 mg of talc powder with a spatula and a tenfold spore serial dilution was carried out up to 10^{-5} notional dilution. *A. thaliana* plants were inoculated with 5 mg of the spore dilution. Inoculation was done while the plants were inside their isolated propagator compartments through a small opening present on the side of transparent lid which covered the plant. The openings were made when the propagator was assembled, but sealed with sticky tape until needed. The spore-talc mix was taken on a small spatula and then the mix was dispersed into the air above the plant by a pulse of air ejected from a syringe through the syringe needle. After inoculation air circulation was stopped and all openings in the isolated propagator were sealed for one day to provide higher humidity.

2.5.2 Lettuce

Inoculation was done at the seedling stage when the plants were growing on seedling trays. The seedling trays were kept inside a cardboard box as a settling tower and the spores were dusted by tapping 15-20 days old *B. cinerea* cultures above the plants. The box was kept closed after spore dusting for 15 min to allow spores to settle. The whole seedling tray was then covered with a transparent polyethylene bag for one day to provide high humidity.

Chapter 3 Establishment of symptomless infection in *Arabidopsis thaliana*

3.1 Introduction

The necrotrophic fungus *B. cinerea* also causes symptomless infection in some host plants. Recent glasshouse studies with lettuce (*Lactuca sativa*) and primrose (*Primula vulgaris*), and field samples of some weeds such as Arabidopsis (*Arabidopsis thaliana*), Dandelion (*Taraxacum agg*), Yarrow (*Achillea millefolium*) and Groundsel (*Senecio jacobaea*) have shown that *B. cinerea* can cause symptomless infection in these host plants (Barnes 2002; Rajaguru 2008; Shafia 2009)

In order to study the non-symptomatic infection of *B. cinerea* a supply of reliably infected plants is needed. Therefore, it is necessary to find out suitable controlled conditions to produce non-symptomatic infection. This chapter summarises experiments carried out to find out the growth stage of *A. thaliana* that is optimum to produce non-symptomatic infection.

The plant *A. thaliana*, a member of the family Brassicaceae, is being used as a model plant for a wide range of basic and applied research in plant biology because of relatively small size, short life cycle, small genome and the possibility of carrying out mutation studies. The genome has been fully sequenced and the function of about 25000 genes has been discovered or inferred. A mature *A. thaliana* plant has a rosette of small leaves and a main stem terminating in an inflorescence. Fertilized flowers develop into fruit called siliques, containing 30-60 seeds. The commonly used *A. thaliana* ecotype (Columbia-1) reaches maturity in around six weeks under continuous light at 25 °C with good nutrition (Weigel & Glazebrook, 2002). Over the last 20 years studies on *A. thaliana* has contributed immensely to explaining the evolution and underlying mechanisms of disease resistance and susceptibility.

A. thaliana has been an excellent model for answering fundamental questions in plant microbe interactions.

A. thaliana is one of hundreds of plant hosts susceptible to infection by the necrotrophic fungus *B. cinerea*. *A. thaliana* has been used in several studies of the pathogenicity of *B. cinerea*. Windram *et al.* (2012) studied high resolution time series of gene expression profiles from a single *A. thaliana* leaf during necrotrophic infection by *B. cinerea*. Approximately one-third of the *A. thaliana* genome is differentially expressed during the first 48h after infection.

Govrin & Levine (2002) studied the role of systemic acquired resistance (SAR) in *B. cinerea* infected *A. thaliana*. *B. cinerea* induced necrotic lesions and camalexin biosynthesis. It failed to induce SAR mediated resistance against a virulent strain of *Pseudomonas syringae*, or against subsequent *B. cinerea* infection. Beneloujaephajri *et al.* (2013) attempted to find out the role of calcium in production of reactive oxygen species (ROS) and wound induced resistance in *A. thaliana* during *B. cinerea* infection. They found that following wounds, increase of calcium concentration in cytosol precede a ROS burst in the same location. Weiberg *et al.* (2013) found that some *B. cinerea* small RNAs (BcRNAs) can silence *A. thaliana* genes involved in immunity. The fungal pathogen transfers virulent sRNA effectors into host plant cells to suppress host immunity and achieve infection. Recently, Dobón *et al.* (2015) identified four transcription factors (TFs) from *A. thaliana* that limit pathogen spread. An *A. thaliana* mutant defective in any of these TFs displayed increased disease susceptibility to necrotic infection by *B. cinerea*, and a general activation of non-immune host processes that contribute to plant disease susceptibility.

There are various methods to diagnose infected plants: visual assessment of the symptoms, lesion diameter measurement or spore counting. However, these methods are not suitable for detection at early stages of necrotic infection or detection of symptomless infection (Gachon

& Saindrenan, 2004). Plating out the tissue samples on selective medium or detection based on fungus DNA can be effective ways to find out symptomless infection.

Real time polymerase chain reaction (RT-PCR) is a sensitive and reliable method to monitor fungus in plant tissue. DNA of a specific target organism is both detected and quantified by measuring the intensity of amplification specific fluorescence generated during the reaction (Brouwer *et al.*, 2003). Real time PCR can be performed using different chemistries, such as TaqMan or SYBR Green 1 dye. In the TaqMan system a fluorogenic probe specific to the DNA target anneals between the PCR primers; upon amplification the probe is cleaved by the 5'-3' exonuclease activity of Taq polymerase releasing fluorescence. SYBR Green 1 relies on a fluorescent dye that intercalates in a sequence non-specific fashion with the double stranded DNA amplified during the PCR (Suarez *et al.*, 2005).

The most commonly used DNA region targeted to design primers for PCR based identification and detection of plant pathogens is nuclear ribosomal DNA (rDNA). This region contains multiple repeats of 18S, 5.8S and 28S rRNA genes separated by two internal transcribed spacers (ITS1 and ITS2) and one intergenic spacer (IGS) (White *et al.*, 1990).

In the case of *B. cinerea* a range of potential target sequences is available for real time PCR amplification such as ITS sequences, sequence characterised amplified region (SCAR) markers, the gene sequence for β-tubulin, actin and cutinase A. Suarez *et al.* (2005) found that for *B. cinerea* identification and quantification primers designed with IGS region of rDNA are very specific and more sensitive than primers designed with gene coding for β-tubulin or SCAR markers.

In a previous study, sampling of wild growing, apparently healthy, *A. thaliana* on selective medium revealed that the plant can harbour *B. cinerea* symptomlessly (Shafia, 2009). Therefore, I attempted to produce symptomless infection of *A. thaliana* by *B. cinerea* in laboratory conditions. As mentioned earlier, *A. thaliana* is used as a model plant to study the

interaction between various pathogens because of its short life cycle and the ease with which molecular studies and microscopy can be done. Severity of disease development may vary with different isolates of a fungus species in a host pathogen interaction, so I also screened different isolates of *B. cinerea* for their ability to cause non-symptomatic infection on *A. thaliana*.

3.2 Materials and Methods

3.2.1 Growth stage of *A. thaliana* producing maximum symptomless infection

3.2.1.1 Growing *A. thaliana* plant

A. thaliana seeds were surface disinfected and then kept at 4 °C to stimulate germination (stratification). After four days seeds were sown on compost in the isolated propagator as described in chapter 2 general methodology.

3.2.1.2 Inoculation of *B. cinerea*

Inoculation was done in two different ways; as seed inoculation and plant inoculation.

For seed inoculation, spore suspension was prepared in sterile distilled water and the concentration was adjusted to 800 spores/µl. Seeds kept on water soaked filter papers for stratification at 4°C for four days were arranged as groups of 15 and then 1 µl of spore suspension was added at the centre of the group of seeds. The spore droplet completely covered the group of spores. The seeds were sown on compost after 4 – 6 hours. Some seeds were also placed on BSM plates and incubated to check that spores had adhered to the seed surface.

Plant inoculation was done at different stages of rosette growth of *A. thaliana* using a spore dusting method. *B. cinerea* spores were collected from 15-20 day old cultures by using a cyclone spore collector. 10 mg spores were thoroughly mixed with 90 mg of talc powder with a spatula and a tenfold spore serial dilution was carried out up to a notional 10^{-7} dilution. Inoculation was done through a small opening present on the transparent lid which covered the plant (already made on the side of the lid, but sealed with sticky tape after sowing until needed). The spore-talc mix was taken on a small spatula and then the mix was dispersed into the air above the plant by a pulse of air ejected from a syringe through the syringe needle. Finally the small opening was again sealed with sticky tape.

The optimum spore dilution that evenly distributed spores on the inoculated surface was determined by dusting the spore dilutions on BSM plates instead of *A. thaliana* plants in isolation propagators instead of *A. thaliana* plants. The plates were incubated at 20°C in UV light and visible Botrytis colonies were counted. A notional 10^{-5} spore dilution leading to about 9 spores / cm^2 spore density on BSM was used in plant inoculation experiments.

A. thaliana plants were inoculated with 5 mg of the 10^{-5} spore dilution prepared as above at different stages in rosette growth. The first inoculation was on the seventh day of sowing (4-6 leaves stage). Further inoculations were made for three weeks at three day intervals until before flowering, therefore all inoculations were done at the rosette growth stage. They were named as 7, 10, 13, 16, 19, 22 and 25 day after sowing; seed inoculation was named as 0 day after sowing. Each inoculation was considered as a separate treatment. The experiment was blocked in time. In the first block each treatment was repeated twice, with two control (18 isolation pots); in the second, there were 4 replicates and 2 controls (34 isolation pots). For the control, only talc powder was dusted.

3.2.1.3 Sampling of *A. thaliana* plant on Botrytis selective medium

Sampling was done 10 days after the last inoculation; plants were in their fifth week of growth after sowing, and they had erect flowering structures. Plants were uprooted, then dissected into aerial and root portions. The root was washed in running water to remove compost and other debris. The aerial part was further dissected into stem, rosette leaves, stem leaves and flowers. Apparently healthy (i.e. uniformly green and undamaged) tissues were used for further experiments. Two stem pieces each about 2 cm long, two stem leaves, three rosette leaves, two inflorescences and about 2 cm piece of root were sampled on Botrytis Selective Medium (BSM) with and without surface disinfection. Tissue disinfection was carried out by treating with 20% bleach (1% NaOCl) for 2-3 min. Plates were incubated at 20°C with 16 hour/day UV light, and the plates were observed under dissection microscope

for characteristic sporulating structures and brown colouration of medium. The remaining plant samples were kept in -80°C until use for DNA extraction.

3.2.1.4 Quantification of *B. cinerea* in plant samples

Quantification of *B. cinerea* in infected tissues was done by quantitative polymerase chain reaction (qPCR) protocol.

DNA extraction was carried out using Plant Mini Kits (Qiagen, Germany). Stems and stem-leaves of plants inoculated at 0, 10, 16 and 22 day after sowing and their respective mock inoculated plants were used for the DNA extraction. DNA was extracted separately from stem pieces about 10 cm long, from the base, and the leaves attached on it. The plant tissues were ground in liquid nitrogen using mortar and pestle, and then extraction was carried out according to the protocol provided by the manufacturer. The DNA extracts were stored at -20 °C until used for further studies.

DNA extraction was also carried out from pure *B. cinerea* and *Trichoderma* sp. cultures; mycelium collected from six days old culture was ground into a fine powder and DNA was extracted with QIAGEN Plant Mini Kits.

The total DNA concentration in each extract was measured using a Spectrophotometer (Nano Drop LITE, Thermo Scientific, Waltham, USA). In order to check the quality of extracted DNA agarose gel electrophoresis was carried out: samples were run in 1% agarose (Bioline, UK) gel containing ethidium bromide. HyperLadder 1(Bioline, UK) was used as standard.

3.2.1.4.1 Primers

For *B. cinerea* two previously published (Suarez *et al.*, 2005) marker gene sequences were initially selected (Table 3-1), the intergenic spacer (IGS) region of the nuclear ribosomal DNA (primer Bc3) and a species specific sequence characterised amplified region (SCAR) marker (primer Bc2). As plant normaliser, the gene sequence of alpha shaggy kinase (primer iASK) was used as mentioned by Gachon and Saindrenan (2004).

Table 3-1: List of primers used in the experiment

Target sequence	Primer	Primer sequence (5'-3')
SCAR marker (*B. cinerea*)	Bc2	Forward : TTCGTGATTATCACCTGGGTTG Reverse: GCTCCTAGAACGTACGACCACA
IGS marker (*B. cinerea*)	Bc3	Forward : GCTGTAATTTCAATGTGCAGAATCC Reverse: GGAGCAACAATTAATCGCATTTC
Alpha shaggy kinase (*A. thaliana*)	iASK	Forward : CTTATCGGATTTCTCTATGTTTGGC Reverse: GAGCTCCTGTTTATTTAACTTGTACATACC

3.2.1.4.2 Determination of optimum annealing temperature and specificity of primers

Optimum annealing temperature of three primers was tested in PCR. 20 µl of reaction mix contained 10 µl master mix (Taq PCR Master Mix Kit, QIAGEN), 0.8 µl primers (10 µM) and 1 µl DNA sample. PCR cycle conditions were maintained as 94 °C for 2 min initial denaturation, followed by 30 cycles of denaturation at 94 °C for 30 s, with annealing temperatures of 50 °C, 55 °C, 60 °C and 65 °C for 30 s, extension at 72 °C for 30 s and final elongation at 72 °C for 7 min. Each primer set was separately tested with its respective pure DNA samples.

Specificity of each primer was separately tested with pure DNA of *A. thaliana*, *B. cinerea* and *Trichoderma* sp. by amplifying in PCR as conditions mentioned above; the annealing temperature was 60 °C. The reason to check *Trichoderma* sp. was its frequent recovery in non-surface disinfected tissues of *A. thaliana*.

The PCR products were analysed by running DNA in 3% agarose gel having 3 µl of 10 mg/ml Ethidium bromide. HyperLadder V was used as standard.

3.2.1.4.3 qPCR assay

DNA of each test sample was adjusted to 10 ng/ µl concentration. For qPCR reaction, 20 µl reaction samples were prepared by mixing 4 µl DNA solution with 10 µl SYBR Green JumpStart Taq Ready Mix (Sigma-Aldrich) and 0.6 µl appropriate primers (300 nM). The primer Bc3 and iASK were used to amplify target DNA sequences of *B. cinerea* and *A. thaliana*. qPCR reactions were run in duplicate under conditions suitable for all the primers (95 °C for 10 min and 40 cycles of 60 °C for 1 min, 95 °C for 15s) using a Rotor gene 6000 qPCR machine (Corbett Life Science, QIAGEN). After each run, melting curves were acquired by heating to 95 °C for 15s, cooling to 60 °C for 1min and heating to 95 °C at 0.3 °C, before holding at 95°C for 15s with data collection. Quantification used a standard curve technique. 2.5 ng/ µl of pure *B. cinerea* DNA and 20 ng/ µl *A. thaliana* stem or stem-leaf DNA samples were tenfold serially diluted, five times. 4 µl of these diluted samples were amplified in qPCR reactions with conditions mentioned above. Each reaction was carried out in triplicate and three control reactions were carried out with nano-pure water instead of DNA samples.

3.2.2 Comparison of non-symptomatic infection of different isolates of *Botrytis cinerea*

3.2.2.1 *Botrytis cinerea* isolation and characterisation

Five different *B. cinerea* isolates were tested for their propensity to cause non-symptomatic infection. Four of them were isolated in this study from surface disinfected tissues of wild *A. thaliana* plants: from stem, rosette leaf, root and inflorescence of distinct *A. thaliana* plants and subsequently labelled S1, SL1, R1 and F1. The fifth one was the standard *B. cinerea* isolate B05.10, obtained from John Innes Centre, Norwich.

3.2.2.2 Morphology of isolates

Culture morphology and growth rate was studied by growing these isolates on Malt Extract Agar (MEA) medium. 5 µl of spore suspension (1×10^6 spores/ ml) of each isolate was plated out separately as a drop. The plates were incubated in two different conditions; four plates of each isolate were incubated in dark at 20 °C for sclerotia development, four more plates of each isolate were incubated in 16 hours day light (combination of UV and white light) at 20 °C to induce sporulation. At 24 hour intervals the radial mycelial growth was measured.

3.2.2.3 Detached leaf inoculation

This experiment was done to compare the ability of the *B. cinerea* isolates to cause necrotic (symptomatic) infection. In preliminary experiments it was found that a spore suspension in Potato Dextrose Broth (PDB) (Oxoid, UK) can produce higher percentage of leaves with necrotic infections than dry spore inoculation or a spore suspension of the same concentration in water or glucose. Spore suspensions of each isolate were prepared from 20 days old cultures, and the final concentration adjusted to $5\ x10^5$ spores / ml in half strength (1.2 g / 100 ml) potato dextrose broth (PDB). Rosette leaves from 3 week old plants were detached and inoculated with 5µl of spore suspension. The inoculated and control leaves were kept in

humid chambers and incubated at 20 °C in 16 hours day light. For each isolate, 10 replicates were maintained. A control was done by inoculating PDB only.

3.2.2.4 Plant inoculation and sampling on BSM

A. thaliana plants were grown in isolation propagators in a CE room as mentioned in chapter 2. Spores were collected from each isolate separately and serially diluted five times by a factor of 10 in talc powder to get 10^{-5} inoculum dilutions. 5 mg of diluted spores were dusted on plants 21 days after sowing. Controls were dusted with talc powder only. 10 replicates were maintained for each treatment and control. Sampling of plant tissues was done on BSM plates 10 days after inoculation. From each plant, about 2 cm long two stem pieces, three rosette leaves, two stem leaves, a piece of root about 2 cm long and two inflorescences were separately sampled as surface disinfected (SD) and non-surface disinfected (NSD) and placed on BSM plates (Figure 3-1). Plates were incubated at 20 °C in 16 hours day light and observed at 2 day intervals under a dissection microscope for 15 to 20 days. Samples showing both brown coloration of medium and characteristic spore producing structures were considered positive for *B. cinerea* infection.

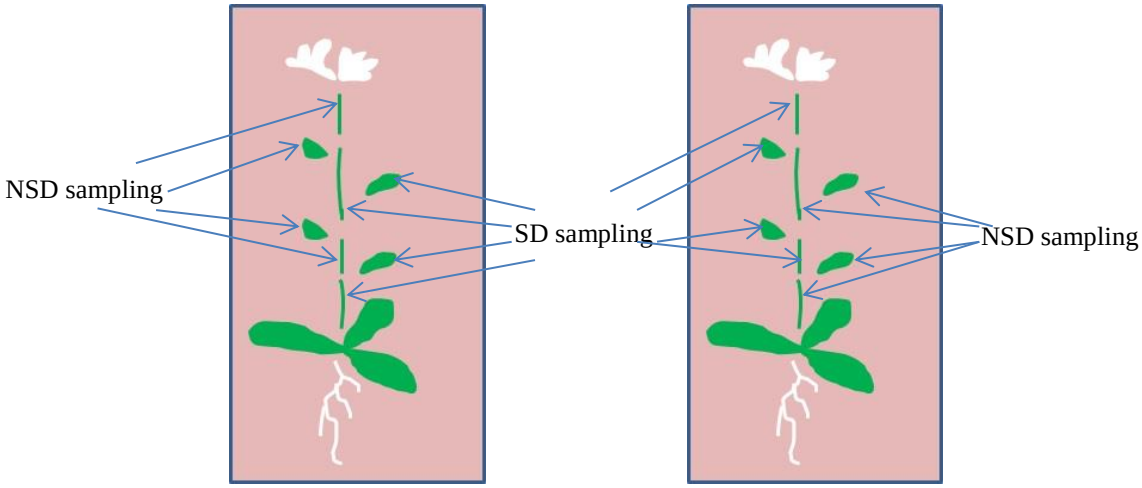


Figure 3-1: The pattern of sampling of stem and stem leaves of *Arabidopsis thaliana* plants inoculated with *Botrytis cinerea* spores. Out of ten plants sampled the first five were sampled as in (a) and the remaining five sampled as in (b), in order to show the spread of fungus along the length of the stem.

3.3 Results

3.3.1 Optimum inoculation time

3.3.1.1 Recovery of *B. cinerea* on Botrytis selective medium

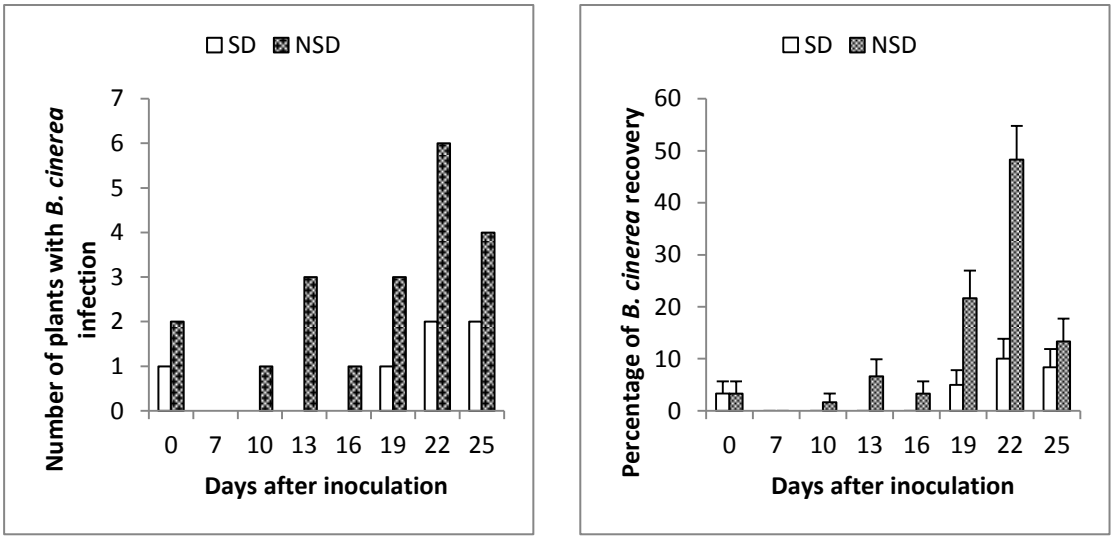
In each treatment 60 surface disinfected and 60 non-surface disinfected plant tissue samples were tested. This includes 6 root pieces, 12 stem pieces, 12 stem-leaves, 12 inflorescence and 18 rosette leaves.

The incidence of *B. cinerea* recovery varied depending on the stage of plant growth at the time of inoculation (Figure 3-2). *B. cinerea* was not recovered from plants inoculated seven days after sowing. In plants inoculated 10, 13 and 16 days after sowing, *B. cinerea* was recovered only from non-surface disinfected tissues. In late inoculated plants, inoculated at 19, 22 and 25 days after sowing and seed inoculated plants, both surface disinfected and non-surface disinfected tissues contained *B. cinerea*. However, recovery was more frequent in non-surface disinfected tissues than surface disinfected. Plants inoculated 22 days after sowing had *B. cinerea* infection in the non-surface disinfected tissues of all plants and in surface disinfected tissues of two plants of six inoculated (Figure 3-2 (a)).

Plants inoculated at 10, 13 and 16 days after sowing and seed inoculated showed infection in less than 10% of 60 non-surface disinfected samples (Figure 3-2 (b)). However, in late inoculated plants more *B. cinerea* was recovered. In particular, plants inoculated 22 days after sowing had about 10% and 50% recovery in surface disinfected and non-surface disinfected tissues respectively.

Rosette leaves not surface disinfected had *B. cinerea* infection in all treatments except in inoculations done 7 days after sowing (Figure 3-3(a)). Inoculation done at 22 days after sowing had the most rosette leaf infection. Late inoculated plants had infection in more plant parts than

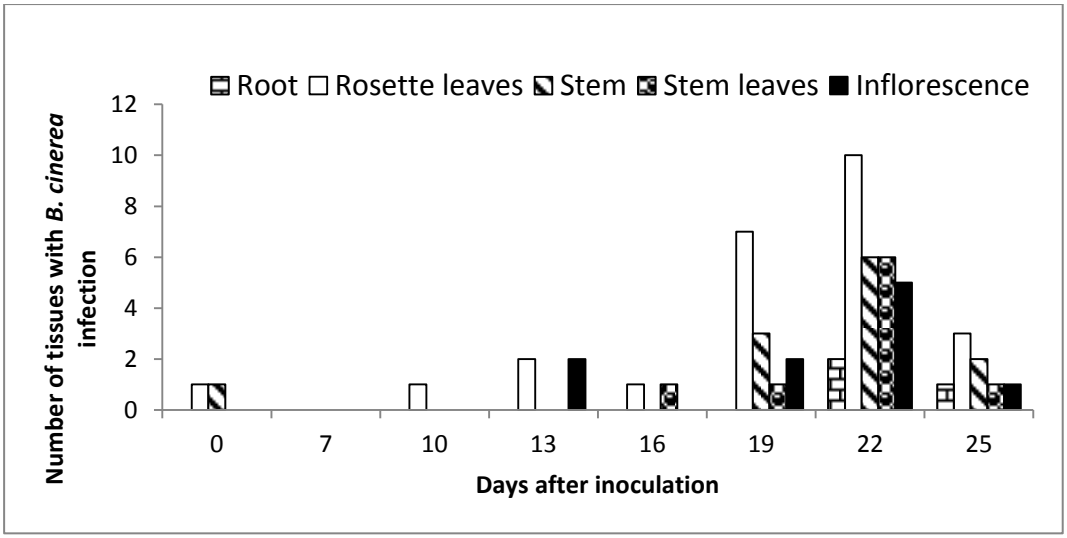
early inoculated plants. Plants inoculated 22 days after inoculation had the most infected samples in all tissue types.



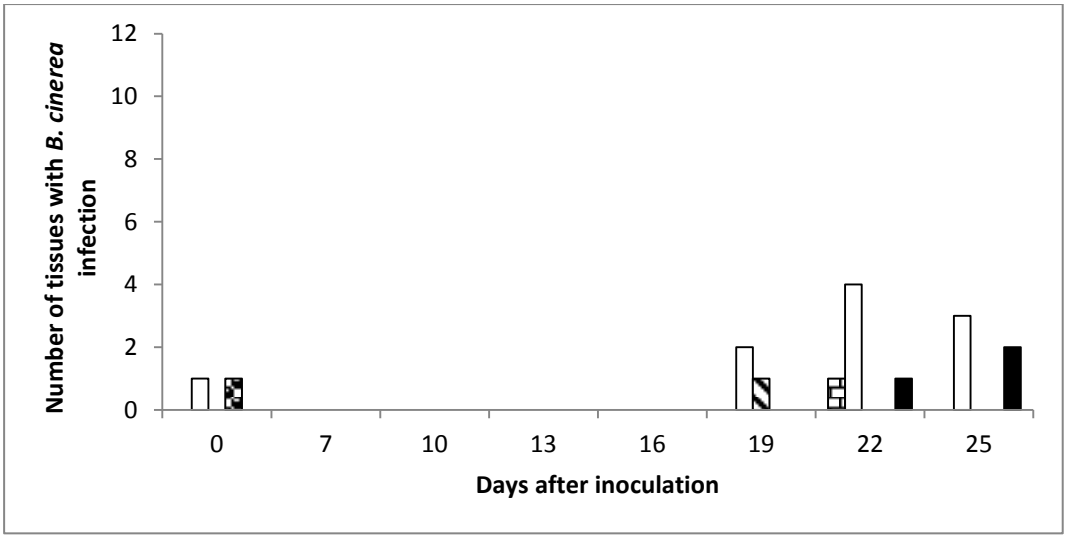
(a) (b)

Figure 3-2: Recovery of *B. cinerea* from surface disinfected (SD) and non-surface disinfected (NSD) tissues sampled from apparently healthy *A. thaliana* plants inoculated at different growth stages (X axis). (a) Number of plants infected with *B. cinerea*, n= 6 at each inoculation time (b) Percentage of tissue samples on which *B. cinerea* grew; error bars denotes standard error.

In surface disinfected tissues very few tissues had infection; even the plants which were inoculated 22 days after sowing revealed *B. cinerea* infection only in samples from 4 rosette leaves, a root and an inflorescence (Figure 3-3(b)) out of the 60 samples tested.



(a)

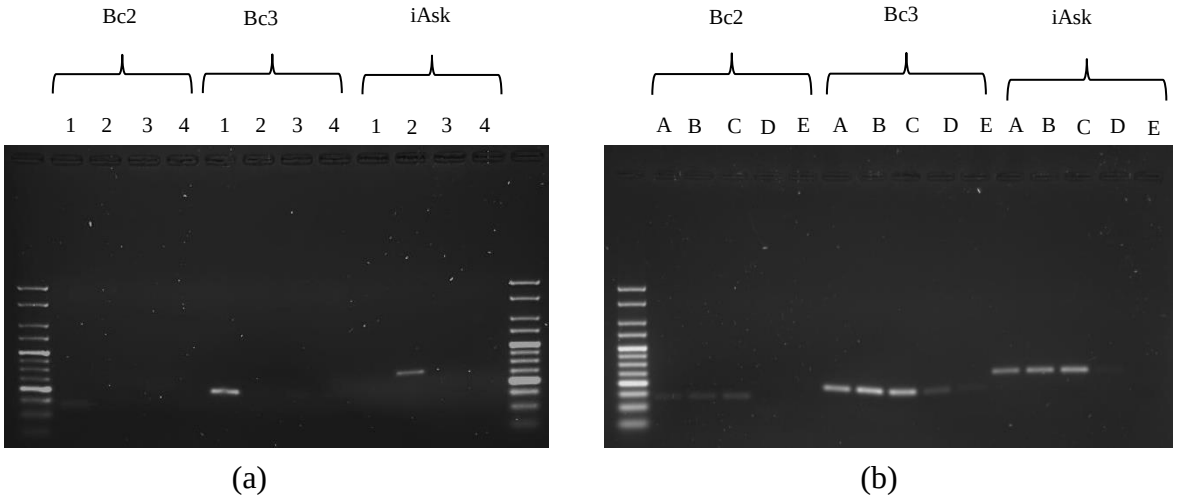


(b)

Figure 3-3: Number of tissues having *B. cinerea* infection in each plant organ in each treatment at different growth stages (X axis). (a) In non-surface disinfected tissues, (b) in surface disinfected tissues. In each treatment 1 root, 3 rosette leaves, 2 stem pieces, 2 stem leaves and 2 inflorescences were sampled from six plants.

3.3.1.2 Quantification by qPCR method

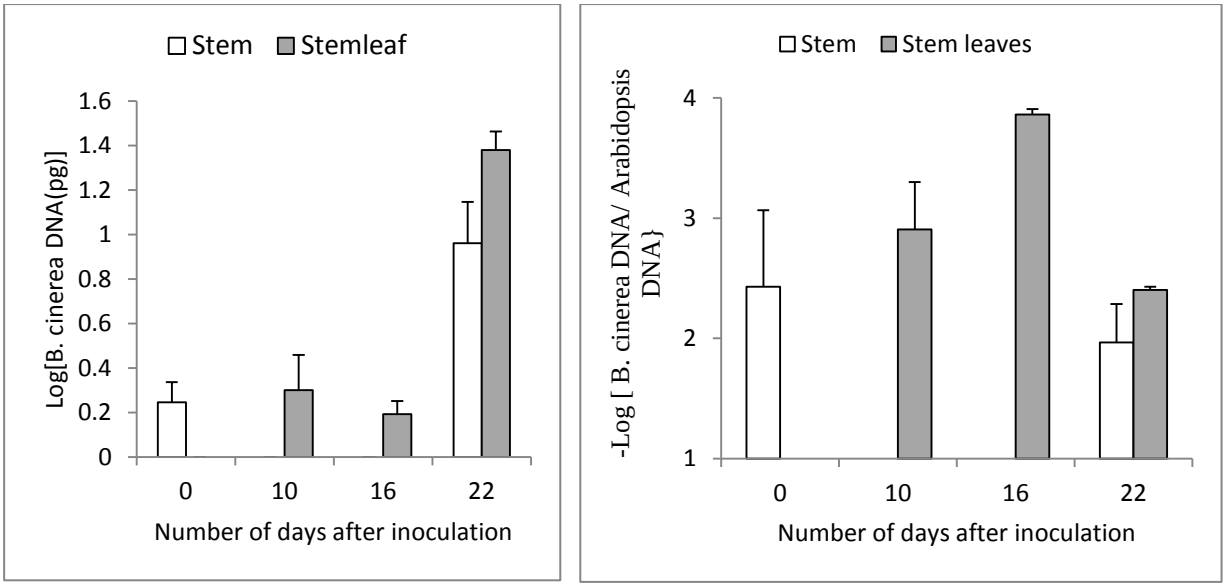
Among the two primer pairs tested for *B. cinerea*, Bc3 and Bc2, the primer pair Bc3 produced bright bands in gel electrophoresis of PCR products (Figure 3-4). This was presumably because the IGS marker had more copies than the SCAR marker. Therefore, Bc3 was used in qPCR amplification. Both *B. cinerea* and *A. thaliana* PCR products produced the brightest bands at 60 °C. Therefore, 60 °C was used as annealing temperature in qPCR.



(a) (b)

Figure 3-4: Gel electrophoresis of PCR amplicons from test DNA samples. (A) Specificity of the three primers Bc2, Bc3 and iASK against DNA of 1- B. cinerea, 2-*A. thaliana*, 3-Trichoderma and 4-control without DNA. (B) PCR products with different annealing temperature A-50 °C, B-55 °C, C-60 °C, D-65 °C, E- control without DNA at 55 °C.

Plants inoculated after 22 day of sowing had more ($p < 0.001$) *B. cinerea* DNA in their stem and stem leaves than plants inoculated at other growth stages (Figure 3-5).



(a) (b)

Figure 3-5: Quantity of DNA in stem and stem leaf of plants inoculated at different growth stage. (a) *B. cinerea* DNA concentration, (b) ratio between *B. cinerea* DNA concentration and plant DNA concentration in respective 40 ng DNA samples, the ratio was negative in value. Two biological replicates were maintained for each treatment. Error bars denote standard error.

3.3.2 Variation in causing symptomless infections in different *B. cinerea* isolates

The radial mycelial growth of the *B. cinerea* isolates was compared when they grew in either complete dark or 16h light day light. At 24h incubation, diameter of radial mycelial growth was similar among the isolates in both growing conditions. However, it varied at 48h and 72h incubation. At 48h incubation the diameter varied greatly among the isolates ($F_{4,30}$ =315, $p<0.001$) among growing conditions ($F_{1,30}$ =43, $p<0.001$) and to a lesser extent due to the interaction of isolates and growing conditions ($F_{4,30}$ =19.10, $p<0.001$) (Figure 3-6, Figure 3-7).

The pattern of initial sclerotia development also varied between isolates (Figure 3-8). All isolates produced rings of sclerotia, however, the size, numbers of rings, and position of ring in the culture varied between isolates (Figure 3-9).

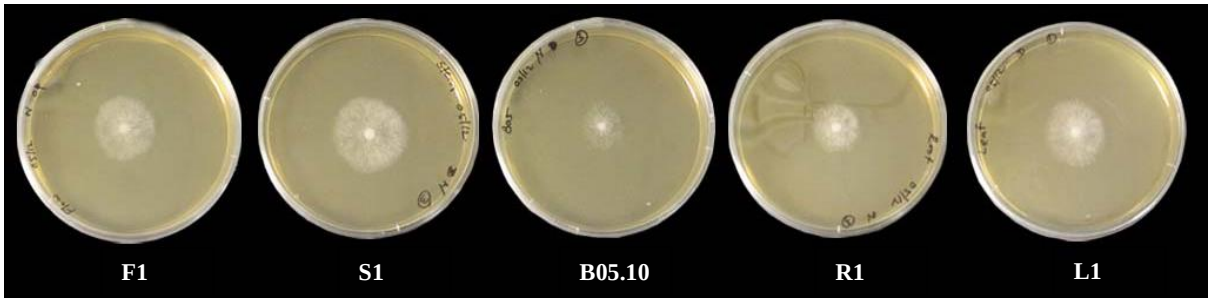


Figure 3-6: Radial mycelial growth of isolates of *B. cinerea* growing in complete dark after 48 h of incubation. The diameter of radial mycelial growth varied among the isolates. F1-flower isolate, S1-stem isolate, R1-root isolate, L1-leaf isolate. The S1and B05.10 isolates showed greatest and smallest diameters respectively**.**

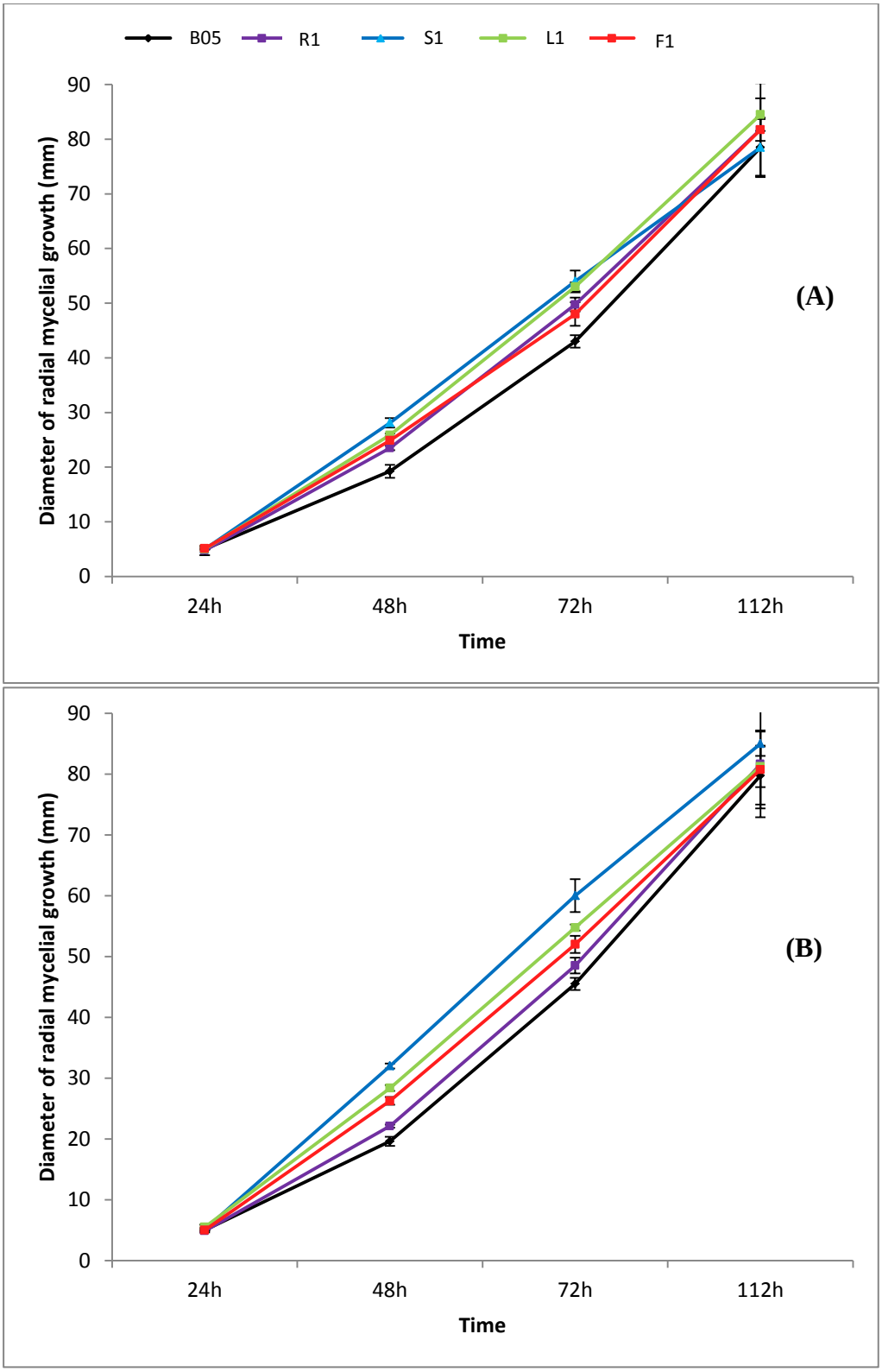


Figure 3-7: Radial mycelial growth of *B. cinerea* isolates growing in (A)16h light period, (B) complete dark. Four replicates were maintained for each isolate.

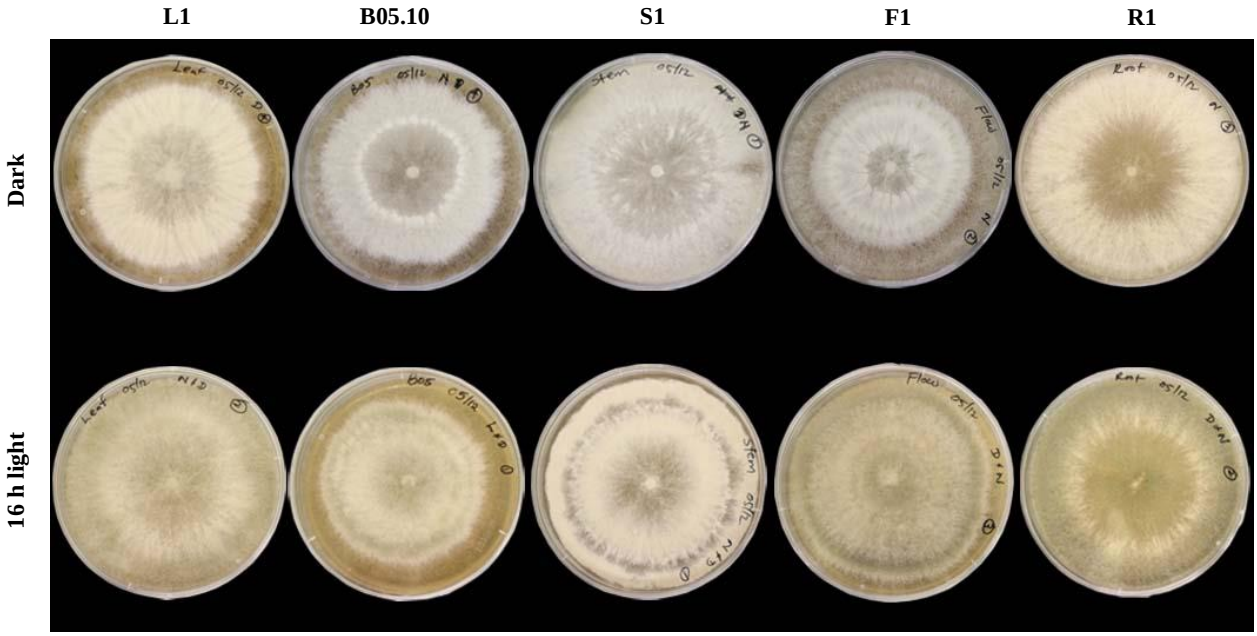


Figure 3-8: Radial mycelial growth of isolates of *B. cinerea* growing in complete dark and 16 hours light after six days of incubation. The isolates starting to produce sclerotia and spores in complete dark and 16h day light conditions respectively.

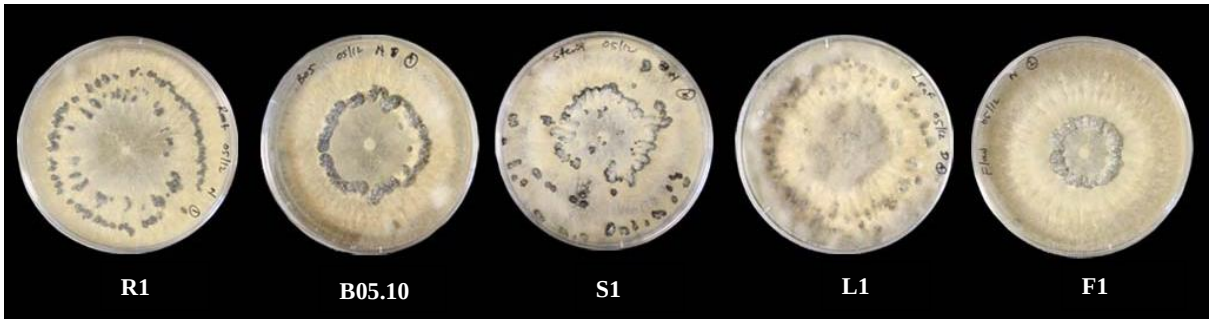


Figure 3-9: Pattern of sclerotial development in isolates of *B. cinerea* after 22 days of incubation in dark. All the isolates tended to produce rings of sclerotia, however, size and arrangement differed.

All isolates caused symptomatic infection on detached *A. thaliana* leaves (Table 3-2) when inoculated in PDB. The speed of symptom development was similar among isolates (chi-square =1.14, 12 d.f., P=1.0), although at 72 hours incubation root and stem isolates showed infection on more leaves than the other three isolates. At 144 hours incubation, all the isolates caused necrotic symptoms in nine or ten leaves out of ten sampled.

Table 3-2: Comparison of necrotic symptom development in detached leaves at different times after inoculation

B. cinerea **isolates**	**Number of leaves having symptom at different times**			
	72h	96h	120h	144h
BO5.10	7	7	8	9
R1	9	9	10	10
L1	6	9	9	9
S1	9	9	9	9
F1	6	7	8	10

The plants inoculated with different isolates of *B. cinerea* revealed that in all cases *B. cinerea* was recovered more often from NSD tissue samples. The proportion of non-symptomatic infection was significantly different between isolates, both NSD samples (chi-square =64.73, 4 d.f., $P<0.001$) and SD samples (chi-square =88.76, 4 d.f., $P<0.001$). Much the most infection was found in B05.10 (Figure 3-10).

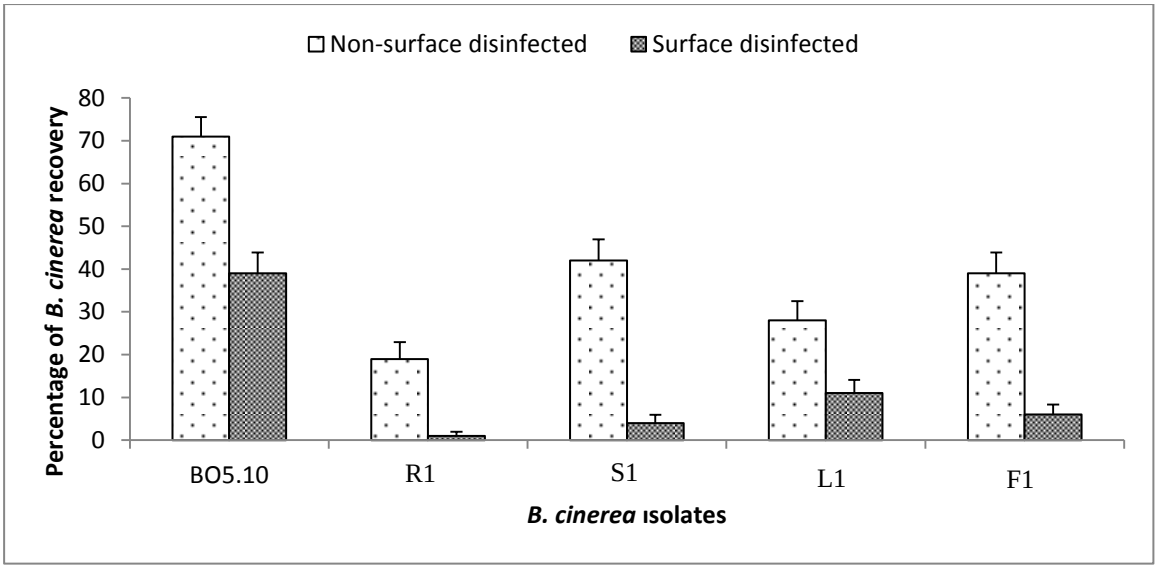


Figure 3-10: Recovery percentage of *B. cinerea* isolates from surface disinfected and non-surface disinfected tissue samples. Each isolate was inoculated on 10 plants, and a total of 100 tissue samples were tested for each isolate and disinfection method. Error bar denotes standard error.

The recovery of isolates varied between plant organs (Figure 3-11). The isolate B05.10 was recovered from rosette leaves, stem, stem leaf and inflorescence; the percentage of recovery of this isolate from surface disinfected and non-surface disinfected tissues was higher than remaining other four isolates. The root isolate was recovered in the lowest percentage from all types of tissue. The rosette leaves were the most infected plant part; all the isolates produce internal and external infection. The least infection was found in root tissues.

In some plants, for example plant numbers 1, 3, 4 and 9, B05.10 was frequently recovered from both NSD and SD tissues (Figure 3-12). In these plants fungus was recovered from stem, stem leaves and flowers, which all formed after inoculation. In plants 2, 5 and 6, B05.10 was mostly recovered from NSD tissues. Repeated random assignment of infected samples to the plants and calculation of the resulting test statistic (Barnes and Shaw, 2003) revealed that multiple *B. cinerea* infections on the same plant occurred more often than expected by chance (for NSD P=0.03 and SD P=0.004).

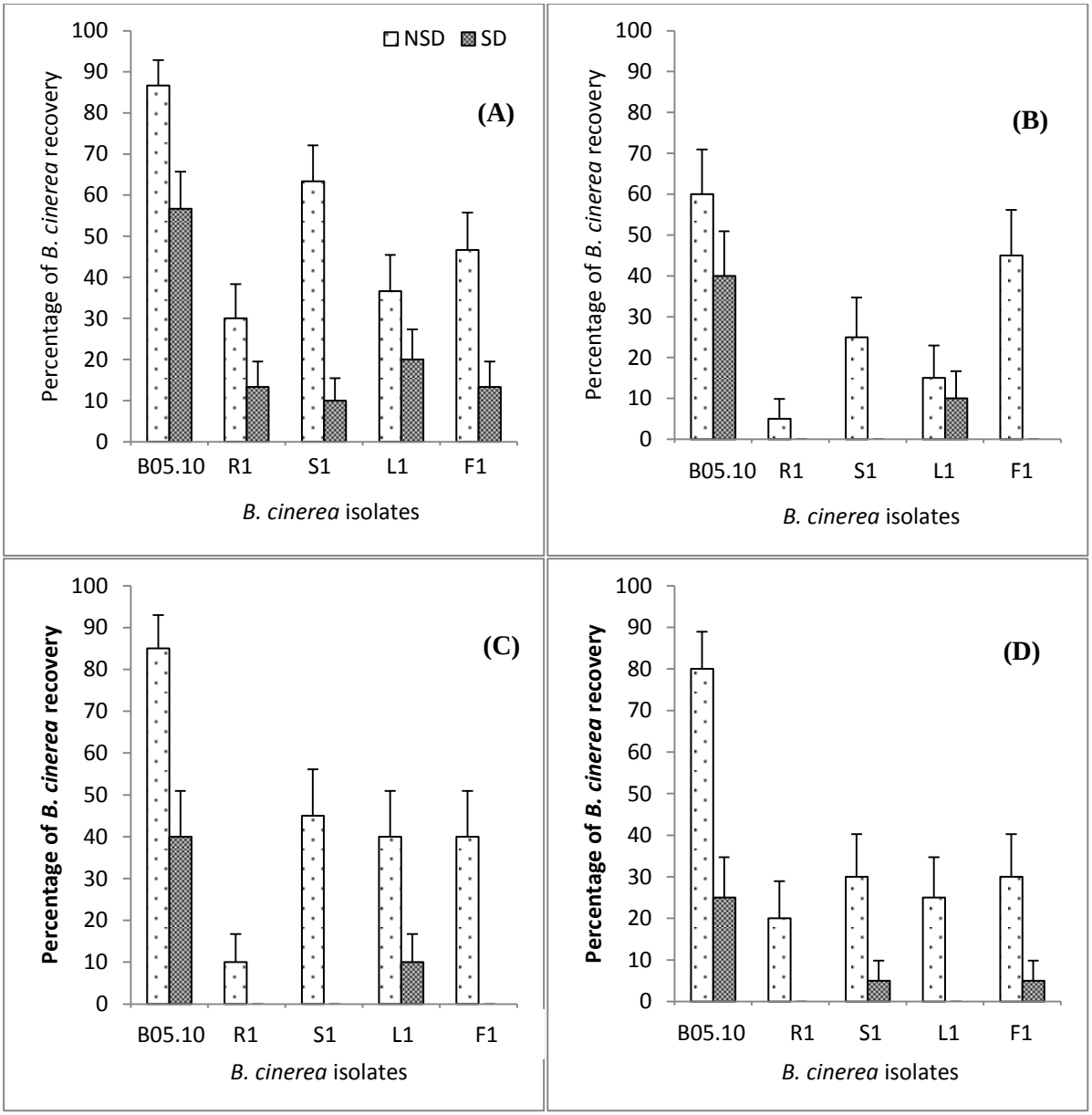
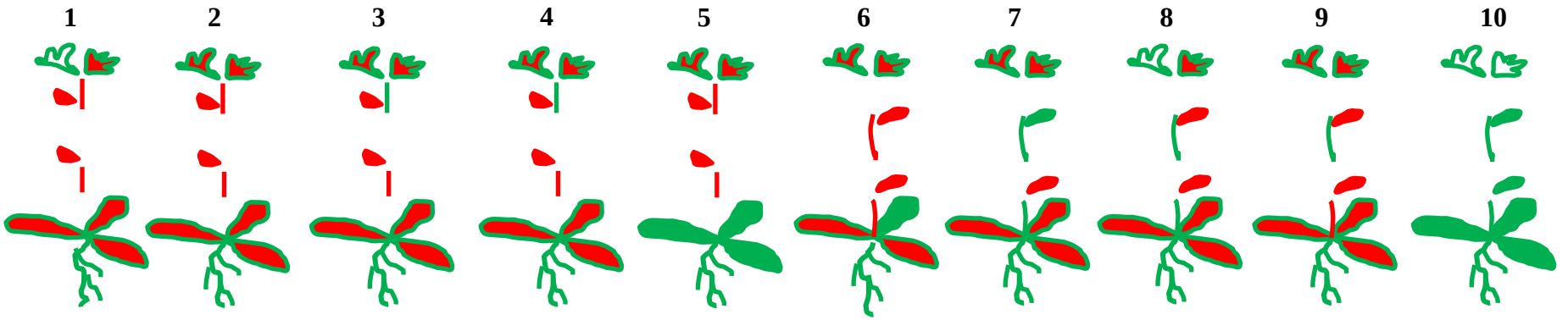
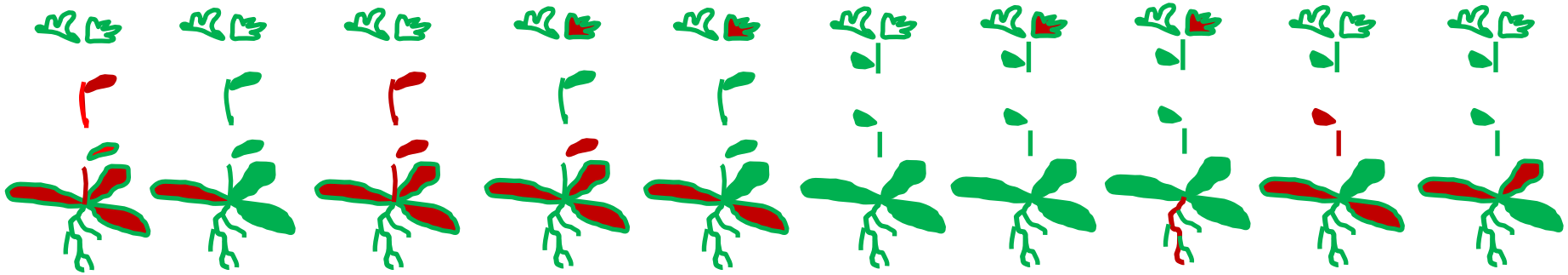


Figure 3-11: Recovery percentage of *B. cinerea* isolates from surface disinfected and non-surface disinfected different plant parts of *A. thaliana*. Recovery varied among the isolates (A) rosette leaves NSD (chi-square =24.98, 4 d.f., p<0.001), SD (chi-square =25.63, 4 d.f., p<0.001), (B) Stem NSD (chi-square =19.05, 4 d.f., p<0.001), SD (chi-square =26.66, 4 d.f., p<0.001), (C) Stem leaves NSD (chi-square =23.30, 4 d.f., p<0.001), SD (chi-square =26.6, 4 d.f., p<0.001), (D) inflorescence (chi-square =20.42, 4 d.f., p<0.001), SD (chi-square =13.21, 4 d.f., p=0.01). Error bars denote standard error.



(A) Non-surface disinfected tissues



(B) Surface disinfected tissues

Figure 3-12: Recovery of isolate B05.10 from ten independently inoculated plants. Non-surface disinfected and surface disinfected tissues were sampled from each plant. Tissues are arranged in the order of sampling and the red colour indicates the tissues having *B. cinerea* infection.

3.4 Discussion

The results confirm that *B. cinerea* can cause non-symptomatic infection on *A. thaliana* plants and during that infection it spreads through the plant body, especially on aerial plant parts.

The present experiments firstly focused to find a suitable growth stage of *A. thaliana* that commonly produced symptomless infection. All plants were inoculated as seed or during rosette growth. *B. cinerea* was subsequently detected in plant parts which formed after inoculation: stem, stem leaf and inflorescence; recovery of *B. cinerea* from one sample from a plant also made it more likely that other samples from the same plant would contain the fungus. This shows that the fungus spreads extensively on or in the plant from inoculated sites. In addition *B. cinerea* was recovered from surface disinfected tissues, which implies internal infection by the fungus.

The *B. cinerea* symptomless infection on *A. thaliana* clearly differs from the quiescent infection expressed in fruits such as grapes and strawberry (*Fragaria* × *ananassa*). In quiescent infection, host defences limit the spread of pathogen, so the pathogen enters a quiescent phase in the host tissue, and remains localized until the host defences decline (Elmer & Reglinski, 2006). But, in symptomless *A. thaliana*, *B. cinerea* grows with the plant and spreads to new developing plant parts.

A. thaliana were grown in isolated propagators with filtered air supplies. The plants had minimal chances to get inoculum from natural environment and through contamination. Barnes & Shaw (2003) studies in symptomless infection of *B. cinerea* in *Primula* x *polyantha*, and later Sowley (2006) in lettuce successfully used similar isolation propagators.

Under natural conditions *B. cinerea* may produce spores, and may spread to newly developing plant parts. Therefore, plants growing in isolated propagators were closely monitored to check that spore bearing structures were absent. Before sampling, plants were thoroughly monitored to check they were free from necrotic lesions; only apparently healthy plants were plated on

selective medium. In the present experiment none of the plants had visible aggressive *B. cinerea* infection while they grew inside the isolated propagators.

Seed borne symptomless infection of *B. cinerea* has been reported in both *Primula* x *polyantha* (Barnes and Shaw, 2003) and in lettuce (Sowley, 2006). In *A. thaliana*, seeds are very minute; the chances of fungus attaching or invading are less. Nonetheless, in the present experiment *A. thaliana* seeds were surface disinfected prior to sowing and some of them were plated on Botrytis selective medium to confirm absence of *B. cinerea*.

Both BSM sampling and DNA quantification revealed that late rosette growth, especially at 22 day of sowing, was the most vulnerable stage for the development of non-symptomatic infection.

In BSM sampling, plant tissue is placed on a selective medium which kills the plant tissue and removes many competitors for the fungus. However, if the fungal structure in the tissue is not viable or if there is competition with a contaminant like *Trichoderma*, then the tissue may not show *B. cinerea* growth. The intensity of surface disinfection is also a critical factor especially for the fungus growing inside the plant body but very close to outer surface.

In BSM sampling a higher percentage of infection was detected in non-surface disinfected tissues than surface disinfected. However, the recovery of infection in non-surface disinfected tissues could originate from *B. cinerea* growing either inside or outside the plant body. On the other hand, surface disinfected tissues may also have had external *B. cinerea* structures on or just below the surface before the disinfection.

Likewise, qPCR quantification gives the amount of *B. cinerea* DNA in the tissue; this includes fungus growing both in and on the plant body. The primer pair BC3 amplify IGS region in rDNA, the region is known to evolve faster and be useful in discriminating more closely related species. Another advantage of IGS assay is that the target DNA is a multi-copy sequence, therefore it can detect even trace amount of fungus (Suarez *et al.*, 2005).

The present experiments were also carried out to test the ability of different pathogenic isolates of *B. cinerea* to cause non-symptomatic infection in *A. thaliana* plants. Four of these isolates were isolated from surface disinfected tissues of wild growing healthy *A. thaliana* plant parts, stem, leaf, root and inflorescence, so they were growing endophytically in their *A. thaliana* host plants. The fifth isolate, B05.10, is a standard *B. cinerea* culture; it has been used in many research studies, and its genome has been sequenced recently.

The comparison of morphology, growth rate and detached leaf inoculations revealed that the isolates were phenotypically distinct. The endopytic isolates did not grow more slowly or show other differences from B05.10 – originally from a necrotic infection. The extent of non-symptomatic infection differed among isolates. Isolate B05.10 had the highest percentage of non-symptomatic infection on *A. thaliana* under the growing and inoculation conditions used.

Based on the observation of individual plants inoculated with isolate B05.10, the fungus mostly preferred to spread on the outer surface of the plant body and in certain points it entered inside the plant body. However, detailed microscopic study is necessary to confirm these observations.

Chapter 4 Amount of mRNA of selected genes of *B. cinerea* during symptomless growth

4.1 Introduction

In order to have successful disease development, a fungus must have evolved strategies to sense the host surface, penetrate and invade plant tissue, and overcome host defences. The pathogen should be capable of perceiving the signals from host plant and respond with the appropriate metabolic activities for disease development. *B. cinerea* is equipped with a range of enzymes and metabolites that allow the fungus to invade host tissue, kill host cells and finally to convert host tissue into fungal biomass (Kars & Van Kan, 2007).

Extensive studies have been done by several research groups to find out the role of signalling genes and pathogenicity genes in *B. cinerea* infection. Most of the findings are based on the studies conducted during the necrotrophic mode of growth of *B. cinerea*. However, several studies have revealed that *B. cinerea* can also cause symptomless and distributed infection in host plants. There is a need to test whether the fungus shows any difference in gene expression profile during necrotrophic mode of growth and symptomless growth. Therefore, the present study tried to quantify expression of selected genes during symptomless infection, and to check whether the pattern of expression was similar or different from well-known necrotic infection.

In *B. cinerea*, several signalling networks, such as the G protein mediated signalling pathway, cyclic adenosine monophosphate (cAMP) signalling, mitogen activated protein kinase (MAPK) cascades and the Ca^{2+}/calmodulin signalling pathway, trigger the expression of appropriate genes (Tudzynski & Schulze Gronover, 2007).

Heterotrimeric guanine nucleotide binding proteins (G-proteins) regulate a variety of cellular functions in eukaryotic cells. These proteins transfer signals between activated cell surface receptors and intracellular effectors (Tudzynski & Schulze Gronover, 2007). In *B. cinerea* three Gα subunit genes, *bcg1*, *bcg2* and *bcg3*, have been identified. Among these genes *bcg1* plays a crucial role in pathogenicity. It controls expression of the genes which are involved in the transition of the infection process from primary infection to secondary invasive growth, the production of phytotoxins such as botrydial and botcinolides, and the secretion of proteases (Gronover *et al.*, 2001; Schumacher *et al.*, 2008). Suppression subtractive hybridization approaches have revealed that, *in planta* about 20 genes are down-regulated in gene *bcg1* defective mutants (*Δbcg1*). This includes genes encoding putative proteases, xylanases and genes involved in secondary metabolism, including botrydial synthesis. The mutants are able to conidiate, penetrate host leaves and produce primary lesions, but failed to produce spreading secondary lesions (Gronover *et al.*, 2001).

Microarray studies revealed that the botrydial gene cluster is controlled by the calcinurin dependent pathway (Viaud *et al.*, 2003). In the Bcg1 mutant some of the defects can be restored by increasing the supply of cAMP. This demonstrates that Bcg1 is involved in a cAMP dependent pathway (Gronover *et al.*, 2001). However, the mutant still had defects in secretion of proteases and botrydial even in the presence of an external cAMP supply. So it is clear that the gene *bcg1* controls another signalling pathway besides the cAMP one.

The cAMP dependent signalling pathway regulates several important processes, such as morphogenesis, differentiation and virulence. Adenylate cyclase, an enzyme coded by gene *bac*, catalyses production of cAMP from ATP. In *Magnaporthe grisea*, adenylate cyclase deletion mutants lost their ability to form appressoria and to penetrate host tissue (Choi & Dean, 1997). In *B. cinerea* the cAMP signalling pathway plays an important role in vegetative growth and pathogenicity. For example, the mutant *Δbac* penetrated the leaves of *Phaseolus*

vulgaris and caused spreading soft rot lesions, but the process was slower than the wild type controls (Klimpel *et al.*, 2002).

RT-PCR studies revealed that in *B. cinerea* gene *bac* expression starts very early in the necrosis development (12h post inoculation) and lasts until at least 48h after inoculation, when spreading soft rot lesions start to enlarge out from primary necrotic spots (Klimpel *et al.*, 2002).

In addition to the G protein α subunit and components involved in the cAMP signalling pathway filamentous fungi usually contain three mitogen activated protein kinases (MAP kinases). In *B. cinerea* MAP kinases are characterized into BMP1, BcSak1 and Bmp3. Bmp1 is responsible for initial stages of infection process, including differentiation of germlings and penetration. *Bmp1* deletion mutants can germinate on plant surface, but do not produce appressoria and have totally lost their ability to penetrate host tissue. Even on wounded plants they are unable to develop necrotic spots (Zheng *et al.*, 2000). Therefore, it is clear that the expression of *bmp1* is essential in disease development for surface sensing and morphogenesis of infection structures.

The BcSak1 mediated pathway allows responses to osmotic and oxidative stresses. The deletion mutant (*Δbcsak1*) is still pathogenic as wild type in *M. grisea* and *Colletotrichum lagenarium*, but in *B. cinerea* the mutant is much less pathogenic (Segmuller *et al.*, 2007).

Calcineurin (CN) is a highly conserved Ca^{2+}/calmodulin protein phosphate. It is crucial for mediating cellular stress responses (Harren *et al.*, 2012). Inactive calcineurin forms a heterodimer, consisting of a catalytic (CNA) and a regulatory (CNB) subunit. Activation occurs when Ca^{2+}/calmodulin binds to the CAN, resulting in conformational changes that release the active site from the auto-inhibiting domain (Schumacher *et al.*, 2008). In the plant pathogen *Sclerotinia sclerotiorum* (closely related to *B. cinerea*), calcineurin plays a major

role in sclerotial development, pathogenicity and cell wall integrity (Harel *et al.*, 2006). *In B. cinerea* cyclosporine A mediated inhibition of calcineurin modified hyphal morphology and prevented the development of infection structures (Viaud *et al.*, 2003).

Harren *et al.* (2012) found that deletion mutation in the gene coding for the catalytic subunit of calcineurin (*ΔbccnA*) caused severe growth defects, no conidia formation and avirulence. cDNA microarray comparison of the gene expression pattern of wild type and the *Δbcg1* mutant, non-treated or treated with calcineurin inhibitor cyclosporin A showed that the production of the phytotoxin botrydial is controlled through cAMP independent signalling pathway of Gα of G protein and Ca^{2+}/ calcineurin dependent signalling pathway (Schumacher *et al.*, 2008).

The first barrier that has to be breached by the fungus during the infection process is the cuticle, a complex mixture of polymeric fatty acids. The fungal cutinases breaks down the cuticle as the first step in the infection process. The *bccutA* coding for cutinase enzyme in *B. cinerea*, is expressed during conidial germination and during penetration into epidermal cells. However, transformants lacking a functional *bccutA* gene behaved similarly to wild type during infection of gerbera flowers and tomato fruits (Van Kan *et al.* 1997).

The second barrier to pathogen invasion is the cell wall. *B. cinerea* produces a variety of cell wall degrading enzymes (CWDEs) to breach the plant cell wall. In particular, *B. cinerea* produces a variety of pectinases, including exo- and endo- polygalacturonases, pectin methylesterases, pectin and pectatelyase (Kars *et al.*, 2005a). The resulting by-products, such as pectin hydrolysates are a source of nutrients to the fungus (Nakajima & Akutsu, 2014). Pectin hydrolysis weakens the cell wall to facilitate penetration and colonization. The various stages in the process are as follows.

Pectinmethylesterases make pectin available for degradation by polygalacturonases and pectatelyases by catalysing the demethylesterification of homogalacturonan (Nakajima & Akutsu, 2014). In *B. cinerea*, the gene *bcpme1* codes for the methylesterase and a mutant with defective *bcpme1* was less virulent on apple fruits, grapevines and *A. thaliana* leaves (Valette-collet *et al.*, 2003). However, Kars *et al.* (2005b) found that the role of *bcpme1* in pathogenicity is a strain dependent feature; the *B. cinerea* isolate B05.10 did not show any reduction in virulence when *bcpme1* was deleted.

Endopoly-galacturonases (PGs) cleave the linkages between D-galacturonic acid residues in non-methylated homogalacturonan and cause cell separation and tissue maceration, resulting in soft rot symptoms. In *B. cinerea* PGs are encoded by at least six *bcpg* genes (*bcpg1* to *bcpg6*). Within this family, the *bcpg1* gene is constitutively expressed and is required for full virulence (Manfredini *et al.*, 2005). In particular, its activity is required during colonization to breach the pectin network through the middle lamella (ten Have *et al.*, 1998). Although the mutant *Δbcpg1* was pathogenic and produced primary lesions similar to wild type, a significant decrease was noticed in growth of the lesion beyond the inoculation spot. *B. cinerea* endo-polygalacturonase 1 (*bcpg1*) also acts as an elicitor of defence responses in grape and bean leaves (Vandelle *et al.*, 2006).

Kars *et al.* (2005a) found that deletion of the *bcpg2* gene resulted in much reduced virulence on tomato and broad bean. Primary lesion formation was delayed by about 24h and the lesion expansion rate was reduced by 50-85%. The protein coded by the gene *bcpg2* is involved in penetration by breaching the pectin network which localized at the anticlinal cell wall.

Xylan is an abundant hemicellulose in plant cell walls. Fungal endo β-1,4-xylanases carry out the initial breakdown. Deletion of the gene in *B. cinerea* had an effect on fungal virulence, delayed secondary lesion development and reduced average lesion size (Brito *et al.*, 2006),

and this was assumed to be due to its xylanase activity. But later Noda *et al.* (2010) found that xylanase has a direct necrotizing activity on plants: the gene *xynll A* contributes to the infection process through its necrotizing activity and not through its xylan hydrolysing activity.

After penetration, *B. cinerea* kills epidermal and mesophyll cells in advance of invasion by infection hyphae (Nakajima & Akutsu, 2014). *B. cinerea* can produce several types of phytotoxic metabolites of low molecular weight. The best studied phytotoxin is botrydial which induces chlorosis and cell collapse, which seem to facilitate both penetration and colonization (Van Kan, 2006). The genes involved in botrydial synthesis have been identified and are organized in a cluster containing at least two cytochrome P450 monooxygenase genes and a terpene cyclase gene. One of the cytochrome P450 monooxygenases is encoded by the *bcbot1* gene (Siewers *et al.*, 2005). The gene *bcbot1* shows maximum expression at the change from primary to secondary lesion. However, deletion of *bcbot1* gene in three standard strains of *B. cinerea* shows that the effect on virulence is strain dependent (Siewers *et al.*, 2005). Therefore, the gene plays a role, but not a critical one, in fungal spreading in the host tissue (Gronover *et al.*, 2004).

The oxidative burst is a common plant response following pathogen infection. However, studies with *B. cinerea* infection on *A. thaliana* and tobacco revealed that *B. cinerea* may need the plant based programmed cell death to achieve full pathogenicity (Govrin & Levine, 2000). In *B. cinerea* infection, reactive oxygen species produced by the fungus also contribute to the oxidative burst. Hydrogen peroxide (H_2O_2) accumulates in the early stages of infection process, both in germinating spores and in the infection cushion (Choquer *et al.*, 2007). *B. cinerea* actively triggers an oxidative burst during cuticle penetration and primary lesion formation. Superoxide dismutase (Bcsod1) plays an important role in evoking the oxidative burst and deletion of the *bcsod1* gene led to reduced virulence on multiple hosts

(Van Kan, 2006; Williamson & Tudzynski, 2007). Plant cell death also occurs through an oxidative burst produced by the host itself as part of programmed cell death in reaction to *B. cinerea* attack.

Nep1-like proteins (NLPs) induce necrosis and ethylene production in dicotyledonous plants. In *B. cinerea* there are two genes, *bcnep1* and *bcnep2*, coding for NLPs. In the host, purified NLPs in low concentration can induce callose apposition, accumulation of reactive oxygen species and ethylene, and the activation of other genes involved in stress and defence response. At higher concentrations they induce cell death at the site of application (Schouten *et al.*, 2008). The two genes seem to have different roles: Arenas *et al.* (2010) detected transcripts of *bcnep1* very soon after fungal inoculation, coinciding with the formation of primary lesions, whereas transcripts of *bcnep2* were detected from the onset of lesion expansion and increased with fungal biomass (Arenas *et al.*, 2010).

In this study I measured the amount of mRNA of some signalling genes (*bcg1*, *bac*, *bmp1* and *bcn*) and pathogenicity related genes (*bcbot1*, *bcnep1*, *bcsod1*, *bcpg1* and *bcpg2*) by RT-qPCR. The choice of these genes was based on their key role in disease development as described in previous paragraphs, availability of sequence information to design specific primers, and most importantly these are single copy genes in *B. cinerea* genome. The amount of mRNA was quantified at several times after infection in symptomless host plants (*A. thaliana* and lettuce). I also compared the amount of mRNA of those genes during symptomless infection and symptomatic necrotrophic infection. Tissue samples for the symptomatic necrotrophic infection of lettuce were collected from two sources: one was direct necrotrophic infection by inoculation with a spore suspension; the second was delayed necrotrophic infection which developed following the symptomless phase of infection.

4.2 Methodology

4.2.1 *Lactuca sativa* (Lettuce)

4.2.1.1 Growing host plants

Lettuce (*Lactuca sativa*, varieties Tom Thumb and All Year Round) was used as host plant in this study. Commercial seeds were obtained from local garden centre (Wyevale Garden Centre, Winnersh). Two seedling trays were filled with compost (Levington F1 seed and modular compost, UK), and in each tray 80 seeds were sown. The seed germination and initial seedling growth was in controlled environment chambers: 20° C in 12 h day and 18°C in night, RH 65%. The compost was kept wet all the time. Seedling transplantation was done seven days after inoculation (14 days after seed sowing); with each lettuce variety 40 inoculated and 40 non-inoculated seedlings were transplanted into 1L pots filled with potting compost (1L compost + 4g Osmacote). Later the seedlings were moved to a glasshouse, where the temperature was 18° C in night, rising to about 30° C in day time. The plants were watered every day.

4.2.1.2 Fungus inoculation

4.2.1.2.1 Fungus isolate

The isolate B05.10 of *Botrytis cinerea* was used in this experiment. The culture was kindly provided by Dr. Henk-jan Schoonbeek (John Innes Centre, Norwich). The isolate was sub cultured on malt extract agar plates and incubated at 20 °C in 16 hours light in near-UV light incubators.

4.2.1.2.2 Seedling inoculation

At the four leaf stage, one tray of seedlings was dust inoculated with 20 days old *B. cinerea* sporulating cultures; inverted culture plates were tapped uniformly above the seedlings. The

seedlings were covered with transparent polyethylene bags overnight. The average density of spores settled on seedlings was determined: cellulose nitrate discs were placed among seedlings before spore dusting, and the spores settled on the discs counted under microscope. On average 90 ± 10 spores were dusted per mm^2 leaf surface.

4.2.1.2.3 Detached leaf inoculation

B. cinerea spore suspension was prepared from a 20 days old culture, and the final concentration adjusted to $2x10^5$ spores / ml in half strength (1.2 g / 100 ml) potato dextrose broth (PDB) (Oxoid, UK). Healthy lettuce leaves were harvested from 4 weeks old plant and kept in humid transparent boxes. Humidity inside the boxes was maintained at a high level by placing sterilized water soaked paper towels were placed inside the boxes. Eight drops, each with 10 µl of spore suspension, were placed on each leaf at about 2cm intervals. The humid chambers were closed and kept air-tight on the laboratory bench near to the window; the samples were exposed to about 10 h day light and the room temperature ranged from 16 to 23 °C. The experiment was repeated twice.

4.2.1.3 Sample collection

4.2.1.3.1 From symptomless plants

The first sampling was done 2 days after inoculation at seedling stage (six leaves stage); five seedlings were uprooted, and the roots were cleaned in running water and blotted dry. Freeze dried seedlings were stored at -80 °C.

A further three samplings of symptomless tissues were done, after transplantation, as shown in Table 4-1. In each sampling, ten apparently healthy plants were uprooted and leaves were collected as pairs in separate freezer bags. Leaf samples were also collected from control plants. From each leaf about 1.5 cm x 1.5 cm sections were made and placed on Botrytis

selective medium to isolate *B. cinerea* if present. The freeze dried samples were stored at -80 °C.

Ten days after the fourth sampling, 44 days after inoculation, some of the treated plants had necrotic lesions and sporulating mycelium of *Botrytis cinerea*: especially on the older leaves. The infections developed in these plants were considered as necrotic infection developed through symptomless infection. Sampling of these tissues was done from two infected plants.

Table 4-1: Samples collected from lettuce plants inoculated as 2-leaf seedlings with dry spores of *B. cinerea* and grown in greenhouse conditions.

Sampling	Time (days after inoculation)	Leaf sampling for gene expression studies (position of the leaf in order of leaf expansion)
2nd	14	5^{th} and 6^{th}, 9^{th} and 10^{th}
3rd	24	5^{th} and 6^{th}, 9^{th} and 10^{th} , 13^{th} and 14^{th}
4th	34	5^{th} and 6^{th}, 9^{th} and 10^{th} , 13^{th} and 14^{th}, 17^{th} and 18^{th}

4.2.1.3.2 From detached leaves

The leaves inoculated with spore suspension were sampled at 12 h, 24 h and 48 h after inoculation. The portion of inoculated leaf tissue was excised and collected in Falcon tubes. The freeze dried samples were stored at -80 °C.

4.2.2 *A. thaliana*

4.2.2.1 Growing *A. thaliana*

Arabidopsis thaliana (wild type, Columbia ecotype) seeds were surface disinfected in 70% ethanol for 2 min and then 20% bleach (1% NaOCl) for 5 min and finally thoroughly rinsed in sterile water 5 to 6 times. After surface disinfection seed stratification was done at 4 °C for four days.

Individual plants growing in the same growth chamber were isolated from each other and the outside air as explained in chapter 2. The plants were grown in a controlled environment chamber at 22 °C day and 18 °C night temperature, 65% relative humidity, 16 h light and 8 h dark period, 200-250 µmol/m^2/s light intensity. The plants were watered every day sufficiently to keep the compost just moist up to two weeks from sowing, then at two-day intervals. Inside the isolation propagator, the average day time temperature was 26.5 °C and night 18.5 °C; relative humidity in day and night ranged between 80% to 85%. The dew point temperature during the day was about 22 °C and at night about 16 °C and light intensity was between 220 and to 170 µmol/m^2/s.

4.2.2.2 Fungus inoculation

The isolate B05.10 of *Botrytis cinerea* was used in this experiment. The culture was kindly provided by Dr. Henk-jan Schoonbeek (John Innes Centre, Norwich). The isolate was sub cultured on malt extract agar plates and incubated at 20 °C in 16 hours light in near-UV light incubators.

4.2.2.2.1 Developing symptomless infection

Inoculation was done using a spore dusting method. *B. cinerea* spores were collected from 15-20 day old cultures by using a cyclone spore collector. 10 mg spores were thoroughly mixed

with 90 mg of talc powder with a spatula and a tenfold spore serial dilution was carried out up to a notional 10^{-5} dilution.

5 mg of diluted spores were dusted on plants 21 days after sowing. Controls were dusted with talc powder only. Inoculation was done through a small opening present on the transparent lid which covered the plant (already made on the side of the lid, but sealed with sticky tape after sowing until needed). The spore-talc mix was taken on a small spatula and then the mix was dispersed into the air above the plant by a pulse of air ejected from a syringe through the syringe needle. Finally the small opening was again sealed with sticky tape.10 replicates were maintained for treatment and control.

4.2.2.2.2 Developing necrotic infection

B. cinerea spore suspension was prepared as for lettuce. Ten healthy *A. thaliana* plants were taken; the plants were 20 days old and growing in controlled environment as mentioned for symptomless infection, but these plants were not connected to a closed air circulation unit. Rosette leaves were inoculated with spore suspension while the leaves have attached to the plants. Two drops, each with 10 µl of spore suspension, were placed on the leaf surface at 2cm interval. Later the plants were covered with transparent lids to provide higher humidity. The experiment was repeated twice.

4.2.2.3 Sample collection

4.2.2.3.1 Sampling of symptomless tissues

Sampling was done 10 days after inoculation. The plant was uprooted, rosette leaves, flowering stem, root, stem leaves and flowers were collected in different falcon tubes. Some of the plant parts were sampled on Botrytis selective medium to recover symptomless growing *B. cinerea*. The rest of the samples were freeze dried and stored at - 80 °C.

4.2.2.3.2 Sampling of necrotic tissues

The inoculated rosette leaves were excised from *A. thaliana* plants at different time points; 3h, 6h, 12h, 24h and 48h. The samples were collected in falcon tubes, freeze dried samples were stored at - 80 °C.

4.2.3 RNA extraction and cDNA preparation

4.2.3.1.1 Necrotic infection

Lettuce leaves sampled at 12 h, 24 h and 48 h after spore suspension inoculation were used for the RNA extraction. The leaves were detached before inoculation. For *A. thaliana*, 3h, 6h, 12h, 24h and 48h post inoculated leaf samples were used. The leaves remained attached during the inoculation and sampling periods.

4.2.3.1.2 Symptomless infection

Frozen (-80°C) plants were selected for RNA extraction based on the recovery of *B. cinerea* from symptomless tissues on botrytis selective medium at the time of freezing.

Lettuce

Samples from the variety Tom Thumb were used because of its early response to symptomless infection. From each time point (2 days, 14 days, 24 days, 34 days and 44 days after inoculation) two different plants which had internal *B. cinerea* infection in most of the leaves were selected and used for the RNA extraction. For plants in 2^{nd}, 3^{rd} and 4^{th} samplings, RNA was extracted from leaves at different leaf positions as detailed in Table 4-1 (page 65).

A. thaliana

RNA was extracted from rosette leaves and stem samples. At each time point two plants that had the most internal infections, were used as biological replicates.

The RNA extraction was done by RNeasy plant mini kits (Qiagen, Hilden, Germany) following the manufacturer's protocol. DNase treatment was carried out in two stages. The first one was done by column treatment during the extraction procedure using Pure-link DNase kits (Life Technologies, USA). After extraction, Turbo DNase kits (Life Technologies) were used for further purification. cDNA preparation was carried out using the High capacity RNA-to-cDNA kit (Applied Biosystems, USA). Each reaction used 1500 ng of total RNA.

4.2.4 qPCR quantification

Quantification was done by using qPCRBIO SyGreen Mix Hi-ROX kit (PCR Bio-systems, UK). The cDNA concentration of four signalling genes and five pathogenicity genes were determined using the gene *bcrpl5* as housekeeping gene (Table 4-2). The gene coding for β-tubulin was also tested as housekeeping (comparator) gene not involved in pathogenesis, but it was not detected consistently in symptomless samples, especially when the samples contained a lower amount of fungus. Therefore, only the gene *bcrpl5* was used as housekeeping gene. In each qPCR reaction 20 µl of reaction mix contained 10 µl master mix (qPCRBIO SyGreen Mix Hi-ROX), 0.8 µl of forward primer (10 µM), 0.8 µl of reverse primer (10 µM), 4 µl of cDNA sample (10 times diluted from original cDNA sample) and 4.4 µl of nuclease free water. qPCR assays for the lettuce samples and *A. thaliana* samples were carried out separately. qPCR reactions were carried out in a partially balanced block experimental design to reduce experimental error; the reactions for each technical replicate of

a sample were carried out in separate 96 well plates, and in each plate a subset of genes were tested using all the test extracts, so as to balance comparisons between genes over the whole experiment. Each plate included cDNA from all symptomless samples and necrotic samples of two biological replicates. The amounts of housekeeping gene (*bcrpl5*) and a random combination of four genes out of tested nine were quantified in each plate. The assays were carried out in triplicate using the following cyclic conditions: 95 °C for 2 min, then 40 cycles of 95 °C for 15s, 60 °C for 30s, using StepOnePlus™ Real-Time PCR System (Applied Biosystems, USA). After each run, melting curves were acquired by heating to 95 °C for 15s, cooling to 60 °C for 1min and heating to 95 °C at 0.3 °C, before holding at 95°C for 15s with data collection.

The cyclic threshold (Ct) value generated in each reaction revealed the copy number of mRNA of the respective gene in the sample. The Ct values are inversely proportional to the amount of mRNA in the sample. The ΔCt value of each gene of interest (signalling gene or pathogenicity related gene) per each sample was calculated using following equation.

ΔCt value of a gene = Ct value of housekeeping gene (*bcrpl5*) – Ct value of the gene

The mean ΔCt value for a gene was calculated by averaging the ΔCt values of two biological replicates of a sample.

4.2.5 Data analysis

Concentrations of mRNA were estimated relative to *bcrpl5*, using the restricted maximum likelihood (REML) commands of Genstat (VSNI, Wallingford, UK). The REML approach is a particular form of maximum likelihood estimation which does not base estimates on a maximum likelihood fit of all the information, but instead uses a likelihood function calculated from a transformed set of data, so that nuisance parameters have no effect. The transformation also allows consistent estimation of fixed effects when the design is not

completely balanced because of missing values or constraints on block size. The model used included random effects from technical (assay) replicates, differences between plants and differences between runs. Fixed effects were mRNA sequence nested within type of gene ('housekeeping', pathogenicity signalling, and pathogenicity factor), type of *B. cinerea* growth (necrotic, symptomless, symptomless leading to necrosis), and time. Appropriate subsets of the data were analysed where necessary.

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Table 4-2: List of primers used for the qPCR

Gene	Gene product	Accession numbers[1]	Sequence (5'-3')	Amplicon size (bp)
Bcg1	Alpha subunit of G1 protein	Y18436.1	F- CAAGATGCTTCTTCTTGGAG R- TGATTGGACTGTGTTGCTGA	139
Bmp1	Mitogen-activated protein kinase 1	AF205375.1	F- GCTTATGGTGTTGTCTGCTC R- TAGCTTCATCTCACGAAGTG	120
Bac	Adenylate cyclase	AJ276473.1	F- GGTGAAGACGGATAGATCAAGTAG R- CTCCCGTGGGGACACATTAG	121
Bcn	Calcineurin beta subunit	KC935338.1	F- GTCGAATCCTCTAGCTACCAGAA R- GAATGCGCTGAGTCCACTG	97
Bcsod1	Superoxide dismutase	AJ555872.1	F- ATTGAGCGTCATTGGCCGTA R- TGGACTCTTCGTTCTCTCCC	77
Bcpg1	Endo-Polygalacturonase 1	EF195782.1	F- ACTCTGCTGGAGATGCTGGT	97

			R- TAGCGAGACAGTAATCTTGG	
Bcbot1	Botrydial	AY277723.2	F– TTATGCCGCACTCCACGAGA	103
			R- TCCAGAGGAGTAGACCTCAT	
Bcnep1	Necrosis- and ethylene-	DQ211824.1	F- GTAATGGTAACACCAGTGGT	96
	inducing protein 1		R- AGCCACCTCGGACATAGGTT	
*Bcrpl5**	Ribosomal protein of		F-GATGAGACCGTCAAATGGTTC	
	large subunit		R- CAGAAGCCCACGTTACGACA	
*Bcpg2**	Endo-Polygalacturonase 2		F– GGAACTGCCACTTTTGGTTAC	
			R- TCCATCCCACCATCTTGCTC	

* cited from Zhang & Kan (2013). All primer pairs were designed to cross an exon- exon junction, except Bcpg1where no suitable introns were available.

[1]NCBI Accession numbers of DNA sequences.

4.3 Results

4.3.1 Lettuce

4.3.1.1 Incidence of *B. cinerea* in inoculated symptomless lettuce plants

In cultivar Tom Thumb the frequency of symptomless infected tissue samples was initially much greater than that in cultivar All Year Round, but the position was reversed by 34 days after inoculation (Tables 4-3, 4-4, 4-5).

Table 4-3: Number of lettuce samples, taken 14 days after inoculation, from which *B. cinerea* grew on selective medium

	Tom Thumb		All Year Round	
#Plant part	Non-surface disinfected	Surface disinfected	Non-surface disinfected	Surface disinfected
5th leaf	10*	3	7	1
7th leaf	10	5	8	2
9th leaf	10	4	7	1
Root	10	1	8	0

*The values are number of plants having symptomless infection out of a sample of 10 plants

Table 4-4: Number of lettuce samples, taken 24 days after inoculation, from which *B. cinerea* grew on selective medium

Tissue	Tom Thumb		All Year Round	
	Non-surface disinfected	Surface disinfected	Non-surface disinfected	Surface disinfected
9th leaf	7*	9	5	5
11th leaf	7	7	6	5
13th leaf	7	7	4	4
Stem	10	10	5	5
Root	8	1	7	2

*The values are number of plants having symptomless infection out of a sample of 10 plants

Table 4-5: Number of lettuce samples, taken 34 days after inoculation, from which *B. cinerea* grew on selective medium

Tissue	Tom Thumb		All Year Round	
	Non-surface disinfected	Surface disinfected	Non-surface disinfected	Surface disinfected
11th leaf	6*	0	10	2
13th leaf	4	0	8	1
15th leaf	3	0	5	1
17th leaf	2	1	7	2
Stem	6	4	9	7
Root	6	0	6	1

*The values are number of plants having symptomless infection out of a sample of 10 plants

4.3.1.2 Relative amount of mRNA in necrotic infections (direct necrotic infection) on detached lettuce leaves

4.3.1.2.1 Overall relative amounts of mRNA

The relative mRNA amounts of signalling and pathogenic genes analysed in this experiment (Table 4-2) varied (Figure 4-1). Among the signalling genes, the highest and lowest amounts of mRNA were detected for the genes *bcn* and *bac* respectively; there was no detectable difference between the mRNA level of *bcg1* and *bmp1*. Among the pathogenesis-related genes, the amount of *bcpg1* was highest. The mRNA coding for necrosis and ethylene inducing protein (*bcnep1*) was least abundant: about eight fold less than the housekeeping gene. There was no difference between the amounts of mRNA of genes coding for *bcbot1* and *bcpg2*; but they were less than the amount of mRNA of superoxide dismutase (*bcsod1*).

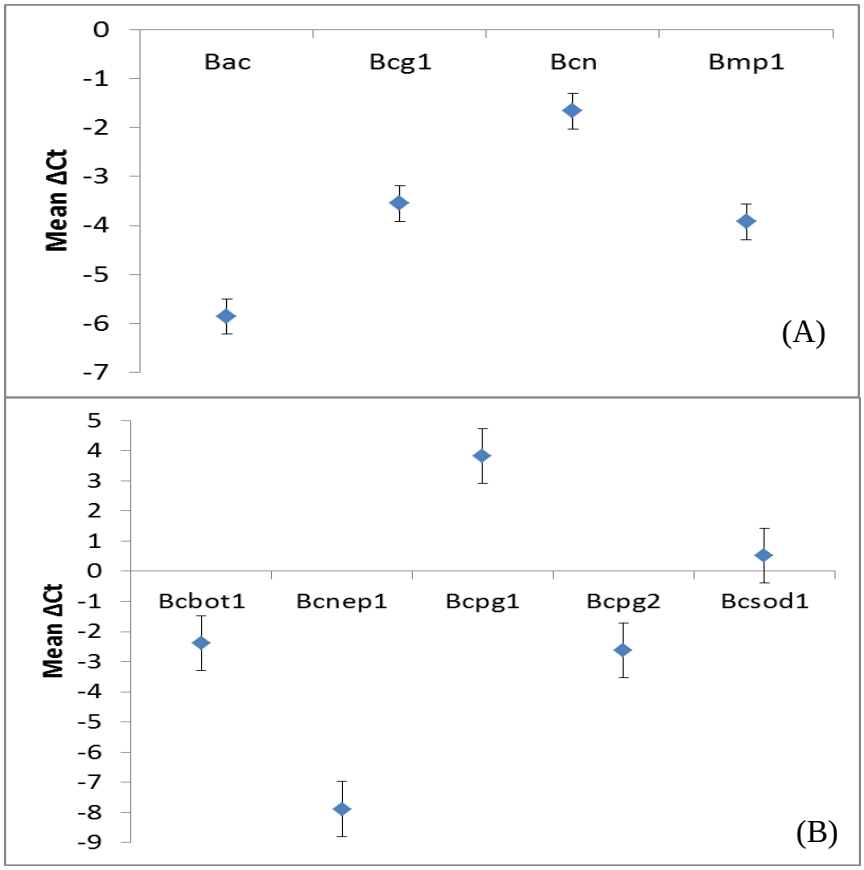


Figure 4-1: The relative amount of mRNA of signalling (A) and pathogenicity (B) genes in necrotic infection of detached lettuce leaf. The relative concentration of mRNA was calculated as the concentration difference between the housekeeping gene and gene of interest. The values are averages of three different time courses in two biological replicates, with three technical replicates in each. The mRNA concentrations differed significantly (A) $P<0.001$, s.e. = 0.36, (B) $P<0.001$, s.e. = 0.9, from REML analysis of the entire experiment.

4.3.1.2.2 Amount of mRNA at different time points in necrotic infection

The mRNA of signalling genes did not vary greatly between different time points in necrotic infection (Figure 4-2). For pathogenesis related genes the relative amount of mRNA varied substantially between time points (Figure 4-3). The amount of *bcnep1* decreased steadily with time. *Bcpg2* declined substantially after 24h. There were no notable differences in relative mRNA quantity of *bcbot1*, *bcpg1* and *bcsod1* at different time points.

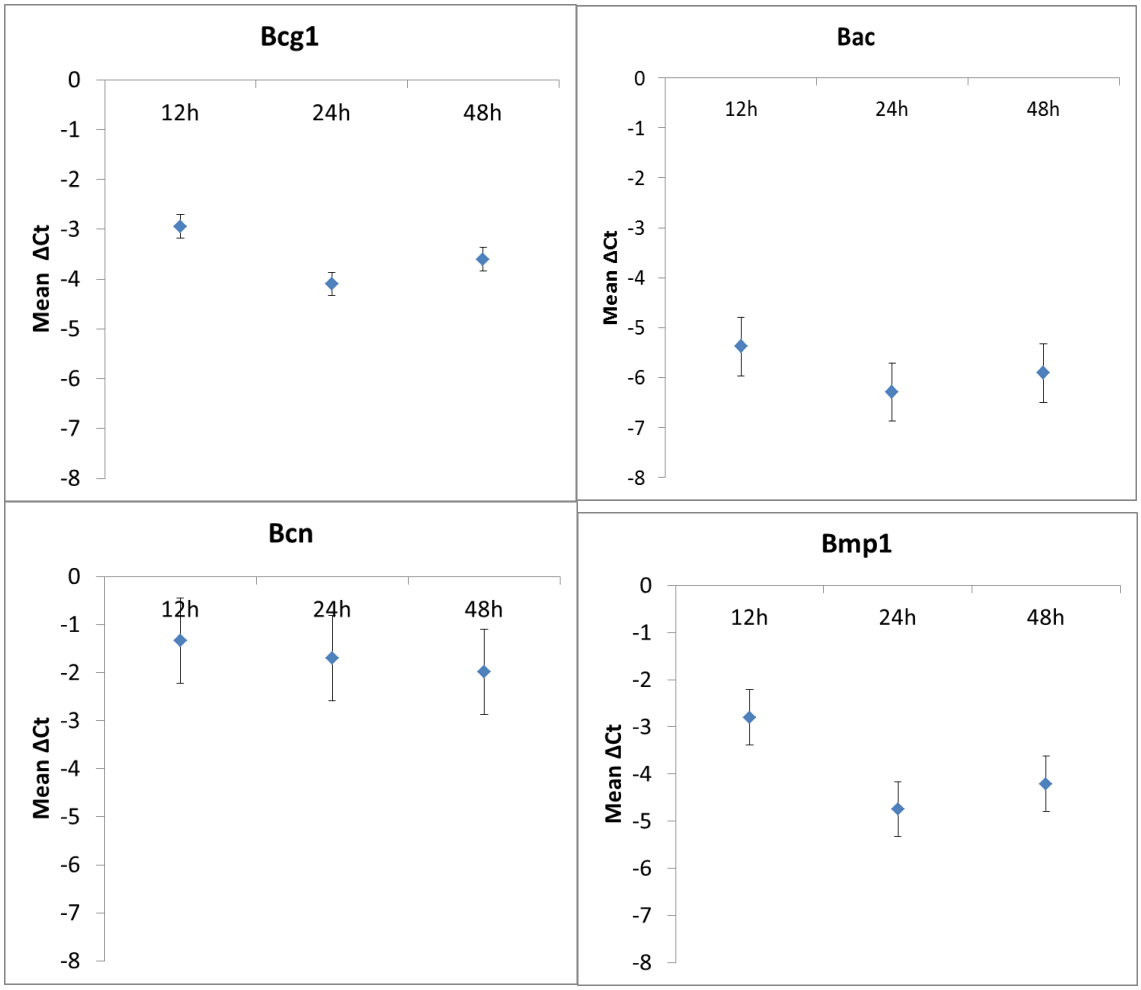


Figure 4-2: Relative concentration of mRNA of signalling genes at different time points in detached necrotic leaves of lettuce. The mean ΔCt values were calculated as the difference between concentration of mRNA of housekeeping gene and gene of interest for time point. The values are average of two biological replicates, with three technical replicates in each. Bac, P=0.6, s.e. = 0.58; Bcg1, P=0.08, s.e. = 0.23; Bcn, P=0.9, s.e. = 0.9; Bmp1, P=0.2, s.e. = 0.58, from REML analysis.

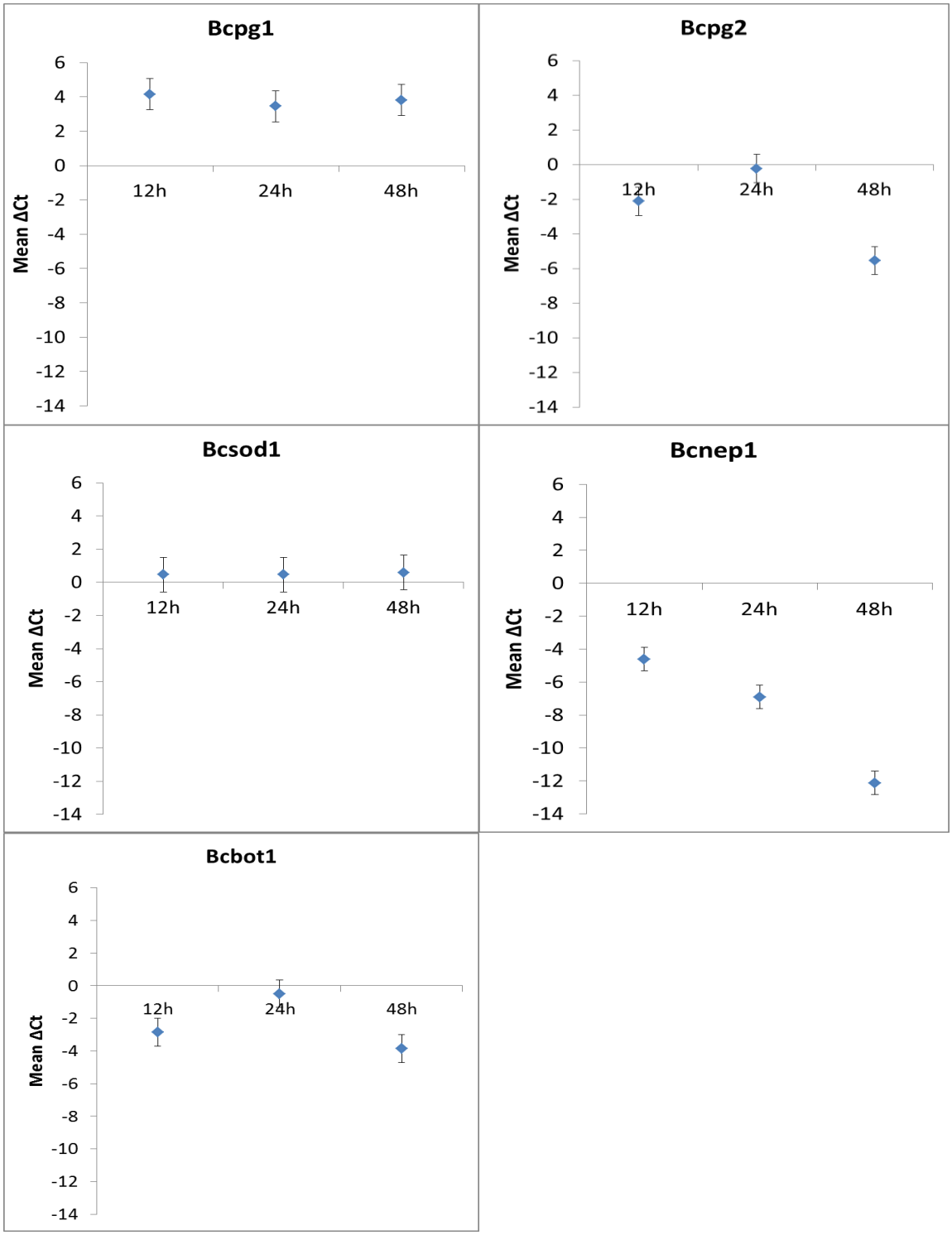


Figure 4-3: Relative concentration of mRNA of pathogenicity genes at different time points in detached necrotic leaves of lettuce. The mean ΔCt values were calculated as the difference between mean concentration of mRNA of housekeeping gene and gene of interest of each time point. The values are average of two biological replicates, with three technical replicates in each. Bcpg1, P=0.86, s.e. = 0.9; Bcpg2, P=0.04, s.e. = 0.8 Bcsod1, P= 0.99, s.e. = 1.0; Bcnep1, P=0.01, s.e. = 0.7; Bcbot1, P= 0.14, s.e. = 0.8, from REML analysis.

4.3.1.3 Relative amounts of mRNA in symptomless infection of lettuce.

Relative mRNA amounts of signalling and pathogenicity genes were measured in symptomless leaf samples collected from different leaf positions in lettuce plants sampled at different time points. Figure 4-4 represents a general model that can help a reader to easily understand the charts in following pages. The results have been presented as mean ΔCt values of two biological replicates. Samples used for the analysis: (A) whole seedlings at two days post inoculation; (B) 5th and 6th leaves of plants sampled at 14 days post inoculation (DPI), 24 DPI and 34 DPI; (C) 9^{th} and 10^{th} leaves of plants sampled at 14 DPI, 24 DPI and 34 DP; (D) 13^{th} and 14^{th} leaves of plants sampled at 24 DPI and 34 DPI; (E) 17^{th} and 18^{th} leaves of plants sampled at 34 DPI.

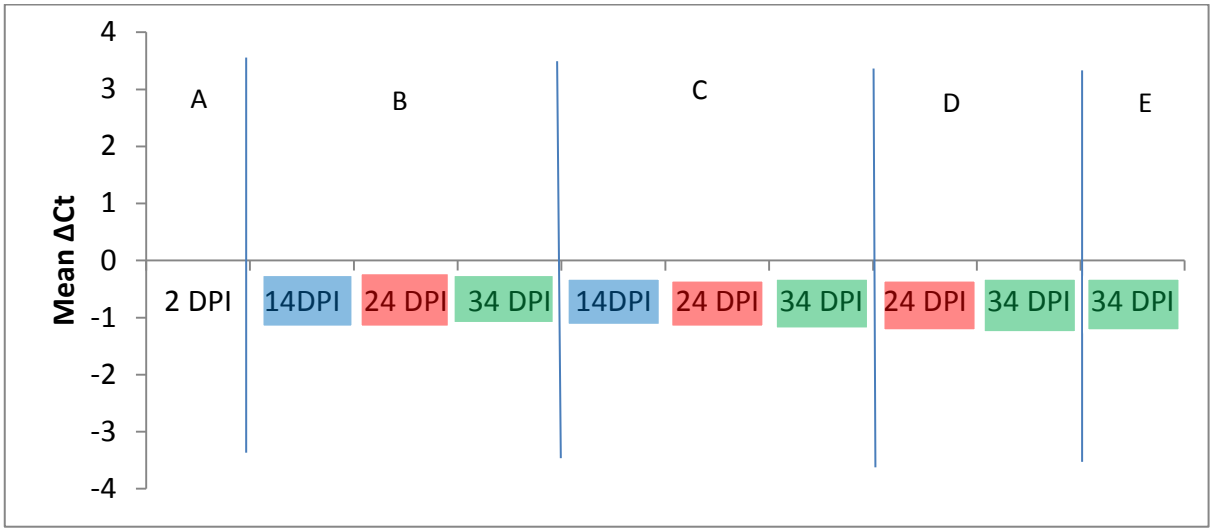


Figure 4-4: A model chart describe type of tissues and sampling days to understand the charts in following results section. A-E different samples used for analysis: (A) whole seedlings; (B) 5th and 6th leaves; (C) 9th and 10th leaves (D) 13th and 14th leaves (E) 17th and 18th leaves. DPI- day after inoculation/sampling days. Leaf samples collected from the same plants are coded with same colour.

In leaf position 5 & 6, mRNAs coding for all signalling genes were detected at all-time points (Figure 4-5); but there were no differences in relative amount of mRNA between genes. The mRNA coding for gene *bcg1* was detected in all leaf positions and at all-time points; however, with the prolonged time after inoculation and increasing distance from initial inoculation site the detection level declined. The mRNA of genes *bac*, *bcn* and *bmp1*were not detected or were detected only in one biological replicate in leaf position 9 &10 and above.

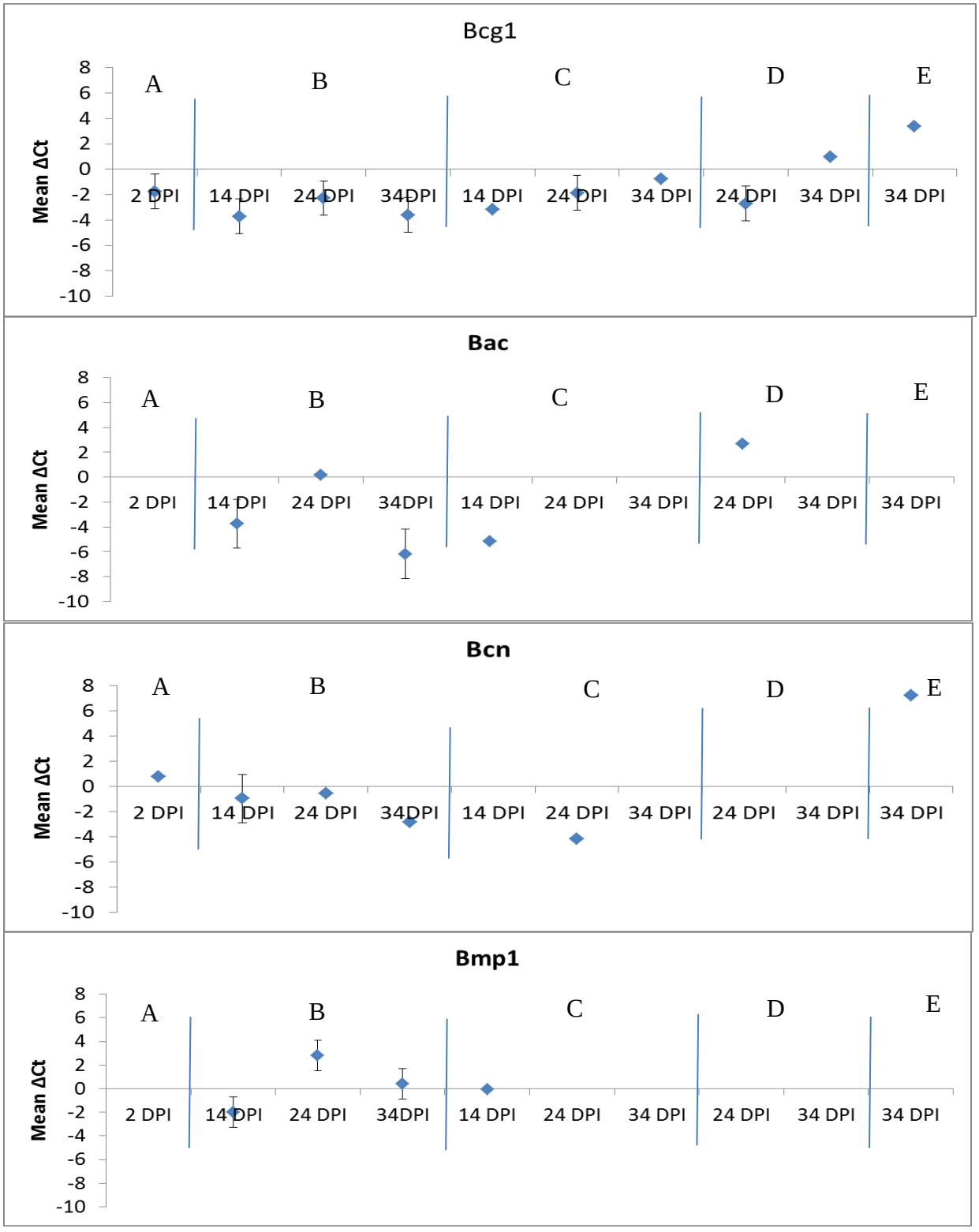


Fig. 4-5: Relative amount of mRNA of four signalling genes from *B. cinerea* at different time points in different leaf position in symptomless lettuce plants inoculated at the seedling stage with dry spores. The mean ΔCt is the difference between the mean mRNA concentration of the housekeeping gene Bcrpl5 and the gene of interest at each time point. At each time point leaf samples were analysed from two biological replicates in which isolation indicated presence of *B. cinerea*. No standard error is shown if the mRNA was detected only in one biological replicate. Statistical analysis was done using REML with plate, replicate and plant as random effects and leaf

position and time point after inoculation as fixed effects. DPI – Days post inoculation, (A) two days post inoculation, whole seedling having 4-5 leaves; (B) 5th and 6th leaves, (C) 9^{th} and 10^{th} leaves, (D) 13^{th} and 14^{th} leaves, (E) 17^{th} and 18^{th} leaves. Bcg1, s.e. = 1.5; Bac, s.e. = 1.9; Bcn, s.e. = 1.9; Bmp1, s.e. = 1.3, from REML analysis.

The mRNAs of all pathogenicity genes were detected in all samples of leaf position 5 & 6 at all-time points: the level of amount of mRNA did not vary with time (Figure 4-6). The mRNA coding for *bcbot1* was not detected in all tested samples. The polygalacturonase (*bcpg1* and *2*) coding mRNA were detected in all leaf position at all-time points; however, in leaf position 9&10 and above the mRNAs were detected only in one biological replicate. The mRNA of gene *bcnep1* was above the detectable level in newly formed leaves and did not show a clear time-trend where it was detectable. Superoxide dismutase coding mRNA (*bcsod1*) was detected in all samples of leaf position 5 & 6 and 9 & 10; in young leaves it was detected in late time points (34 DPI). Within a leaf position there was no clear time trend, but the concentration dropped in later produced leaves.

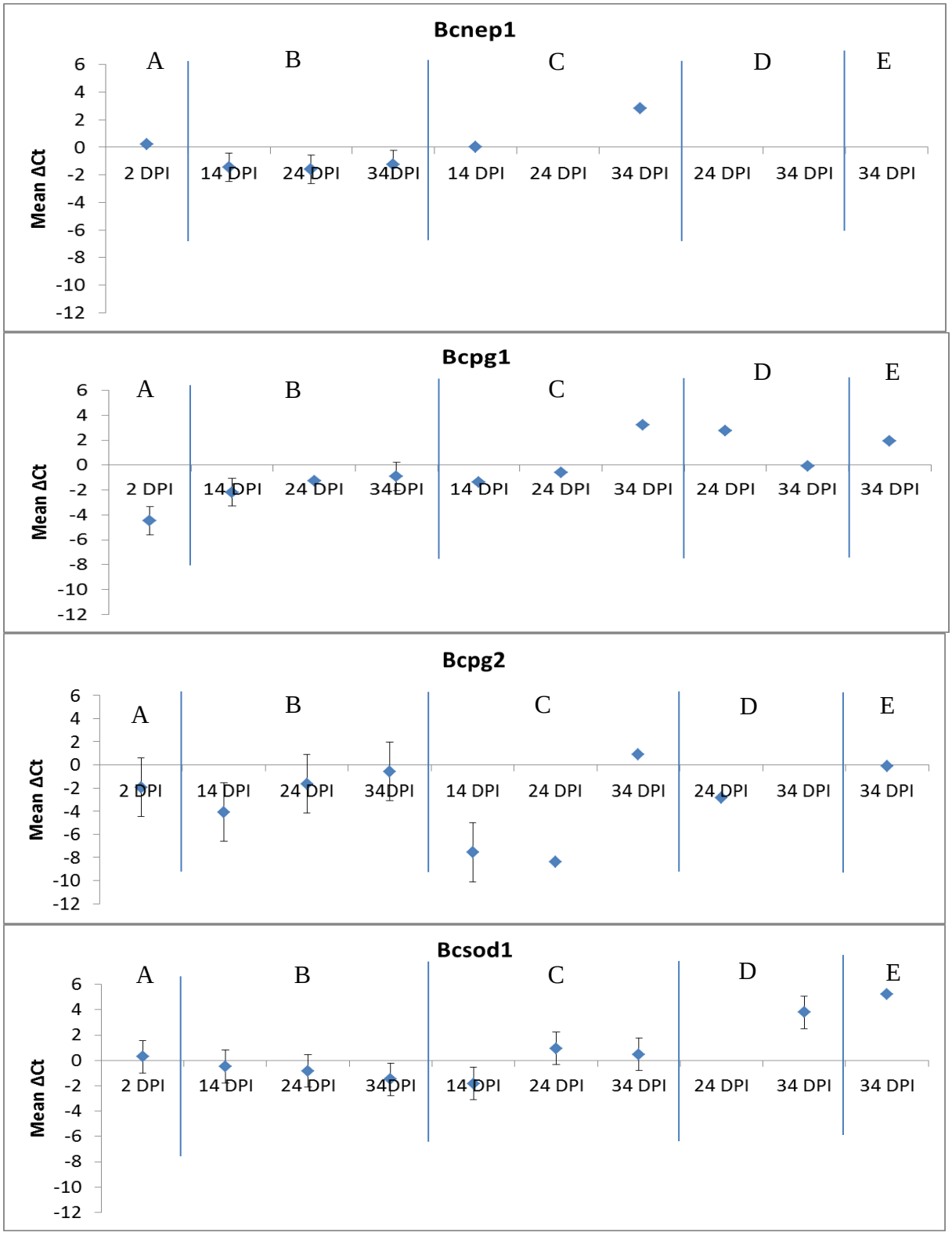


Figure 4-6: Relative amount of mRNA of four pathogenicity genes from *B. cinerea* at different time points in different leaf position in symptomless lettuce plants inoculated at the seedling stage with dry spores. The mean ΔCt is the difference between the mean mRNA amount of the housekeeping gene Bcrpl5 and the gene of interest at each time point. At each time point leaf samples were analysed from two biological replicates in which isolation indicated presence of *B. cinerea*. The standard error is based on differences between biological

replicates. Statistical analysis was done using REML with plate, replicate and plant as random effects and leaf position and time point after inoculation as fixed effects. DPI – Days post inoculation, (A) two days post inoculation, whole seedling having 4-5 leaves; (B) 5th and 6th leaves, (C) 9^{th} and 10^{th} leaves, (D) 13^{th} and 14^{th} leaves, (E) 17^{th} and 18^{th} leaves. Based on REML analysis: Bcnep1, s.e. = 1.0; Bcpg1, s.e. = 1.1; Bcpg2, s.e. = 2.5; Bcsod1, s.e. = 1.4. There was no difference ($P>0.05$) in mRNA concentration of Bcnep1, Bcpg1and Bcpg2 based on leaf position and time. The mRNA concentration of Bcsod1 varied significantly ($P=0.049$) based on leaf position.

4.3.1.4 Relative amount of mRNA in necrotic infection developed through symptomless infection (delayed necrotic infection).

The symptomless infected plants showed necrotic infection in leaves 40 days after inoculation. Comparison of relative amount of mRNA showed significant (P<0.001) variation between genes (Figure 4-7). mRNA of all the signalling genes were detected at similar amounts. Pathogenicity genes showed very large differences in mRNA amount: the mRNA amount of *bcpg1* and *bcsod1* were similar to signalling genes, and much greater than *bcpg2* and *bcnep1*. The mRNA coding for *bcbot1* was detected only in one biological replicate; it was in very low amount.

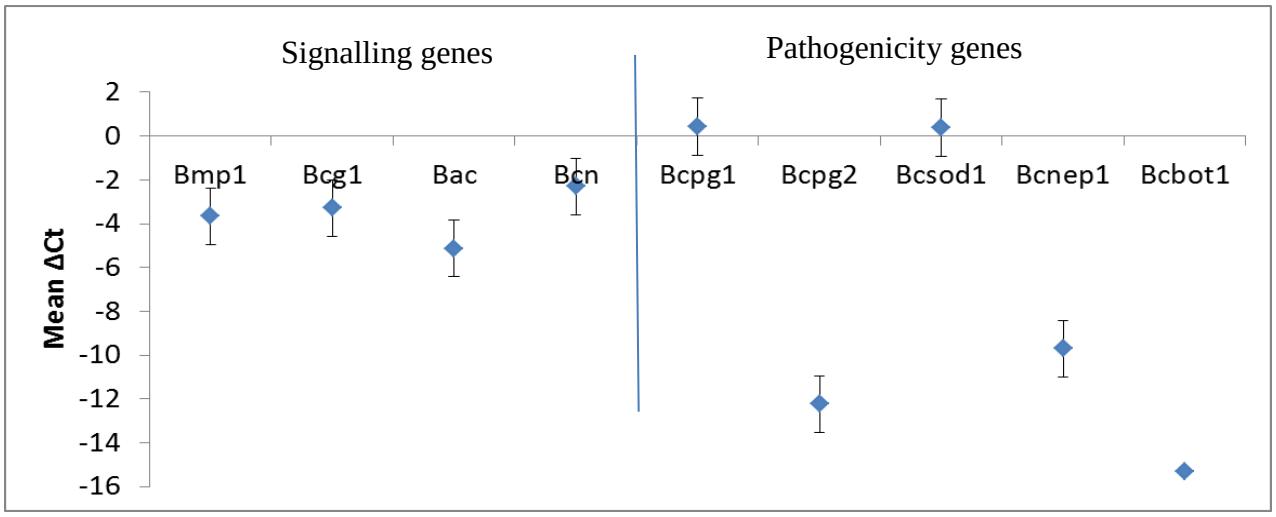


Figure 4-7: Relative amount of mRNA of signalling and pathogenicity genes in necrotic infection of uninoculated lettuce leaves around 40 days after inoculation of the plant. The mean ΔCt is the difference of mean mRNA concentrations between housekeeping gene *bcrpl5* and the gene of interest. The values are average of two biological replicates, with three technical replicates in each. P<0.001, s.e. = 1.3.

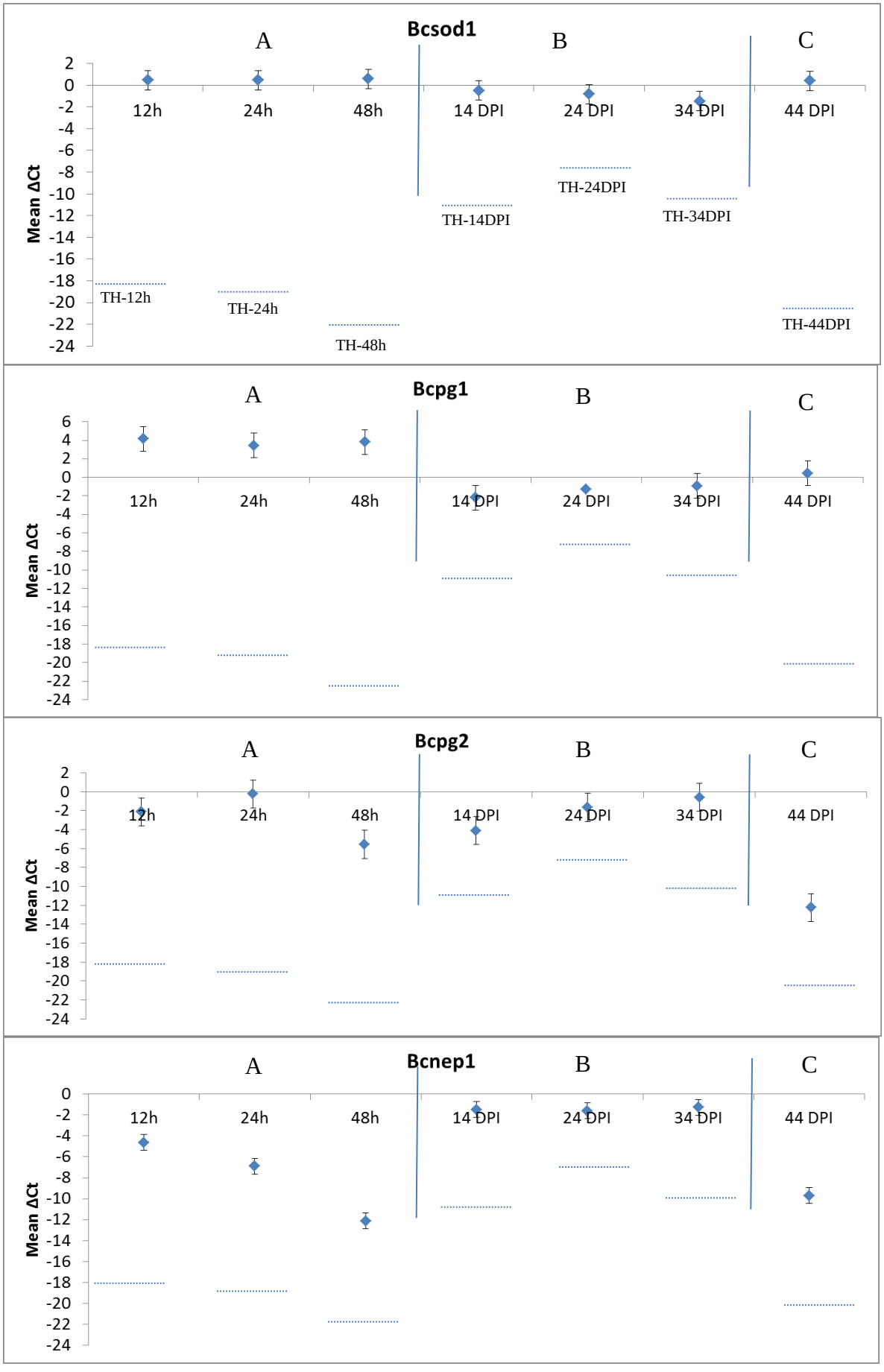
4.3.1.5 Comparison of relative amount of mRNA in different infection types

Relative amount of mRNA of some pathogenicity related genes differed substantially at different time points in necrosis infection and at different time points in 5^{th} and 6^{th} leaf of symptomless infection (Figure 4-8).

The number of transcripts of *bcnep1*varied significantly ($P<0.001$): in symptomless infection more mRNA was detected than in necrotic infection developed by either direct or delayed infection. There was no difference in *bcnep1* mRNA concentration between late stage (48 h) of necrotic infection developed directly or delayed.

The relative mRNA amount of *bcpg2* showed significant ($P=0.008$) differences at different time points: at the late symptomless stage (34 DPI) more transcripts were detected than in late stage (48h) of directly infected necrotic samples, which in turn had higher amount than in delayed necrotic infection.

There was a marginal difference ($P=0.05$) in mRNA amount of *bcpg1* between infection types; the amount was slightly higher in direct necrotic infection than other two kinds of infection. There were no differences in mRNA amounts of *bcsod1* among the tested conditions. The mRNA coding for *bcbot1* was detected in direct and delayed necrotic infections; but not in symptomless infections. The amount of mRNA of *bcbot1* was much greater in directly infected lesions than delayed necrotic infection.


Bcsod1
A
B
C
Mean ΔCt
12h
24h
48h
14 DPI
24 DPI
34 DPI
44 DPI
TH-12h
TH-24h
TH-48h
TH-14DPI
TH-24DPI
TH-34DPI
TH-44DPI
Bcpg1
Bcpg2
Bcnep1

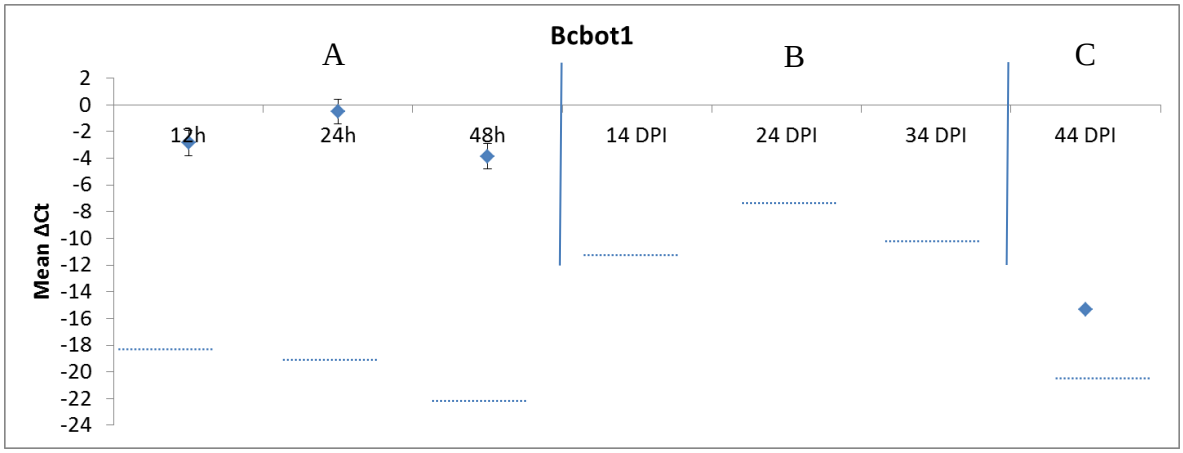


Figure 4-8: Relative amount of mRNA of pathogenicity genes at different time points in direct necrotic infection (A), in symptomless infection in 5th and 6th leaf of lettuce plants (B) and in necrotic infection developed through symptomless infection / delayed necrotic infection (C) . DPI- days post inoculation, TH- threshold level. Threshold level was calculated as the difference between the mean Ct value of housekeeping gene and the detectable maximum Ct value of the reaction, which was always 40. Bcsod1, P=0.58, s.e. = 0.89; Bcpg2, P=0.008, s.e. =1.5; Bcpg1, P=0.05, s.e. = 1.3; Bcnep1, P<0.001, s.e. = 0.75; Bcbot1, P=0.007, s.e. = 0.9, from REML analysis.

No significant differences were detected in the amount mRNA of the signalling genes *bcg1*, *bac* or *bcn* (Figure 4-9). The late symptomless stages, 24 DPI and 34 DPI, had a greater (P=0.004) amount of mRNA transcripts of gene *bmp1* than other samples; no difference was detected between the two kinds of necrotic infection.

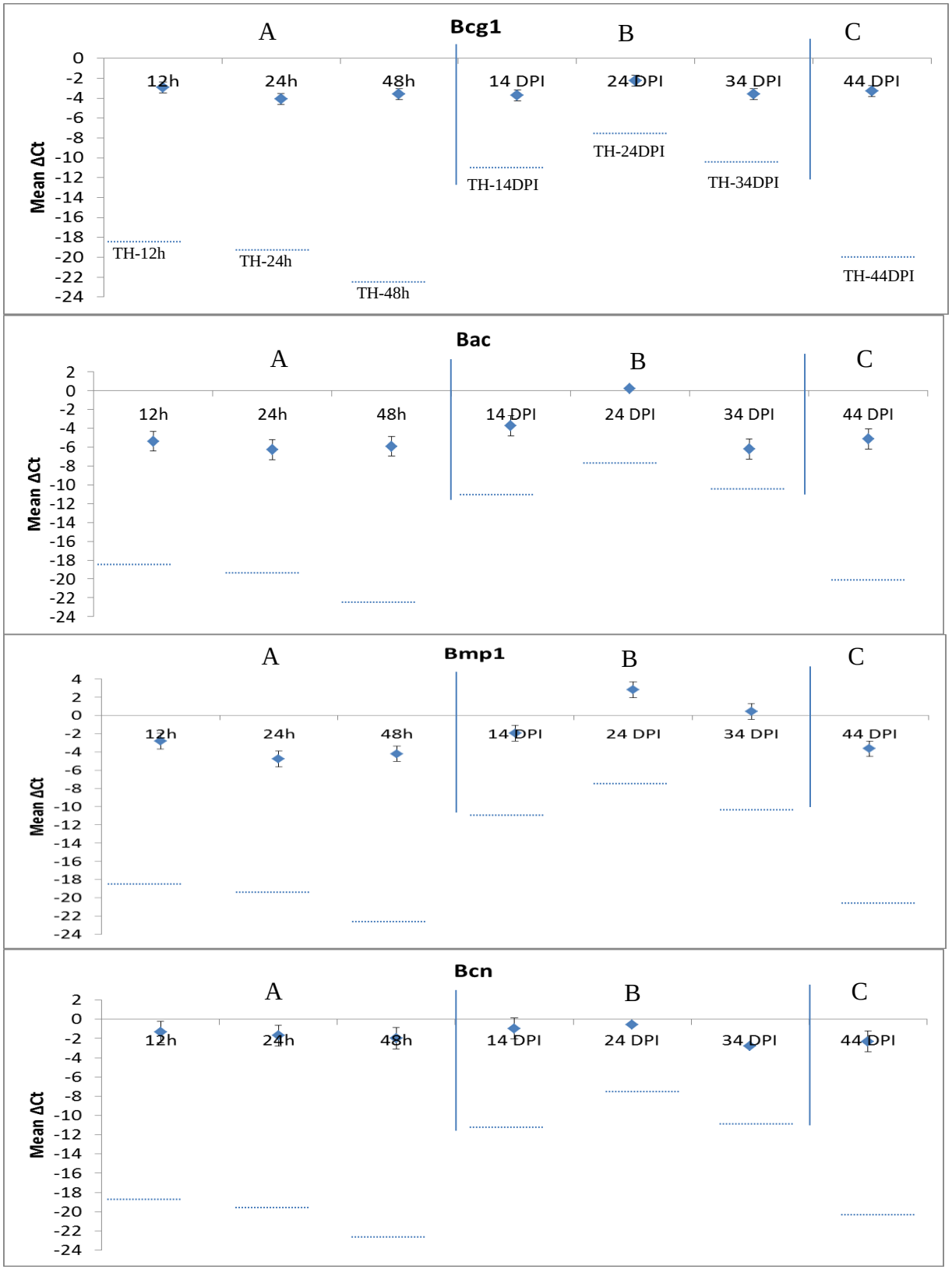


Figure 4-9: Relative mRNA amount of signalling genes at different time points in direct necrotic infection (A), in symptomless infection in fifth leaf of lettuce plants (B) and in necrotic infection developed through symptomless infection / delayed necrotic infection (C) . DPI- days post inoculation, TH- threshold level. Threshold level was calculated as the difference between the mean Ct value of housekeeping gene and the

detectable maximum Ct value of the reaction, which was always 40. Bcg1, P=0.4, s.e. = 0.55; Bac, P=0.09, s.e. = 1.1; Bmp1, P=0.004, s.e. = 0.86; Bcn, P=0.8, s.e. = 1.1, from REML analysis.

4.3.2 *A. thaliana*

4.3.2.1 Recovery of symptomless growing *B. cinerea* on Botrytis selective medium

B. cinerea was recovered from surface disinfected (SD) and non-surface disinfected (NSD) plant parts of *A. thaliana*. The percentage of recovery from SD and NSD samples was highest in rosette leaf samples followed by stem leaf and stem (Figure 4-10).

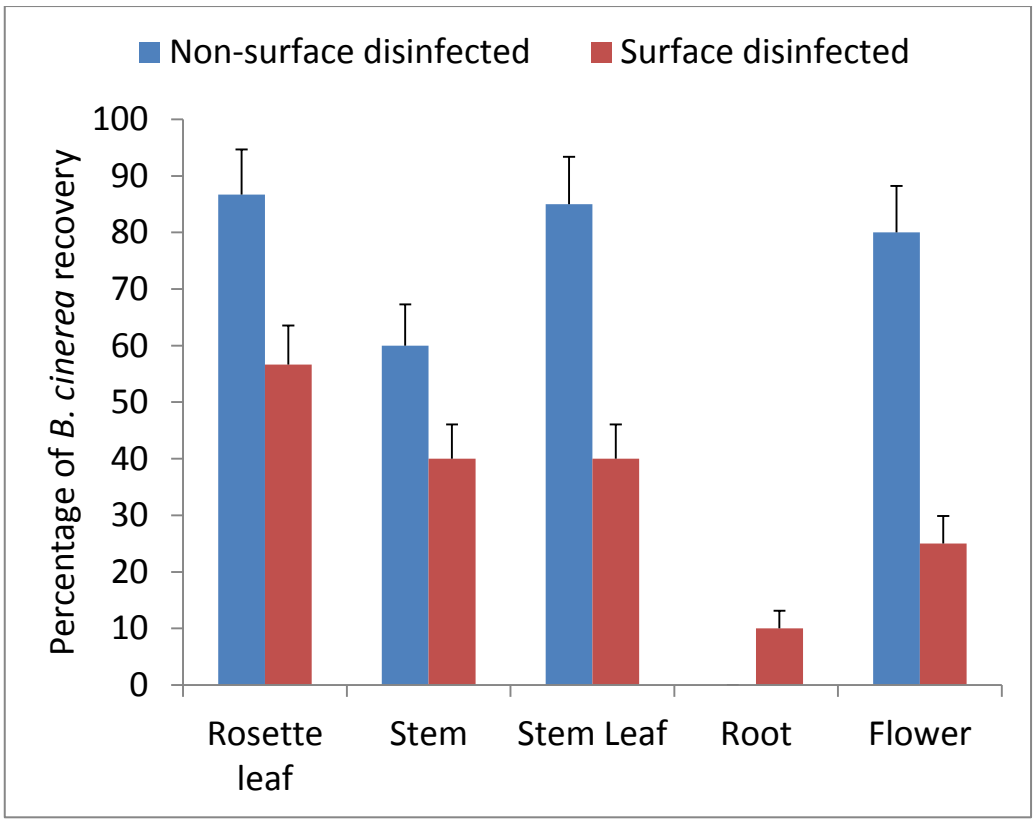


Figure 4-10: Percentage of *B. cinerea* recovery from different plant parts of *A. thaliana*. Ten plants were sampled and from each plant ten plant parts (2 rosette leaves, 2 stem pieces, 2 stem leaves, 1 root piece and 2 flowers) were plated on selective medium.

4.3.2.2 Relative mRNA amounts of selected genes in necrotic infection

4.3.2.2.1 Overall relative amounts of mRNA of signalling genes

The mRNA level of the selected signalling genes relative to the housekeeping gene *bcrpl5* was compared in necrotic infections on attached leaves (Figure 4-11). The relative amount of *bac* was less (P<0.001) than the other genes. There was no detectable difference between relative amount of *bcg1* and *bmp1*, but both were significantly higher than *bcn*.

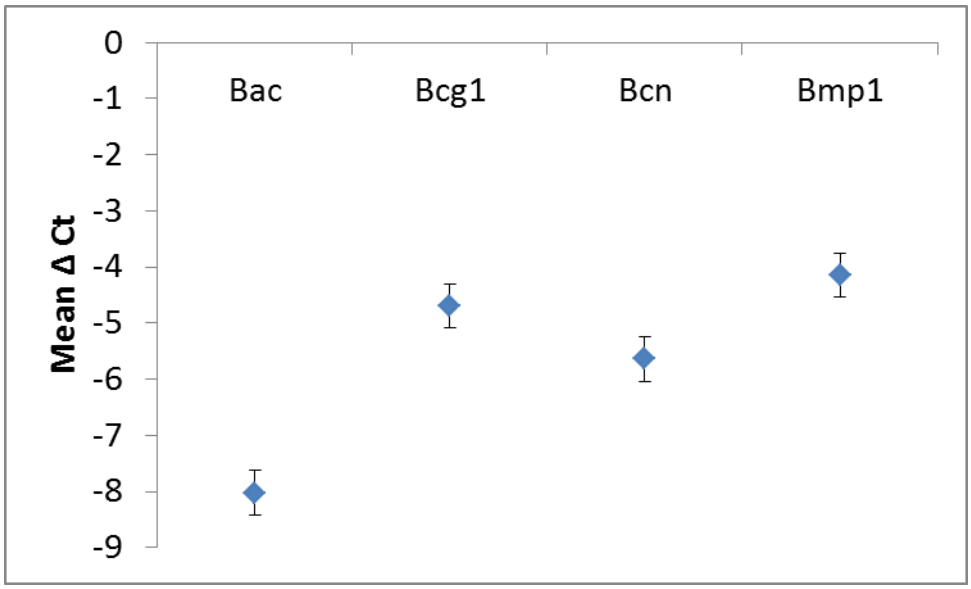


Figure 4-11: Average amount of mRNA of signalling genes in necrotic leaf tissues relative to Bcrpl5. The difference in mRNA concentration between each signalling gene and housekeeping gene is given as Mean ΔCt. The values are average of measurements at five different times in two biological replicates, with three technical replicates in each, s.e = 0.39. The mRNA concentration of these genes differed significantly, P<0.001.

4.3.2.2.2 Overall relative amount of mRNA of pathogenicity genes

The amount of mRNA of the selected pathogenicity genes, standardised relative to *bcrpl5* differed greatly in necrotic leaf tissues (P<0.001) (Figure 4-12). The mRNA of *bcsod1* was most abundant. Amounts of *bcbot1* and *bcnep1* in necrotic samples were about 2000 to 15,000 fold lower while amounts of *bcpg1* mRNA were intermediate.

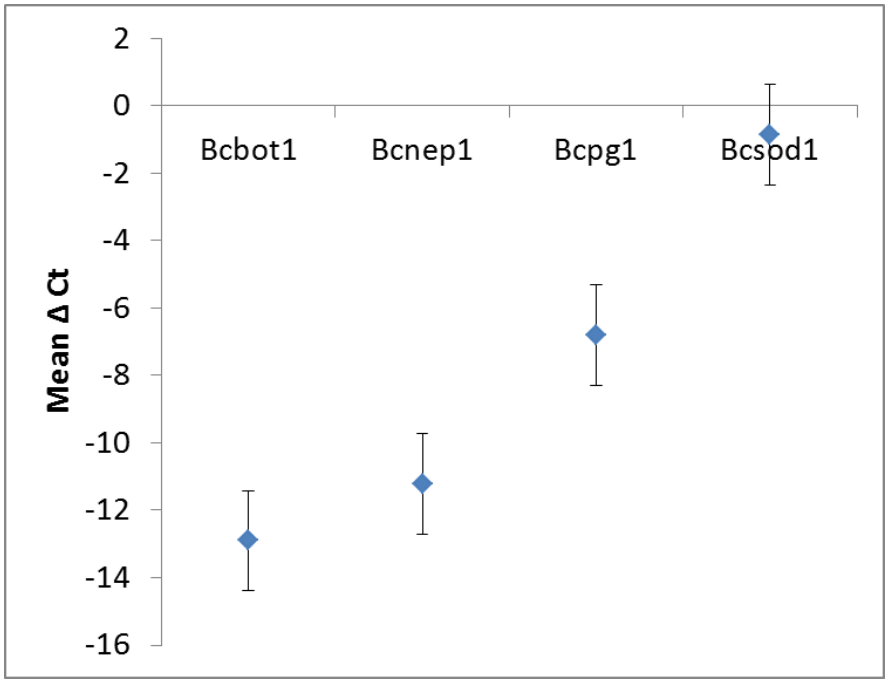


Figure 4-12: Average amount of mRNA of pathogenicity genes in necrotic infection. The mean ΔCt value is the difference in mRNA concentration between *Bcrpl5* and gene of interest. The values are average of five different time courses in two biological replicates, with three technical replicates in each, s.e = 1.5. The mRNA concentration of these genes differed significantly, P<0.001.

4.3.2.3 Relative amount mRNA at different time points in necrotic infection.

The relative amount of mRNA of the signalling genes was compared at different time points as necrotic infections developed on attached leaves (Figure 4-13). The genes *bmp1*, *bcg1* and *bac* showed similar patterns: there was no difference in mRNA amount from 3h post inoculation to 24h post inoculation, but by 48h post inoculation the level of mRNA increased 4-8 fold. The mRNA concentration of *bcn* did not vary detectably over time.

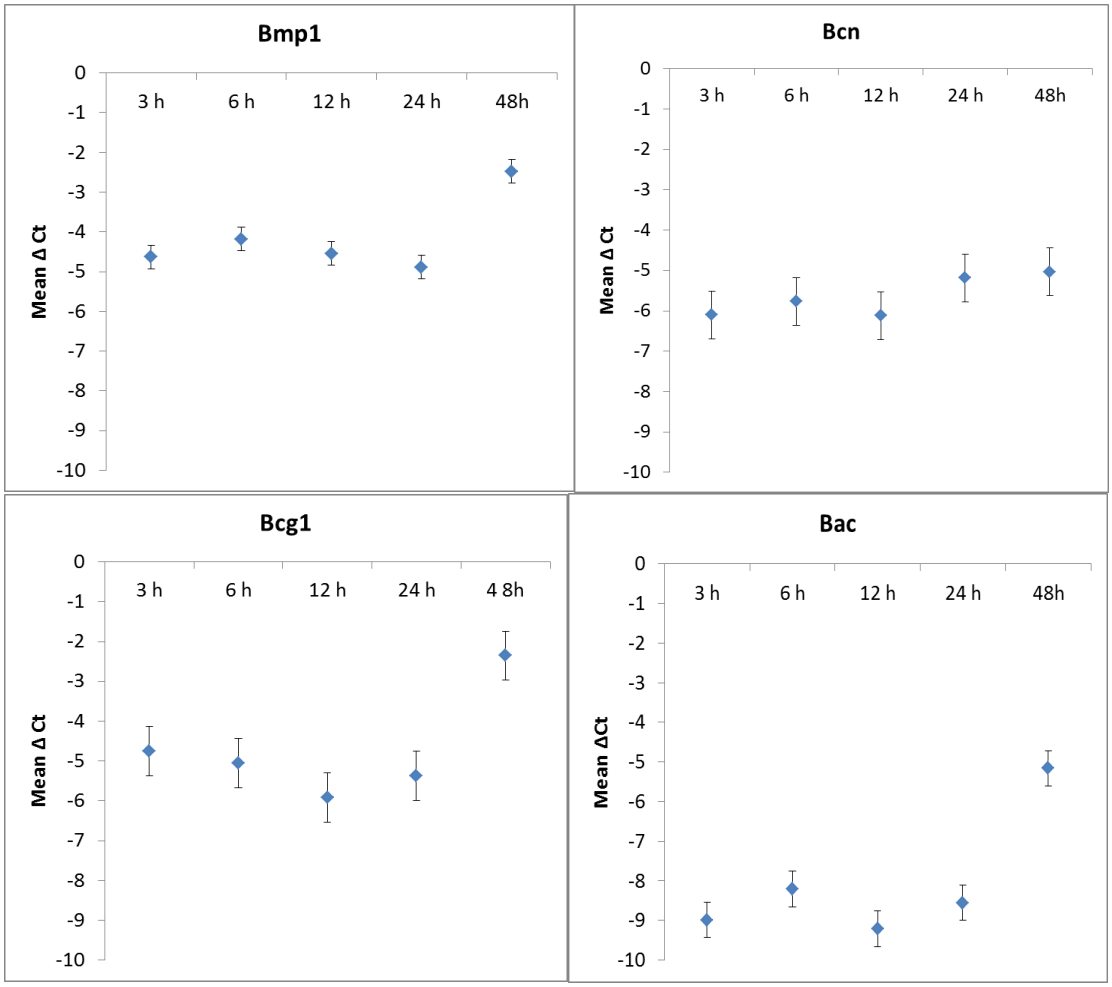


Figure 4-13: Relative amount of mRNA of signalling genes at different time interval in necrotic infection. The mean ΔCt value is the difference in concentration of mRNA between housekeeping gene and gene of interest in each time point. The values are average of two biological replicates, with three technical replicates in each. Bmp1, P=0.01, s.e. = 0.3; Bcn, P=0.6, s.e. = 0.6; Bcg1, P=0.055, s.e. = 0.62; Bac, P=0.007, s.e.= 0.45, from REML analysis.

Changes in amount of mRNA of pathogenicity genes followed the same general pattern as the signalling genes, with the most marked changes in the 48 h samples. This pattern applied to *bcpg1*, *bcnep1* and *bcbot1* (Figure 4-14). From 3h to 24h, variation was slight. *Bcnep1* and *bcbot1* were detected in very low amount at 3h to 24h inclusive; in particular, before 48h the mRNA coding for *bcbot1* was produced about 30,000 fold lower than the housekeeping gene, and it was detected only in one biological replicate of 3h and 12h samples. The relative amount of mRNA of *bcsod1* was much higher than the other genes at all-time points. The level of *bcsod1* mRNA amount rose fairly steadily from 3h to 48h.

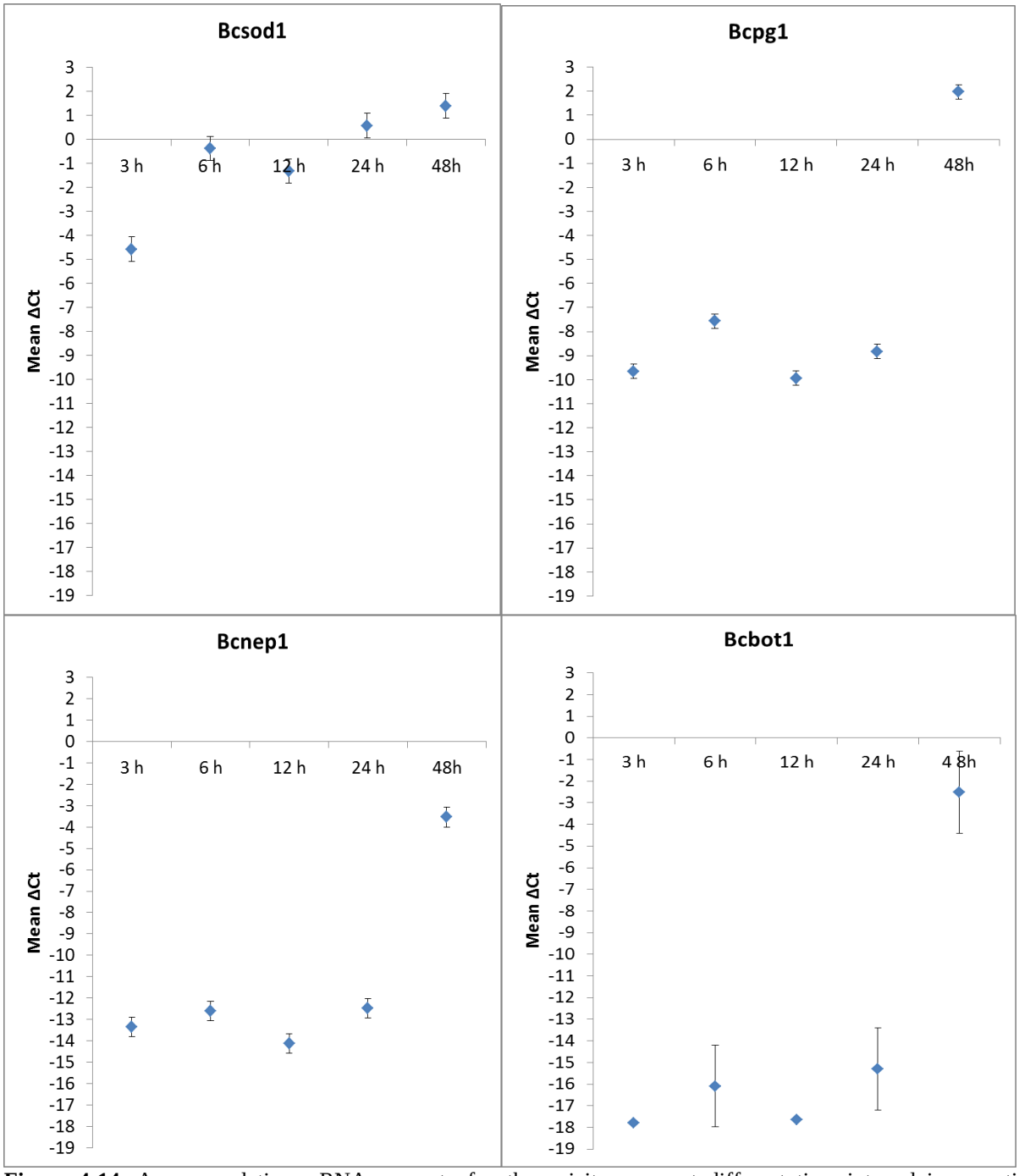


Figure 4-14: Average relative mRNA amount of pathogenicity genes at different time interval in necrotic infection. The mean ΔCt value is the difference in concentration of mRNA between housekeeping gene and gene of interest in each time point. Except for Bcbot1, the values are average of two biological replicates, with three technical replicates in each: In Bcbot1, mRNA was detected only in one biological replicate in 3h and 12h samples. Bsod1, P=0.003, s.e. = 0.5; Bcpg1, P<0.001, s.e. = 0.3; Bcnep1, P<0.001, s.e. = 0.5; Bcbot1, P=0.03, s.e.= 1.9, from REML analysis.

4.3.2.4 Relative amount of mRNA of some selected genes in symptomless infection

4.3.2.4.1 Overall relative amount of mRNA of signalling genes in leaf and stem samples

The relative mRNA level of genes *bcn* and *bmp1* did not show differences between leaf and stem samples from symptomless *A. thaliana*. However, mRNA coding for G1 protein was about 20 fold greater in stem samples than leaf samples (P=0.01) (Figure 4-15). mRNA of the gene *bac* was not detected from symptomless samples.

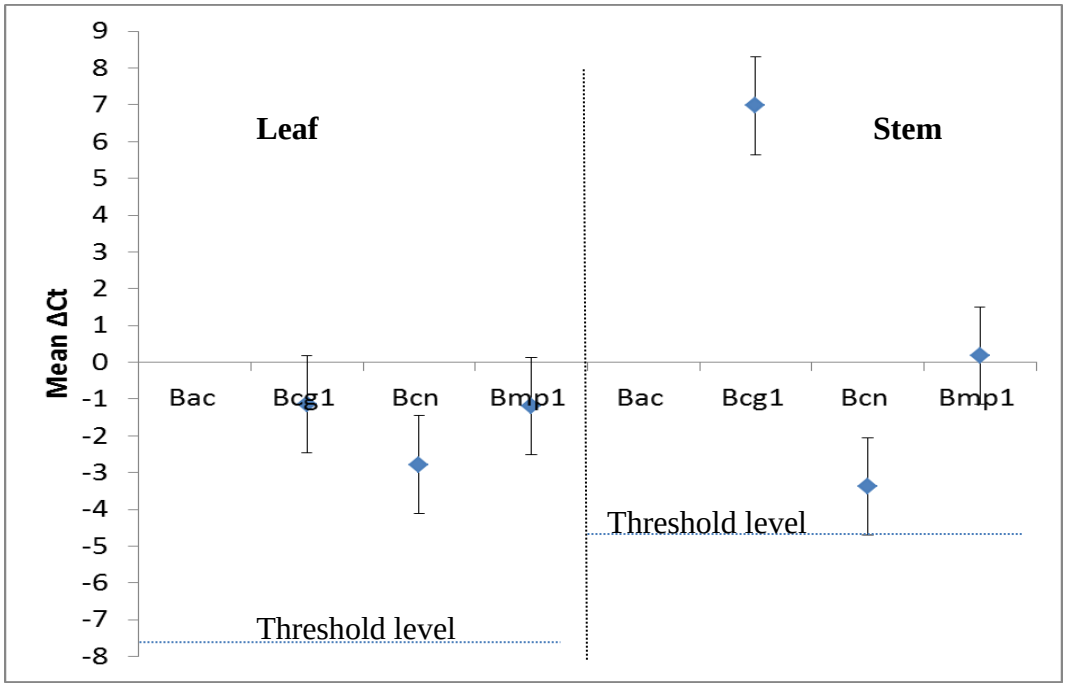


Figure 4-15: Relative amount of mRNA of signalling genes in rosette leaves and flowering stems of symptomless *A. thaliana*. The mean ΔCt value is the difference in concentration of mRNA between housekeeping gene and gene of interest. The values are average of two biological replicates, with three technical replicates in each: Threshold level was calculated as the difference between the mean Ct value of housekeeping gene and the detectable maximum Ct value of the reaction, which was always 40. P=0.01, s.e. = 1.3.

4.3.2.4.2 Overall relative amount of mRNA of pathogenicity genes in leaf and stem samples

The mRNA of the genes *bcnep1* and *bcbot1* was not detected in any samples from symptomless plants. The relative amount of mRNA of the remaining pathogenicity genes did

not show significant variation between leaf and stem samples of symptomless plants (P=0.16) (Figure 4-16).

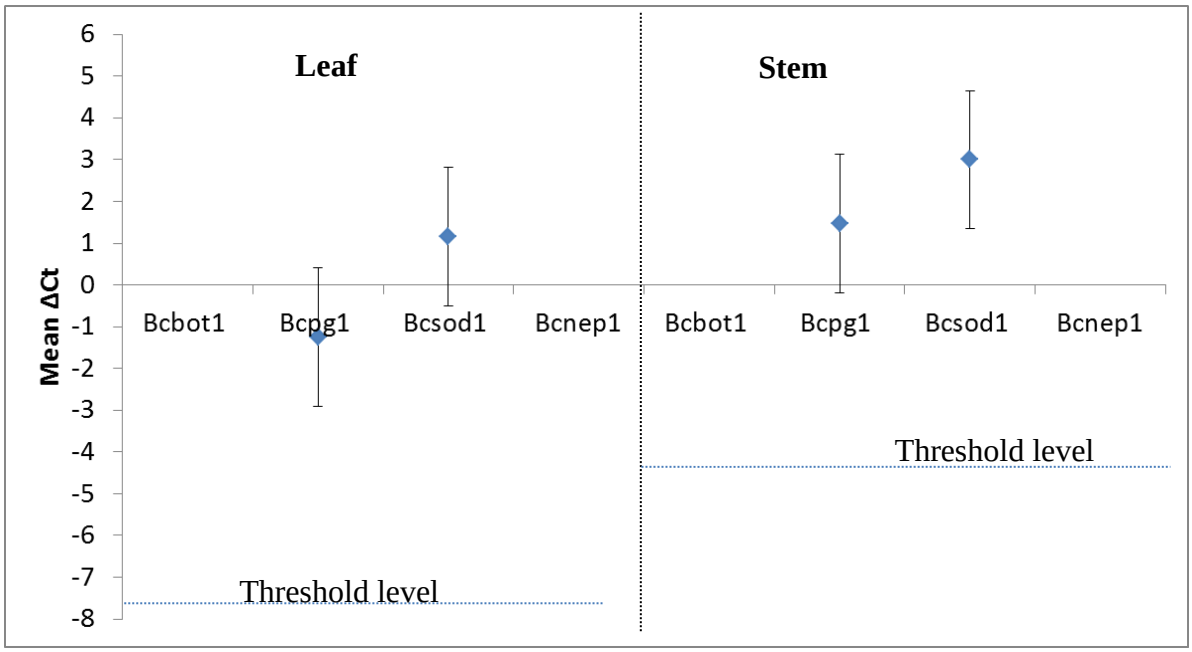


Figure 4-16: Relative amount of mRNA of pathogenicity genes in rosette leaves and flowering stem tissues of symptomless Arabidopsis plants inoculated at 20 days old with a dry suspension of *B. cinerea* spores. The mean ΔCt value is the difference in amount of mRNA between the housekeeping gene Bcrpl5 and the gene of interest. The values are average of two biological replicates, with three technical replicates in each. The mRNA level of Bcnep1 and Bcbot1 were below detectable level at all times. Threshold level was calculated as the difference between the mean Ct value of housekeeping gene and the detectable maximum Ct value of the reaction, which was always 40. P=0.16, s.e. = 1.7.

4.3.2.5 Changes in relative amount of mRNA of selected genes over time

4.3.2.5.1 Comparison of signalling genes

There were distinctive differences in the pattern of relative mRNA amounts of signalling genes between necrotic leaves and symptomless leaves and stem of *A. thaliana* (Figure 4-17). Relative amounts of transcripts of *bcg1* and *bmp1* were greater in stem samples than in any necrotic stage. The mRNA level of *bcn* was slightly greater in symptomless leaves and stems than in any necrotic stage. The transcripts of gene *bac* were not detected in samples from symptomless infections.

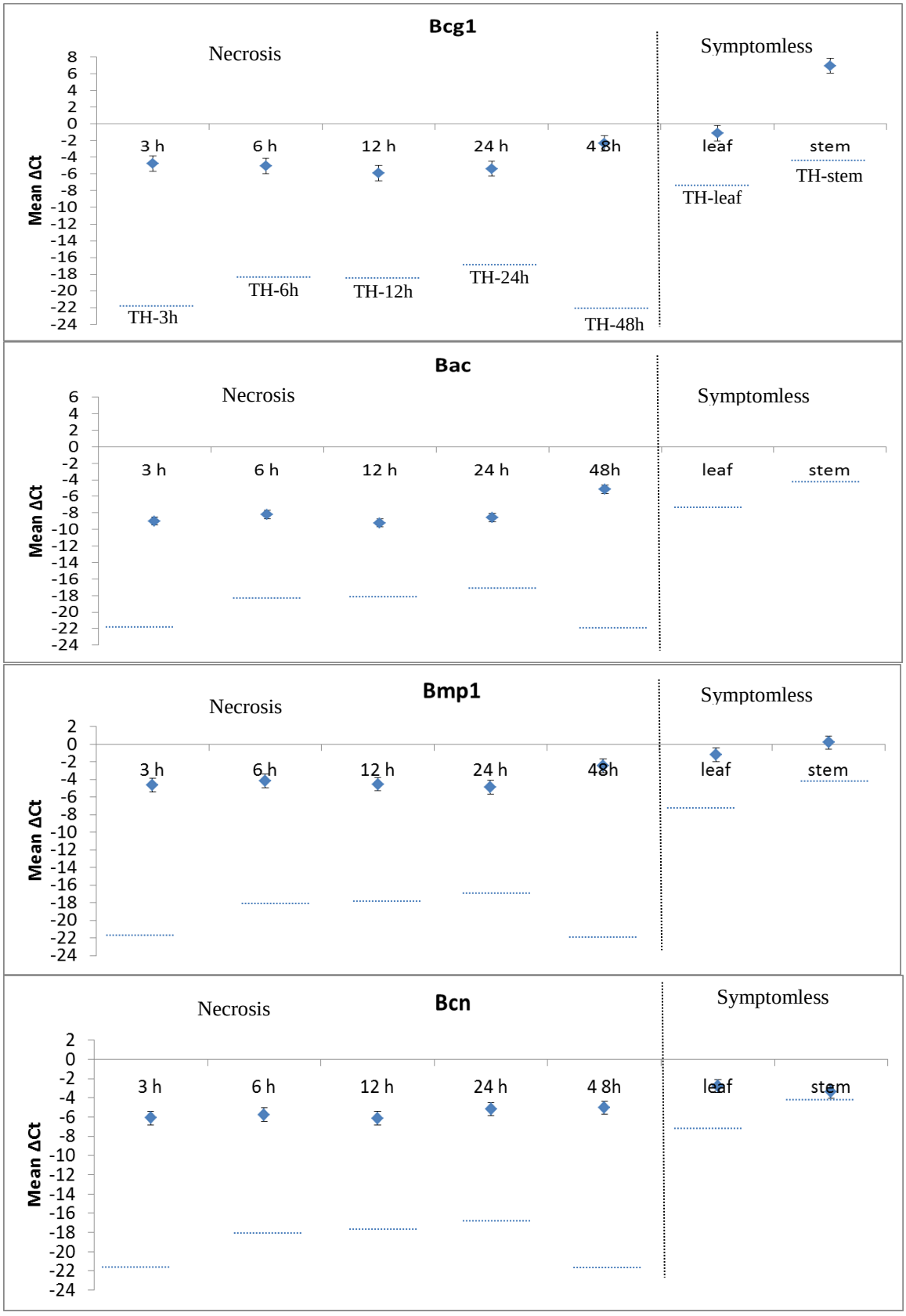


Figure 4-17: Comparison of relative amount of mRNA of signalling genes at different time points in necrotic leaves and in symptomless leaves and stem of *A. thaliana*. The mean ΔCt value is the difference in concentration of mRNA between housekeeping gene and gene of interest. TH- threshold level: threshold level was calculated

as the difference between the mean Ct value of housekeeping gene and the detectable maximum Ct value of the reaction, which was always 40. Bcg1, P<0.001, s.e.=0.92; Bmp1, P=0.01, s.e.= 0.77; Bcn, P=0.06,s.e.=0.71; Bac, P<0.001, s.e.=0.5, from REML analysis.

4.3.2.5.2 Comparison of pathogenesis genes

Detectable mRNA of pathogenesis related genes also showed distinctive differences between necrotic leaf tissue and symptomless leaves and stem samples (Figure 4-18). The mRNA of *bcnep1* and *bcbot1* were produced in necrotic samples (Figure 4-14) but at very low concentration until 48h post infection. They were not detected in any of the symptomless samples. Before 48 h, the relative amount of mRNA coding for polygalacturonase (*bcpg1*) and superoxide dismutase (*bcsod1*) were substantially greater in symptomless samples than in necrotic samples. The mRNA amount of *bcsod1* was slightly higher in symptomless than necrotic samples.

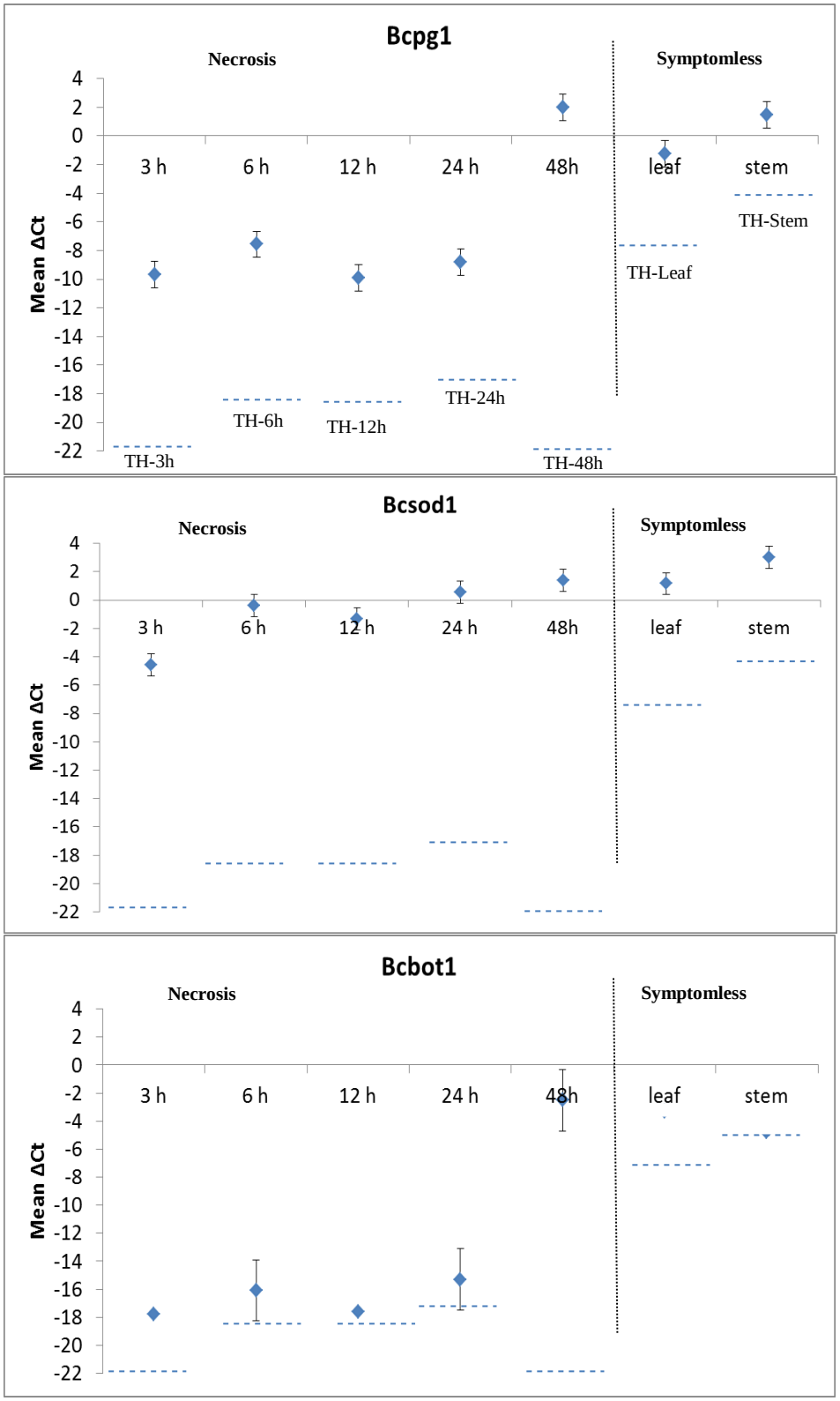


Figure 4-18: Comparison of relative amount of mRNA of pathogenesis related genes at different time points in necrotic leaves and in symptomless leaves and stem of Arabidopsis. The mean ΔCt value is the difference in amount of mRNA between housekeeping gene and gene of interest. TH- threshold level: threshold level was calculated as the difference between the mean Ct value of housekeeping gene and the detectable maximum Ct value of the reaction, which was always 40. Bcpg1, $P<0.001$, s.e.= 0.92; Bcsod1, $P=0.004$, s.e.=0.78; Bcbot1, $P=0.006$, s.e.=2.2, from REML analysis.

4.4 Discussion

The experiments reported here show that the physiological state of *B. cinerea* in a symptomless, systemic growth phase is quite distinct from that of a developing necrotic infection, but still involves activation of a subset of genes believed to be involved in pathogenesis or protection from plant defences. This generalization applies across both host species, but there are also substantial differences between infection of lettuce and of *A. thaliana*.

In this experiment I used two varieties of lettuce, Tom Thumb and All Year Round, to study symptomless *B. cinerea* growth. The choice of varieties was based on a previous experiment carried out by the Sowley (2006); these two varieties showed more consistent symptomless infection than other varieties tested. In the present study, these two varieties behaved differently in the plating out experiment; the incidence of detectable symptomless infection started from the early stages of plant growth in Tom Thumb and then declined, but increased during the growth of All Year Round. This may be due to the difference in the leaf arrangements in each plant; from the beginning, leaves develop very closely and compactly in Tom Thumb but in All Year Round leaves are arranged loosely at the beginning and only in late stages become close to each other.

In lettuce, the number of transcripts were quantified according to both age of leaf and leaf cohort in order to generate a complete profile; this gives information about development within a single leaf position and differences between different leaf positions at a single time point. Even though the fungus was recovered from almost all leaf tissues plated on selective medium, the transcripts were not equally easily detected. A clear profile of selected mRNA transcripts was obtained for the leaf samples (leaf position 5^{th} and 6^{th}) collected near to the

inoculation site (leaf position 1st to 4th), but not in leaf samples collected more distally from the inoculation site. This might be due to less RNA in the samples.

Several genes have been used as normalizer/housekeeping genes in RT-qPCR quantification; for example, genes coding for actin, β-tubulin and elongation factor. In this experiment, the gene coding for the large subunit of ribosomal protein (*bcrpl5*) was used as a housekeeping gene on the recommendation of Dr. Jan Van Kan (Zhang & Kan, 2013), based on his personal analysis with several housekeeping genes.

In lettuce necrotic infection, the amount of transcripts of the selected genes varied with time. The amount of mRNA of signalling genes reduced from 12 h to 48 h after inoculation. In symptomless samples there was variation over time and with leaf position at a single time. Samples collected from the leaf position 5th and 6th had easily detectable amount of mRNA but in higher leaf positions this was not the case, perhaps due to a reduced amount of fungus in those samples. Therefore, results of 5th and 6th leaves are best to use for the comparative analysis of symptomless infection and necrotrophic infection. The amount of mRNA of signalling genes was similar for genes *bcg1*, *bac* and *bcn* in both types of necrotic infection (necrotic lesions developed following spore suspension inoculation and necrotic lesions developed from symptomless tissues) and in the symptomless samples. But in symptomless samples the gene *bmp1* slightly increased from 14 days to 34 days after inoculation and was slightly higher at all-time points than in necrotic samples. The gene *bmp1* has been identified as essential for the initial stages of infection such as surface recognition, infection structure morphogenesis, and penetration (Zheng *et al.*, 2000). The presence of higher amount of mRNA of the gene *bmp1* may indicate that the fungus continuously attempts to initiate infection but that certain factors such as plant inhibitors do not allow the fungus to move to the next stage in the infection process.

During necrotrophic infection on lettuce, the number of transcripts of genes *bcpg1* and *bcsod1* remained relatively constant from 12 hpi to 48 hpi. A similar lack of trend was noticed in symptomless samples in leaf position 5th and 6th from 14 dpi to 34dpi. The relative amount of mRNA of the gene *bcsod1* was also similar in necrotic samples and symptomless samples. This suggests that the conversion of superoxide radicals into H_2O_2 may happen more intensively in symptomless samples as it does in necrotrophic infection. One of the plant responses to incompatible pathogen infection is an oxidative burst which involves a rapid, transient and localized production of reactive oxygen species. However, physical damage to the host tissues by an oxidative burst assists the colonization of necrotrophic pathogens, such as *B. cinerea* (Govrin & Levine, 2000). Therefore, dismutation of (fungal or host) superoxide, or generation of H_2O_2 (or both) are important for pathogenesis, and are common in necrotrophic infection. The similar observation in symptomless infection and necrotrophic infection indicates that the fungus is growing in a condition that evades the plant induced oxidative burst.

Transcripts of the gene *bcpg1* were detected relatively less in symptomless than in necrotrophic samples. Endopolygalacturonase acts in two ways during the infection process: it plays important role in spreading lesion development by degrading cell wall pectin (ten Have *et al.*, 1998), but it acts as an elicitor to provoke defence response in plants (Vandelle *et al.*, 2006). The reduced amounts of *bcpg1*in symptomless infection could therefore be causally related to fungal growth without visible necrosis lesion in the host.

The transcript concentration of genes *bcpg2* and *bcnep1* declined from 12 hpi to 48 hpi in necrotic samples. But in symptomless samples of leaves at 5th and 6th leaf positions *bcpg2* showed a slight increase and *bcnep1* remained at a similar level from 14 dpi to 34 dpi. The protein coded by the gene *bcpg2* is essential for penetration and primary lesion formation but contributes less to lesion expansion/colonization of fungus in plant tissue (Kars *et al.*, 2005a).

This is consistent with the reduced amount of *bcpg2* mRNA in necrotic lesions after 24 h. But in symptomless samples a slight increase was noticed with time. This may be due to the development of microscopic necrotic lesions or reflect primary lesion development not triggering necrosis. However, the necrotic samples which developed from symptomless infection had much lower levels of mRNA than in other two conditions. In the present experiment mRNA level of *bcnep1* greatly reduced from 12 hpi to 48hpi in necrotising infections. Arenas *et al.* (2010) found that during infection on detached tomato leaves transcripts of *bcnep1* gene increased at early stages of infection until the formation of primary lesion, but then declined. This is similar to findings in the present experiment. The constant higher relative level of transcripts of *bcnep1* in symptomless samples suggests that either the fungus was attempting to develop primary lesions on healthy leaf but failing, presumably due to plant defence activation, or that the mRNA was not translated or the protein was not exported.

The mRNA of the gene *bcbot1*, a gene involved in the synthesis of the phytotoxin bortydial, was detected in necrotic samples but not in symptomless samples. It has been report that the gene *bcbot1* shows its maximum expression at the change in the lesion from primary to secondary lesion (Gronover *et al.*, 2004). This is consistent with the measurements obtained in necrotic samples developed by inoculation of spore suspension, but not with measurements of the necrotic samples which developed through symptomless infection. This could be because host defence in the older tissues was less effective and necrosis can develop without excreted botrydial. In symptomless samples the transcripts were not detected, consistently with the lack of damage to host tissues.

In *A. thaliana*, leaves were inoculated while they were attached to the plant to develop necrotic lesions. In general *A. thaliana* is a less favourable host plant for *B. cinerea* than lettuce or *Primula*. The wild Arabidopsis sampling (Chapter 6) for *B. cinerea* showed lower

incidence of plants harbouring *B. cinerea* than recorded in other hosts. In the present experiments necrotic lesions started to develop after 24 hpi and the spreading lesions started to develop at about 48 hpi, more slowly than infection of detached lettuce leaves. This is consistent with the numbers of transcripts of most of the tested genes remaining more or less the same until 24 hpi.

Tissue sampling for the symptomless infection was done only at one time point after inoculation, 10 dpi, because of the speed with which the plants flowered. Symptomless rosette leaves and stem samples were used for the analysis. In symptomless samples, the relative amount of mRNA of the signalling genes, *bcg1*, *bcn* and *bmp1* was similar or slightly higher than in the 48 hpi necrotic samples. The results in *A. thaliana* leaf samples at 48 hpi were similar to the result observed in 12 hpi samples of lettuce, and the stage of disease development also similar in both cases. The presence of similar or slightly higher levels of mRNA of the rest of the signalling genes in symptomless tissues of lettuce and *A. thaliana* indicates that these signalling pathways may function as in necrotic infection. However, the major difference between *A. thaliana* symptomless and necrotic samples was that the transcripts of gene Bac were not detected. It has been reported that defects in the expression of gene Bac may lead to slow development of spreading soft rot lesions (Klimpel *et al.*, 2002), which is consistent with the symptomless infection. The difference between symptomless *A. thaliana* and lettuce could indicate differences in the way in which symptomless infection arises in the two hosts.

The transcripts of pathogenicity genes *bcnep1* and *bcbot1* were not detected in symptomless *A. thaliana* samples, but they were detected in necrotic samples and the amount in 48 hpi *A. thaliana* samples were similar to the 12 hpi lettuce samples. Again the absence of these two gene transcripts correlates with the absence of necrotic lesions in the tissue samples.

In conclusion, mRNA of the signalling genes tested, except the gene *bac*, had similar amounts in both symptomless infection and in necrotic infection (at the time of spreading lesion development) in lettuce and *A. thaliana*. The amounts of transcripts of pathogenicity related genes varied among the type of infection and host plant, but were consistent with the fungus existing in a state in which aspects of the necrotising infection process were switched off, and defences against host generation of active oxygen species were strongly activated.

Chapter 5 Microscopy of symptomless infection caused by *Botrytis cinerea*

5.1 Introduction

Microscope studies of infected plant samples are helpful to identify the causative agent of the disease and provide visual evidence of the physical interaction expressed by the host and pathogens. Fungal foliar infections mostly occur in a series of events beginning with a spore landing on the plant surface, followed by spore germination, penetration of plant tissue and colonization of fungus in plant tissue (Agrios, 2005). During this series of processes, structural and morphological developments and modifications occur in both fungus and host plant. The nature of these modifications varies greatly with the mode of fungal infection. Biotrophic fungi, especially show several structural adaptations for the penetration and survival within the plant tissue. Structural changes also occur in plant cells or tissues at the site of infection and in adjacent cell layers due to the direct activities of fungal enzymes and toxins, and by the hypersensitive responses exerted by the plant (Agrios, 2005). Microscopic examination of infected tissues with temporary or semi-permanent mounts is one of the best methods to detect and identify fungal pathogens which cannot grow on artificial media (Narayanasamy, 2011).

Visualization of fungus which is growing inside the plant tissues is not an easy task: it is necessary to clearly differentiate fungal structures from plant cell components. Even though some stains can specifically visualize the fungal structures, auto-fluorescence of plant components, such as chlorophyll and cell walls, interferes with visualization. Thickness of plant tissues and the depth of fungal invasion may also limit microscope studies of endophytic fungi. However, several methodologies have been developed to overcome these limitations and successfully used to visualize fungi *in planta*.

Fungal hyphae and sporulating structures can be visualized in infected tissues using visible light and transmission microscopy by using various stains; tissue clearing prior to staining is essential to get higher contrast. Stains such as trypan blue, cotton blue or acid fuchsin can be used to stain fungus in plant tissues (Vierheilig *et al.*, 2005). For example, intercellular mycelium and haustoria of the fungus *Ustilago scitaminea* were visualized with trypan blue stain (Sinha & Singh, 1982).

Epi-fluorescence microscopy, another useful technique, has several advantages over conventional light microscopy: fluorescence microscope uses much higher intensity light source which excites fluorophores (fluorescent substances) present in the tissue samples; special filters present in the excitation pathway limit the non-specific wavelength light and provide the image with more contrast (Webb & Brown, 2013). Several stains had been used to differentially stain fungal structures from plant tissue in epifluorescence microscopy.

Hood & Shew (1996) developed a KOH- aniline blue technique for fluorescent staining, which provided rapid, simple, and effective documentation of several plant-fungal interactions. Card *et al.* (2013) screened several fluorescent dyes to visualize endophytes *Neotyphodium lolii* and *N. coenophialum*, symbiotic endophytes of the Pooideae grasses *Lolium perenne* and *Festuca arundinacea* respectively. Among the fluorescein-based fluorophores tested (fluorescein diacetate, 5(6)-carboxyfluorescein diacetate, 5-chloromethylfluorescein diacetate and the chitin-binding stain, Calcofluor white) 5-chloromethylfluorescein diacetate produced the greatest intensity of staining of fungal hyphae and gave excellent contrast *in planta*. Knight & Sutherland (2011) found that staining with solophenyl flavine 7GFE (a fluorescent dye) can be used for the rapid visualization of *Fusarium pseudograminearum* hyphae in cereal tissues.

Fluorescent labelled lectins provide a valuable tool for the study of fungus present inside or on the surface of plant tissues. The most promising lectin, wheat germ agglutinin (WGA), is

highly specific for residues of N-acetylglucosamine, the principal component of chitin. Krahmer *et al.* (1986) used WGA coupled fluorochrome fluorescein isothiocyanate (FITC) to visualize wood decaying fungi in *Pinus* spp. In another example of histochemical detection of chitin, WGA-Alexa Fluor 488 conjugate was used to localize the chitin in hyphae of *Magnaporthe oryzae* invading rice leaf sheath cells (Mochizuki *et al.*, 2011).

Immunofluorescence techniques have also been used to localize and follow infection paths of fungal pathogens. Zelinger *et al.* (2004) examined the ultra-structure of the extracellular matrix produced by conidia and germ tubes of *Stagnospora nodorum* on wheat. To visualize the extracellular matrix a combination of lectins and monoclonal antibodies were used. Antibodies specifically recognize surface proteins and carbohydrates of the fungus.

Development of immunological methods for the detection of *Botrytis* has had a relatively long and chequered history. Dewey & Yohalem (2004) and Dewey & Grant-Downton (2015) summarised several immunological methods used to detect and to a lesser extent quantify level of *Botrytis* infection in plants.

Cole *et al.* (1996) studied the infection mechanisms of *B. fabae* with *Botrytis*-specific monoclonal antibody, BC-KH4. Ultrathin sections of *B. fabae* infected bean (*Vicia faba* cv. The Sutton) leaves were Immunofluorescence labelled. Antibody binding was noticed at the surface of both dry and wet conidia. In the presence of aqueous glucose, fluorescent labelling was also detected at the surface of the germ-tubes and throughout the fibrillar matrix material which extended from the periphery of the conidia and germ-tubes. In contrast, the short germ-tubes of dry-inoculated conidia were not immunolabelled with BC-KH4. Cole *et al.* (1998) studied the pectin degradation, a primary event in cell wall breakdown, during *B. fabae* infection on bean (*Vicia faba* cv. The Sutton). Double-labelling experiments using the monoclonal antibody BC-KH4 directed against *Botrytis* matrices and a marker for the plant cell wall (anti-pectin monoclonal antibody-JIM 7 or cellobiohydrolase conjugated to gold)

showed the extension of fungal matrices through modified host walls and degeneration of cytoplasm. Cook *et al.* (2000) used the *Botrytis* specific antibody BC-KH4 to show the active attachment of a potential biocontrol bacterium, *Enterobacter aerogenes*, to the extracellular matrix of germinating hyphae of *B. cinerea*.

The gene specific monoclonal antibody BC-12.CA4, was used to detect and quantify *B. cinerea* in grape juice, wines, strawberries and grape berries (Dewey & Yohalem, 2004).

Nowadays, the genes coding for fluorescent proteins can act as an excellent tool to label and detect pathogenic microorganisms during their infection process. Such proteins include green fluorescent proteins (GFP), yellow fluorescent proteins (YFP) and DsRed protein (Larrainzar *et al.*, 2005). The GFP has been developed into a widely used reporter system allowing the observation of cellular and molecular events in living cells. The most widely used GFP variant is enhanced GFP (eGFP), with the substitutions F64L and S65T in the chromophore region of the original GFP (Leroch *et al.*, 2011). However, one of the major limitations in working with GFP labelled / transformed plant pathogens is that the experimental system needs to be small and well-developed.

GFP has great advantages over other labelling systems; the major advantage is a single living specimen can be monitored continuously over a period of time, this reduces the variation that could arise due to the destructive sampling (Lorang *et al.*, 2001).

Ustilago maydis, the causative agent of corn smut disease, was the first filamentous fungus to be successfully transformed with the *gfp* gene (Spellig *et al.*, 1996). Since then, GFP tagged fungi have been used to reveal various aspects of fungus–host plant interaction. At present, GFP is commonly used as a reporter to detect gene expression and protein localization, in diverse groups of fungal pathogens. Dumas *et al.* (1999) studied the expression of endopolygalacturonase in *Colletotrichum lindemuthianum* during bean infection. The genes coding for the GFP and endopolygalacturonase were fused together and the expression of

GFP was monitored at different stages in the infection process. Gourgues *et al.* (2004) studied the expression of the tetraspanin-encoding gene (BcPLS1) in *B. cinerea* with eGFP transcriptional fusion; BcPLS1 was specifically expressed in conidia, germ tubes and appressoria during host penetration.

GFP also facilitates tracking of fungi *in planta*: to monitor their distribution, and to estimate their biomass. For this reason, whole fungi have been labelled with GFP. Labelling whole fungi with GFP requires strong constitutive expression of *gfp*, which typically implies transforming a cytoplasmically located protein occurring in all fungal morphotypes with no obvious effects on fungal growth or pathogenicity (Lorang *et al.*, 2001).

There have been several studies which successfully visualized fungal pathogens which grow inside the plant using GFP transformed pathogens. Maor *et al*. (1998) labelled *Cochliobolus heterostrophus* with GFP and studied the cytoplasmic and development changes in the fungus, and monitored fungal development on and inside maize leaves. Mikkelsen *et al.* (2001) used GFP to visualize the endophyte *Neotyphodium lolii* in rye grass. Sesma & Osbourn (2004) studied root infection process of rice by GFP transformed *Magnapotha grisea*. Visser *et al.* (2004) observed GFP transformed *Fusarium oxysporum* in tissues of artificially inoculated banana roots.

Confocal laser scanning microscopy (CLSM) facilitates the exploration of microbial habitats and allows the observation of host associated microorganisms *in situ* (Cardinale, 2014). Like epi-fluorescence microscopy, CLSM works by detecting fluorescence: visible light emitted from the sample after excitation by a shorter wavelength. But CLSM has several advantages over conventional epi-fluorescence microscopy. In particular CLSM acquires the fluorescent signal exclusively from the focal plane, as a pinhole excludes out of focus light, and so in CLSM consecutive optical slices along the Z-axis of an image series can be prepared for the projection and three dimensional reconstructions (Cardinale, 2014). Auto-fluorescence of

biological and synthetic substances is usually considered a negative aspect of CLSM images, because in combined images the auto-fluorescence from non-specific structures could interfere the visualization of site of interest (Lo Piccolo *et al.*, 2010). However, auto-fluorescence may be useful for interpretation of the confocal images (Cardinale, 2014).The optical sectioning capability of confocal microscopy is also an advantage in that it affords clear visualization of GFP despite the autofluorescence and light scattering properties of cell walls (Lorang *et al.*, 2001).

The fungus *B. cinerea* is well known as a necrotroph, but also shows symptomless infection in host tissues, as discussed in earlier chapters. There are several microscopic studies which have been done to elucidate the stages in the infection process of necrotrophic or symptomless infection. Williamson & Duncan (1989) studied the infection of black current flowers by *B. cinerea* using scanning electron microscopy. The conidia germinated on stigmas and the hyphae grew throughout the styles, reaching the proximal end of the style. The infection process of *B. cinerea* on rose petals was studied by Williamson *et al.* (1995) using fluorescence microscopy. Germ tubes penetrated the epidermal cells of rose petals and the hyphae entered the intercellular spaces of the petal within 24h. The hyphae were visible passing through cell walls of the mesophyll cells with little evidence of distortion of the cell wall. *B. cinerea* infection on grape flowers was monitored under electron microscopy by Viret *et al.* (2004).

There have been several attempts to produce GFP transformed *B. cinera* and to trace the infection process in host plants. Xiu-Zhen *et al.* (2007) successfully transformed GFP gene into *B. cinerea*. The gene was expressed in hyphae and conidia both *in vitro* and *in planta*, without showing differences in morphology or pathogenicity of the fungus. Leroch *et al.* (2011) generated a codon optimized intron containing genes (*bcgfp1*) coding for the eGFP; the original *egfp* gene showed rather low fluorescence in *B. cinerea* despite being expressed under the control of the strong *oliC* promoter, because it had higher GC content than *B.*

cinerea. In the present experiment, I used the same isolate (Bcgfp1) for the inoculation studies. The isolate Bcgfp1 is a GFP transformed *B. cinerea* isolate B05.10. The isolate B05.10 produced highest amount of symptomless infection in inoculation studies with *A. thaliana* and lettuce (Chapter 3 and 4). In addition to that, spore suspensions of the isolates Bcgfp1 and B05.10 showed similar necrotic lesion development on lettuce and *A. thaliana* leaves.

Studies in the host plants lettuce, *Primula* and dandelion (*Taraxacum agg*) showed that *B. cinerea* can also cause symptomless systemic infection, as discussed previously. A couple of attempts have been made to provide microscopic evidence for symptomless systemic infection by *B. cinerea*. Sowley *et al.* (2010) confirmed the systemic, endophytic infection of *B. cinerea* in symptomless stem, roots and leaf of lettuce using monoclonal antibody BC-12.CA4. Penetration of the antibody into the host tissue was poor. However, immunolabelled mycelium was seen in the interior of plant. Rajaguru (2008) made an attempt to monitor the symptomless and systemic growth of the *B. cinerea* inside the *Primula* plants. *B. cinerea* was labelled with GFP; the gene coding for the GFP was introduced in to the *B. cinerea* by Agrobacterium mediated transformation. Even though the GFP expressing pathogen was isolated from non-symptomatic parts of the plants it was very difficult to visualize the fungus inside healthy plant tissues. However, once leaves started to show symptoms GFP expressing pathogen was observed inside the leaf tissue. The interference of green auto fluorescence from plant tissue, weak expression of GFP inside the healthy plant and trace amounts of fungus inside the tissues may be the main reasons for the invisibility of *B. cinerea* in healthy tissues.

The objective of the experiments in this chapter was to visualize *B. cinerea* which was growing symptomlessly in host plants lettuce and *A. thaliana*. GFP labelled B05.10 isolate (Bcgfp1) was used for the inoculation and the infection process was monitored under CLSM at different time intervals. Some *A. thaliana* were also inoculated with *B. cinerea* isolate

B05.10 and tissue samples were stained with calcofluor white or Wheat Germ Agglutinin linked Fluorescein isothiocyanate (WGA-FITC). The experiment clearly confirmed the presence of *B. cinerea* in healthy plant tissues and has revealed the development stages of fungus in and on the plant tissue.

5.2 Materials and Methods

The experiments with GFP labelled *B. cinerea* were carried out in the appropriate containment in the Biffen building, John Innes Centre, Norwich, UK. The rest of the experiments were done in the University of Reading.

5.2.1 Host plants

Arabidopsis thaliana ecotype col-0 and the lettuce variety Tom Thumb were used as host plants. After four days of stratification *A. thaliana* seeds were sown in Seed and Modular (Clover, Ireland) compost in multi-cell seedling trays. Lettuce seeds were sown in F1 Seed & Modular (Levington, UK) compost in multi-cell seedling trays. After four weeks the seedlings were transplanted into potting compost in large pots. The plants were grown in a growth chamber (Sanyo gallenkamp plc, UK) set to 22 °C day and 18 °C night temperature, 65% relative humidity, 16 h light and 8 h dark period, 80-85µmol/m^2/s light intensity.

5.2.2 Fungus Inoculation

Fungus strains were kindly provided by Dr. Henk Jan Schoonbeek, John Innes Centre, Norwich. *B. cinerea* isolate B05.10 (which has been fully sequenced (Staats & van Kan, 2012)) and its GFP labelled derivative Bcgfp1 were used in this experiment. GFP labelled isolate Bcgfp1 was made in the Department of Biology, University of Kaiserslautern, Kaiserslautern, Germany (Leroch *et al.,* 2011). Spores were collected from three week old culture plates by tapping the plates on a piece of aluminium foil inside the flow cabinet. 5mg of spores were dusted on seedlings under spore settling towers. During each inoculation clean coverslips were placed among the plants and the spore density was measured based on microscopic counting; the spore density was 90 ± 10 spores / mm^2 (n=10).

For the lettuce, inoculation was done at the four leaf stage (including cotyledons). *A. thaliana* was inoculated about 3 weeks after seed sowing, while the plants were at rosette growth stage.

After inoculation plants were covered with a transparent lid for two days to provide higher humidity.

5.2.3 Microscopic examination of tissue samples

Different plant parts of *A. thaliana* and lettuce were collected at different time points, starting from 2 days after inoculation, and examined for the presence of *B. cinerea*. The tissue samples from plants which were inoculated with GFP labelled *B. cinerea* were directly observed under the microscope as water mount samples.

Tissue samples from plants inoculated with B05.10 isolate were stained with calcofluor white (Sigma-Aldrich, USA) by vacuum infiltration method. Stock stain solution was prepared with 5g/l concentration of calcofluor white in sterilized water and the pH was adjusted between 10-11 by adding 1M NaOH. The tissue samples were stained with 100 times dilution of stock stain solution.

Some of the tissue samples from B05.10 inoculated *A. thaliana* were first fixed in ethanol and acetic acid (3:1 v/v) mixture, then washed in phosphate buffer (pH 6.8) and then kept in 10 % KOH for 2h. The samples were stained with Wheat Germ Agglutinin linked Fluorescein isothiocyanate (WGA-FITC) (Sigma, USA). The stain was diluted 500 times in Phosphate buffer and then tissue samples were stained for 1 h. Tissue samples were then counter stained with Propidium Iodide (10µg/ml) (Sigma, USA) for further 1 h. Before and after each staining procedure the samples were washed in phosphate buffer. Finally they were mounted on a slide with citifluor (Agar scientific, http://www.agarscientific.com).

Samples inoculated with GFP labelled *B. cinerea* or stained with calcofluor white were first observed under UV epi-fluorescence microscope (Nikon Eclipse E800, Japan). Slides which appeared of interest were observed under a Leica TCS SP5 ii confocal laser scanning microscope, software LAS AF (Version 2.7.3.9723). Tissue samples stained with WGA-FITC were observed under TCS SP2 Confocal Laser scanning microscope.

The CLSM images were processed with the Fiji application (http://fiji.sc/Fiji): image channels were merged and brightness and contrast were adjusted. Z stacks were combined to produce 3D and animated images. Images were prepared by the combination of different channels: GFP labelled *B. cinerea*, WGA-FITC stained *B. cinerea* isolate B05.10 and sometimes chloroplasts were detected in green channel; auto fluorescing chloroplasts and PI stained plant tissues were detected in red channels; blue channel was used to detect calcofluor white stained *B. cinerea* stain B05.10 and plant cell wall; the bright field image was observed using grey channel.

5.3 Results

The spores were evenly distributed on leaf surface with the spore density of 90 ± 10 spores / mm^2. Two kinds of spores were noted: ovoid shaped macro conidia with average length 10.1 ± 1.5 µm (n=20) and average width 6.2 ± 0.8 µm (n=20), and spherical shaped micro conidia with average size of 6.2 ± 0.7 µm (n=20) (Figure 5-1).

Germinated spores were very few compared to the total number of spores inoculated; the germinated spore density was about 3 spores / mm^2 leaf surface. Germinating spores produced short germ tubes. However, the fungal development was stopped at this stage in most of the spores, and only about 5 spores / leaf showed continued fungal development. There were no abnormal structural changes or visible macroscopic or microscopic plant responses in the plant cells adjacent to spores whose germination had ceased. Successful spore germination and further development was found on the epidermal cell surface, including on or near to the guard cells, intercellular junctions, and also on the trichomes in *A. thaliana*.

5.3.1 In Lettuce

The spores mostly produced single germ tubes, but occasionally more than one germ tube (Figure 5-2). The length of germ tubes and their mode of penetration were not uniform; some spores produced a short germ tube and immediately penetrated the epidermal cell wall or guard cell wall (Figure 5-2A and 5-3); some other spores produced slightly longer germ tubes which grew parallel to the leaf lamina either completely inside the cuticle or exposed to outer surface at certain points. Germ tube growth was also noted at the cell junctions where the germ tube ran parallel to the cell junction below the cuticle layer (Figure 5- 4 to 5-6). The germinated spores, germ tubes and growing mycelium made scars on the cuticle (Figure 5-4B and C).

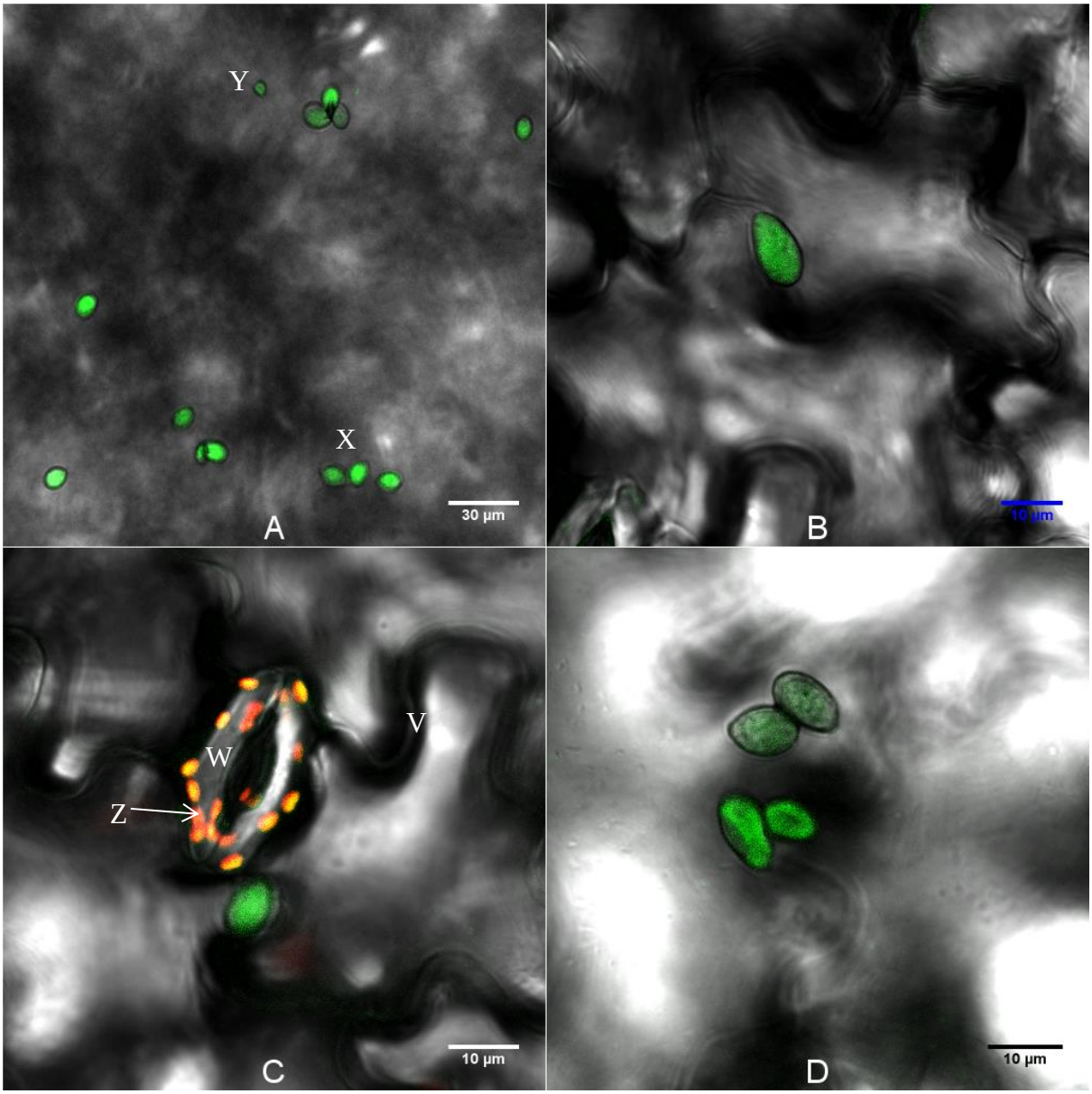


Figure 5-1: Un-germinated spores on lettuce leaf surface, 2 days after inoculation. **A-D** different leaf samples inoculated with GFP labelled *B. cinerea*. The images are combined Z sections of green, red and grey channels. Epidermal cell boundary (**V**), Guard cell (**W)**, Spherical shaped micro conidia (**Y**), ovoid shaped macro conidia (**X**), Chloroplasts (**Z**).

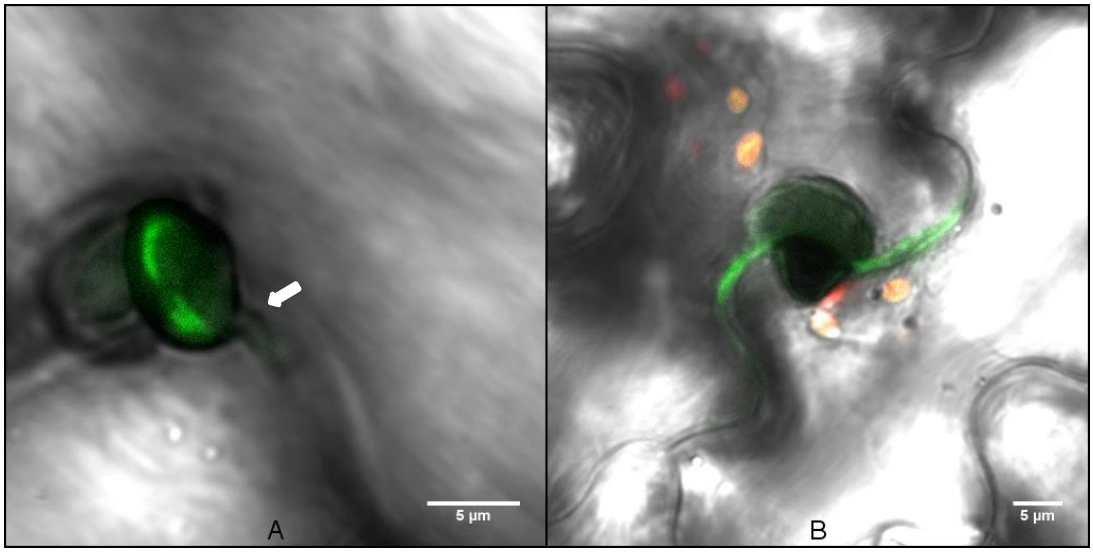


Figure 5-2: Macro-conidia germinated on a lettuce leaf surface 2 days after inoculation. **A** and **B** images from different leaf samples inoculated with GFP labelled *B. cinerea* isolate. Z sections of green, red and grey channels have been merged to produce the images. Short germ tube penetrates cuticle layer immediately bellow the macro-conidium at epidermal cell junction (**A**). A macro-conidium produces two germ tubes which move along the cell junction (**B**).

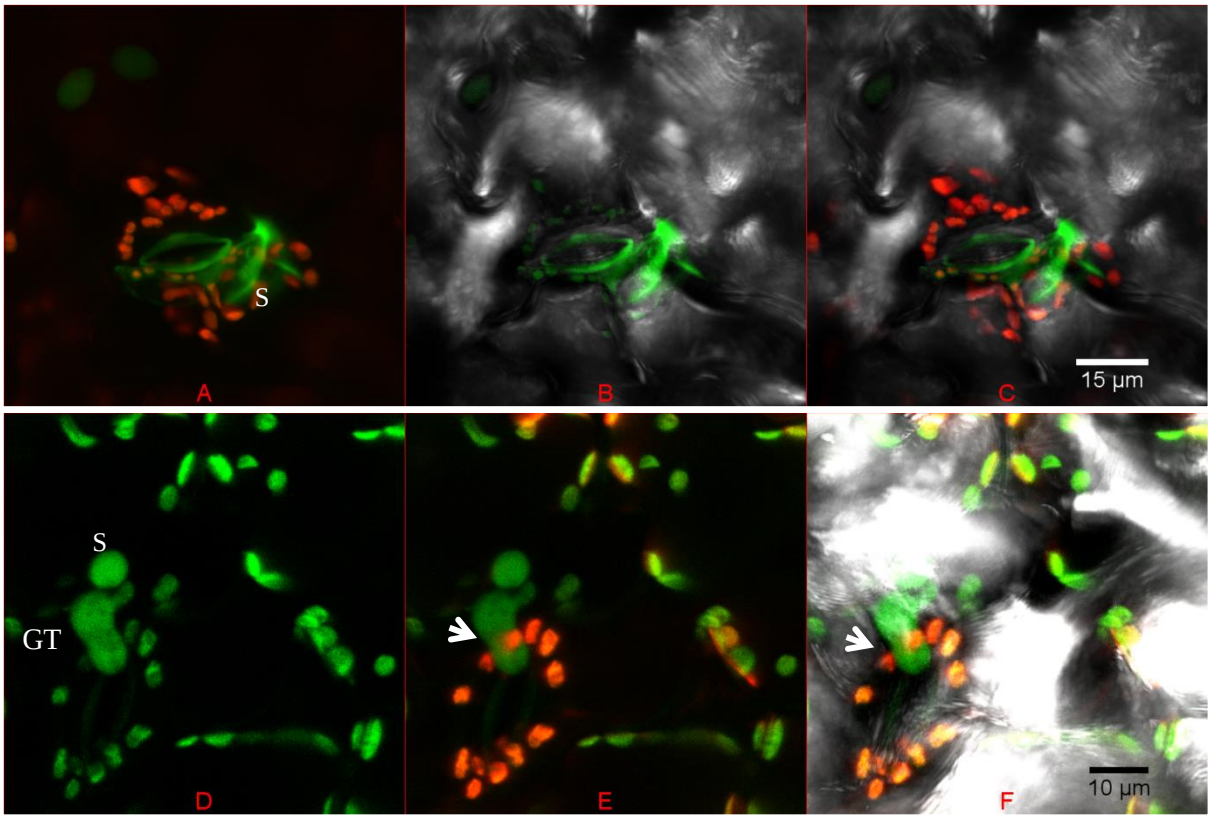


Figure 5-3: Macro-conidia germinating at the guard cells of a lettuce leaf 4 days after inoculation. Images **A-C** and **D-F** are from two different leaf samples inoculated with GFP labelled *B. cinerea*. Images are combination Z sections of green, red and grey (bright field) channels. (**A-C**) Macro-conidium germinates and produces short germ tube very close to the guard cell. (**D-F**) The germ tube of a macro-conidium penetrates a side wall of guard cell and the tip of the germ tube remains inside below the level of chloroplasts (arrow). **S**- macro-conidium, **GT**-germ tube.

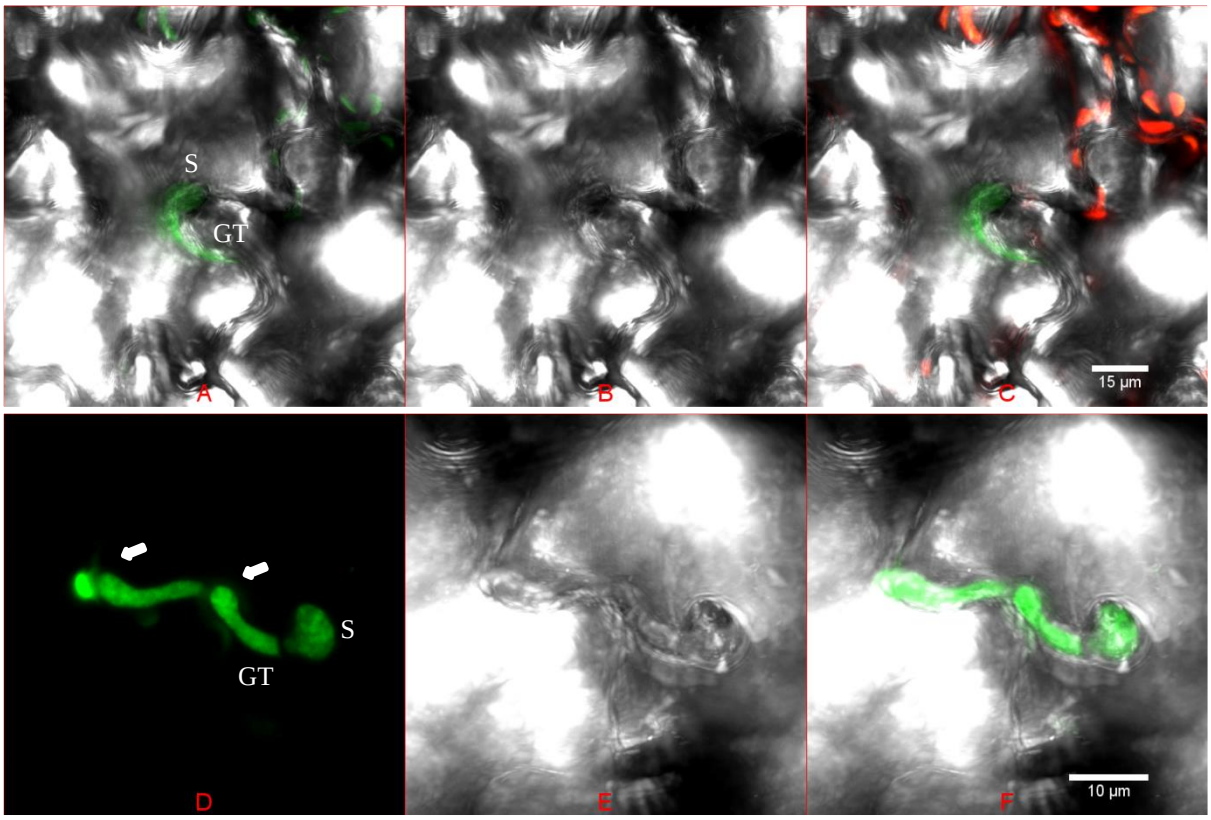


Figure 5-4: Germ tubes growing below the cuticle 5 days after inoculation. Images **A-C** and **D-F** are from two different leaf samples with GFP labelled *B. cinerea* infection and the images are combination of Z sections of green, red and grey (bright field) channels. In **A-C**, Germ tube of a macro-conidium grows below the cuticle layer parallel to the cell junction. In **D-F**, Spore germinates on the epidermal cell surface, the germ tube grows on the outside of cuticle until reach a cell junction, then penetrates cuticle at the cell junction and grows parallel to the leaf surface below the cuticle, finally it comes out from the cuticle until it reaches a junction of three epidermal cells (**D-F**). The arrows indicates site of cuticle penetration. **S**- spore, **GT**- germ tube, and the arrows indicates site of penetration.

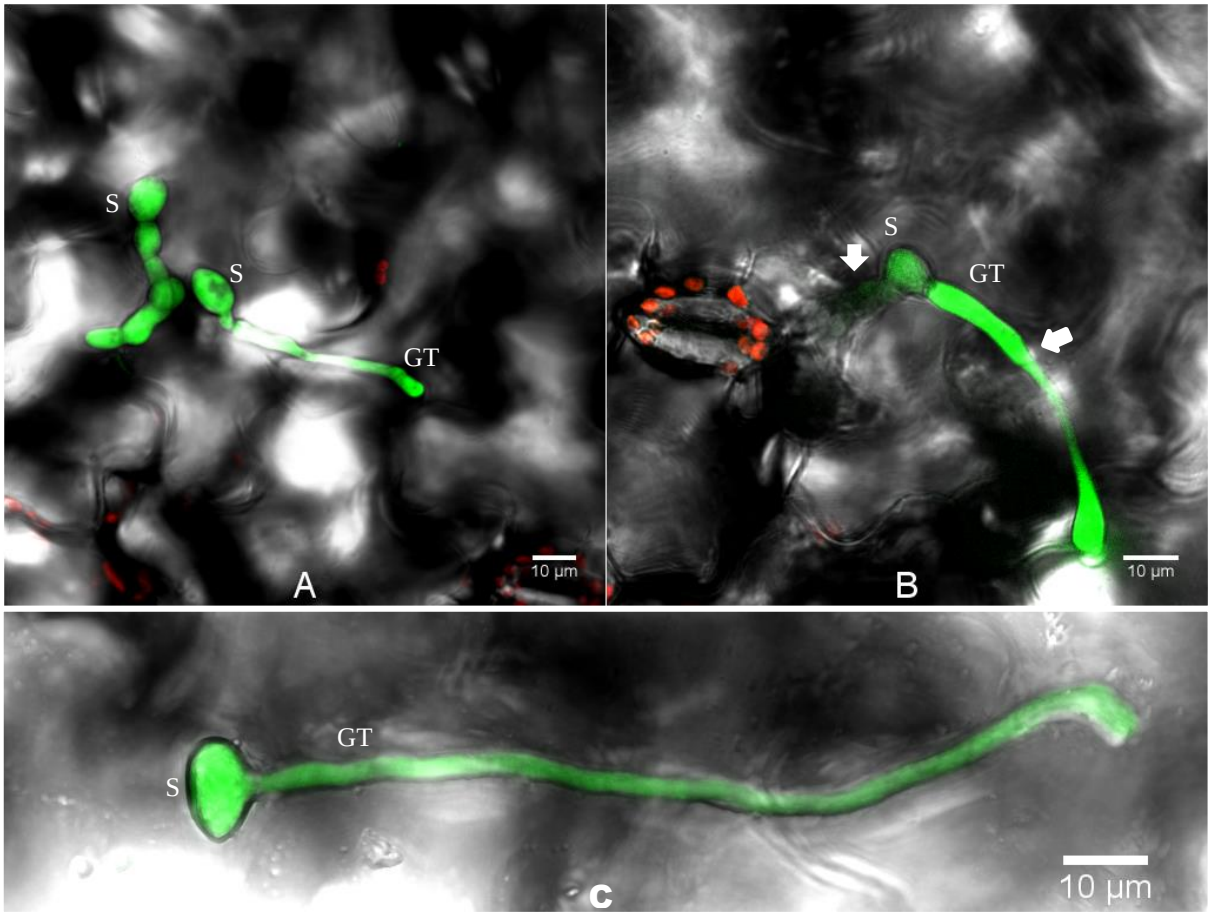


Figure 5-5: Long germ tubes growing on lettuce leaf surface above and below the cuticle layers 10 days after inoculation. Images are combination of Z sections of green, red and grey (bright field) channels. Germ tubes show different kinds of growth on leaf surface; they may grow on the outer surface of cuticle for some time after germination (**A**), they may penetrate the cuticle layer and grow parallel to the leaf surface, then again come out of the cuticle (**B**), or in some others they grow below the cuticle layer for long distance (**C**). **S**- Spore, **GT**- germ tube, and the arrows indicates site of cuticle penetration.

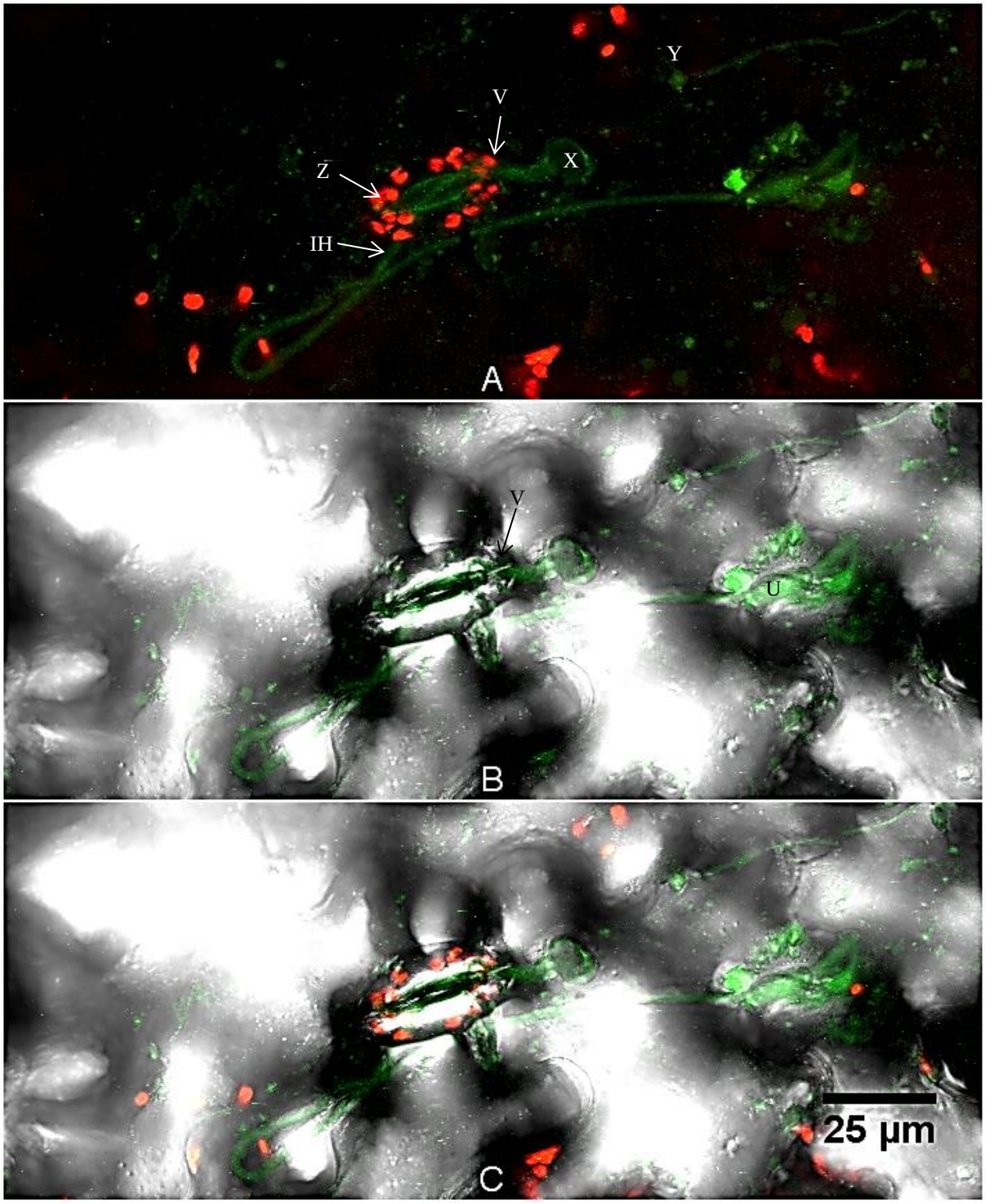


Figure 5-6: Fungal hypha growing inside the guard cells of lettuce 15 days after inoculation. Images (**A**-**C**) are combination of Z sections of different channels (green, red and bright field) of an image. The leaf sample shows symptomless infection caused by GFP labelled *B. cinerea*. Germ tube penetrates the wall of a guard cell (**V**) and grows inside the stomatal opening (**IH**). Later it penetrates the other end of the guard cell wall, and then penetrates the adjacent epidermal cell. The terminal end of the hypha forms a broad structure compared to the hypha on the surface of an epidermal cell (**U**). **X**- macroconidium, **Y**- microconidium.

Both micro conidia and macro conidia germinated and produced hyphae; the hyphae produced from micro-conidia were thin, long, and not often branched, but the macro conidia produced about 3 times thicker hyphae which often branched (Figure 5-7).

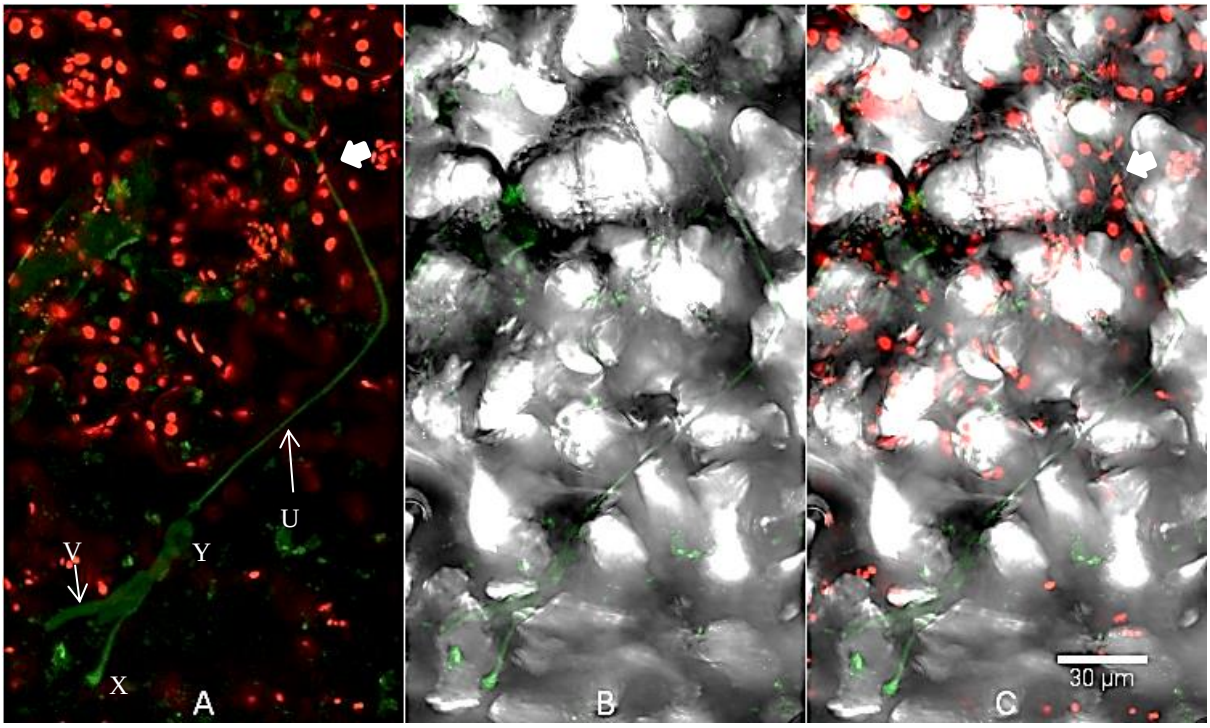


Figure 5-7: Micro conidia and macro conida germinated on lettuce leaf surface 13 days after inoculation. The image was obtained from a lettuce plant having symptomless infection caused by GFP labelled *B. cinerea*, and **A-C** are combination of Z sections of different channels (green, red and bright field) of an image. Micro conidia (**X**) produces thin and long hypha (**U**) compared to the hypha (**V**) of macro conidia (**Y**). The thin hypha grows inside the leaf tissue at the terminal end, possibly below the epidermal layer Indicated by the arrows.

Symptomless lettuce leaf tissues often had auto-fluorescent epidermal cells and guard cells. The wall of these cells fluoresced brightly, and could mimic fungal hyphae growing closely associated with the cell wall. These cells had fewer chloroplasts compared to adjacent cells. The bright field images showed that these cells were apparently damaged, and always associated with un-germinated or germinated spores. Therefore these cells could be microscopic necrotic lesions. These microscopic lesions were always restricted to one or few plant cells and did not increase in size with time (Figure 5-8).

Most of these microscopic necrotic lesions had a green fluorescent, thick fungal inclusion or subcuticular vesicle inside the plant cell, often closely associated with the plant cell wall (Figure 5-8 and 5-9). It had definite margins but not a specific shape. The development of this fungal inclusion often began with the germination of a spore. The germinating spores produced more than one germ tube and the germ tubes or hyphae ran along the plant cell wall. The size of the fungal inclusion increased and at late stages about 2/3 portion of the plant cell was filled by the inclusion (Figure 5-10).

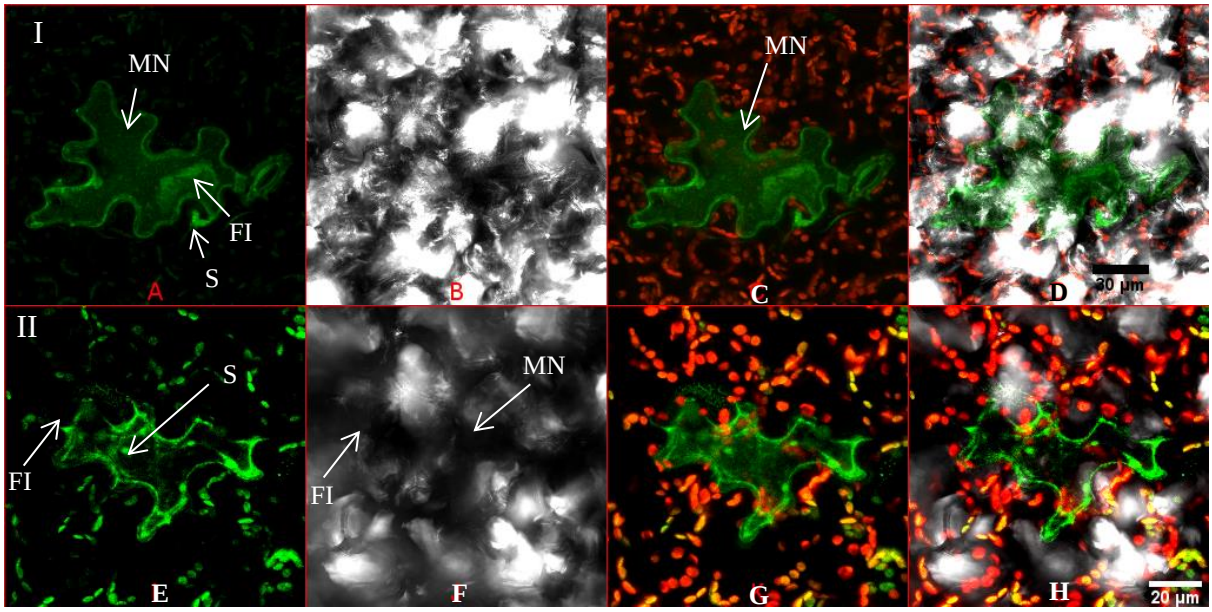


Figure 5-8: Apparently healthy lettuce leaves showing microscopic necrotic lesions 5 days after inoculation. Images in each row (**I-II**) obtained from different leaf samples which were inoculated with GFP labelled *B. cinerea*. The images in every single row represent combination of Z sections of different channels (green, red and bright field) of an image. Green auto fluorescence cell or cells are detected among the healthy cells. These cells often associated with germinated and / or un-germinated spores and a fungal inclusion / subcuticular vesicle which was closely associated with the cell wall. The cell walls of these cells are unevenly thickened, and produced bright fluorescence. **S**- spore, **FI**- fungal inclusion, **MN**- microscopic necrotic lesion.

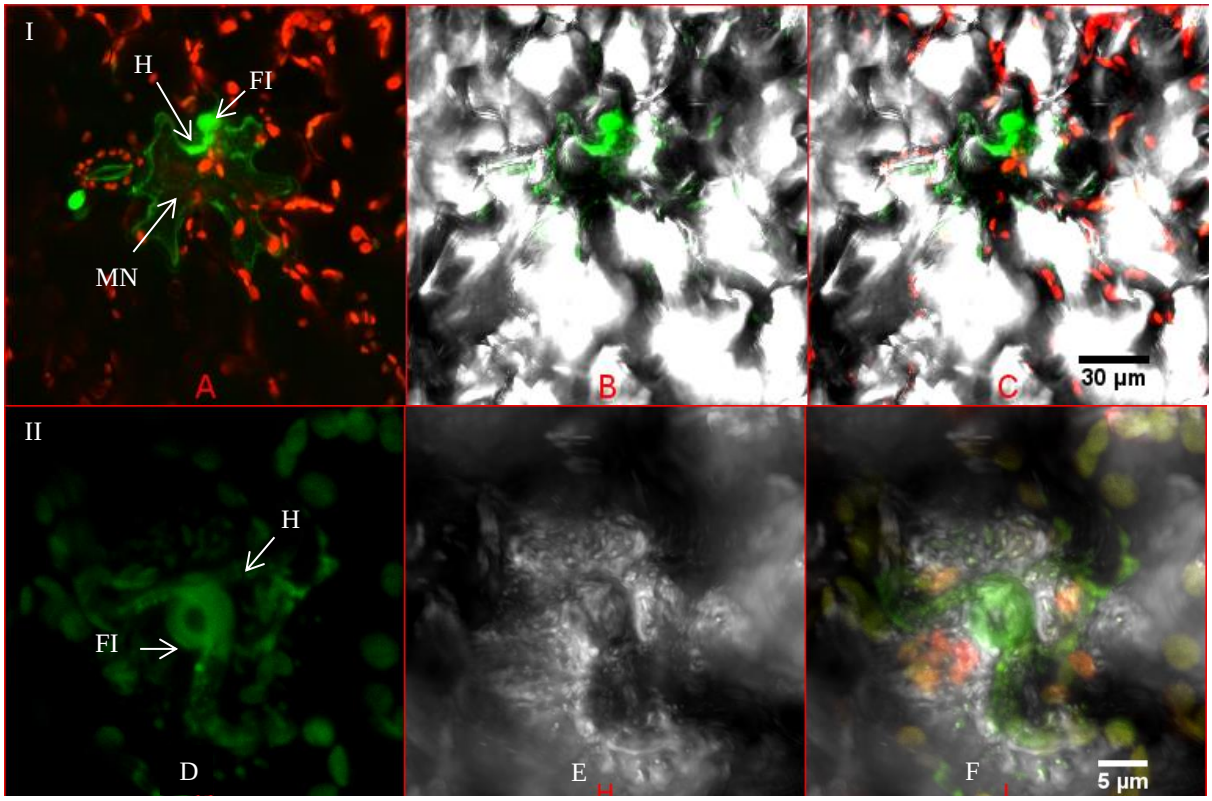


Figure 5-9: Hyphae develop from fungal inclusion in symptomless lettuce leaves 5 days after inoculation. Images in each row (**I-II**) are from three different leaf samples inoculated with GFP labelled *B. cinerea*. The images in every single row represent combination of Z sections of different channels (green, red and bright field) of an image: A-red and green, B-green and bright field, C and F -all three channels, D-green, E-.bright field. The fungal inclusion produces hyphae in the microscopic necrotic lesion, and the newly developed hyphae grow along the cell wall of the infected cell. The bright field image **E** shows the cell damage in the infected plant cell. **S**- spore, **FI**- fungal inclusion, **MN**- microscopic necrotic lesion.

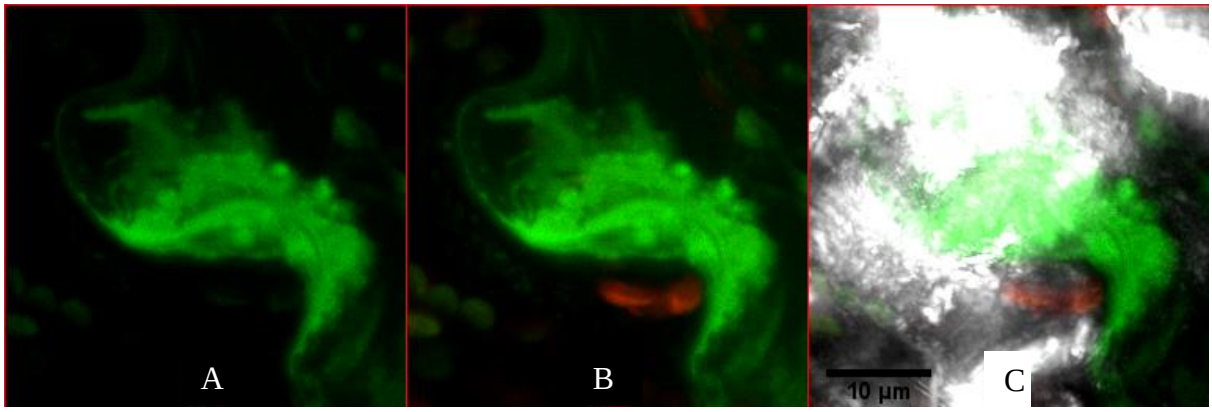


Figure 5-10: Fungal inclusion/subcuticular vesicle inside the plant cell 6 days after inoculation. The inclusion is closely associated with the plant cell wall and apparently it develops below the cuticle layer (**C**). The inclusion does not have a simple shape but it has distinct margins.

The fungal inclusion present in the microscopic necrotic lesions produced hyphae. The fungus spread in and on the healthy leaf tissues as branched mycelium (Figure 5-11 and 5-12). The thickness of hyphae varied in some site of infections; even in a single mycelium the portion which grew inside the tissue was thinner than the portion that growing outside. In some sites of infection the internal mycelium was not clearly detected, but when they appeared on the outer surface they became visible. Therefore, the mycelium appeared to be non-continuous (Figure 5-11). The terminal ends of hyphae mostly produced broad or enlarged structures below the cuticle layer, often branched and appendage-like. These terminal structures penetrated the plant tissues at cell junctions (Figure 5- 11 to 5-13).

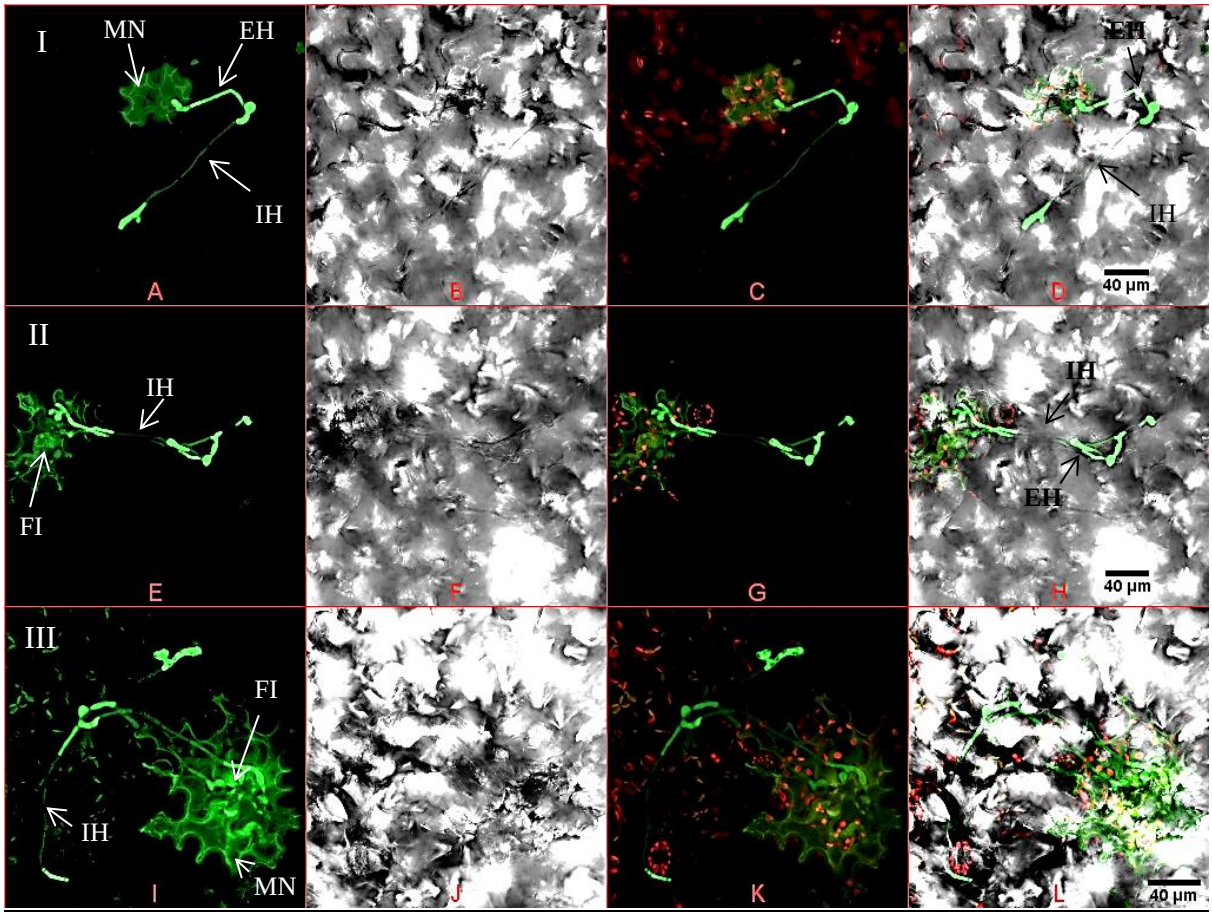


Figure 5-11: Fungal hyphae developing from microscopic necrotic lesions 10 days after inoculation. The images in rows **I-III** are from three different lettuce leaf samples inoculated with GFP-labelled *B. cinerea* 10 days after

inoculation. The fungal inclusions in the microscopic necrotic lesions produce spreading hyphae. The hyphal branches grow parallel to the leaf surface in and out of the plant tissue. The hyphal regions which grow inside the tissue or cuticle produce less florescence than the region exposed to outer surface. **MN**- microscopic necrotic lesion, **IH**– internal hyphae, **EH**- external hyphae.

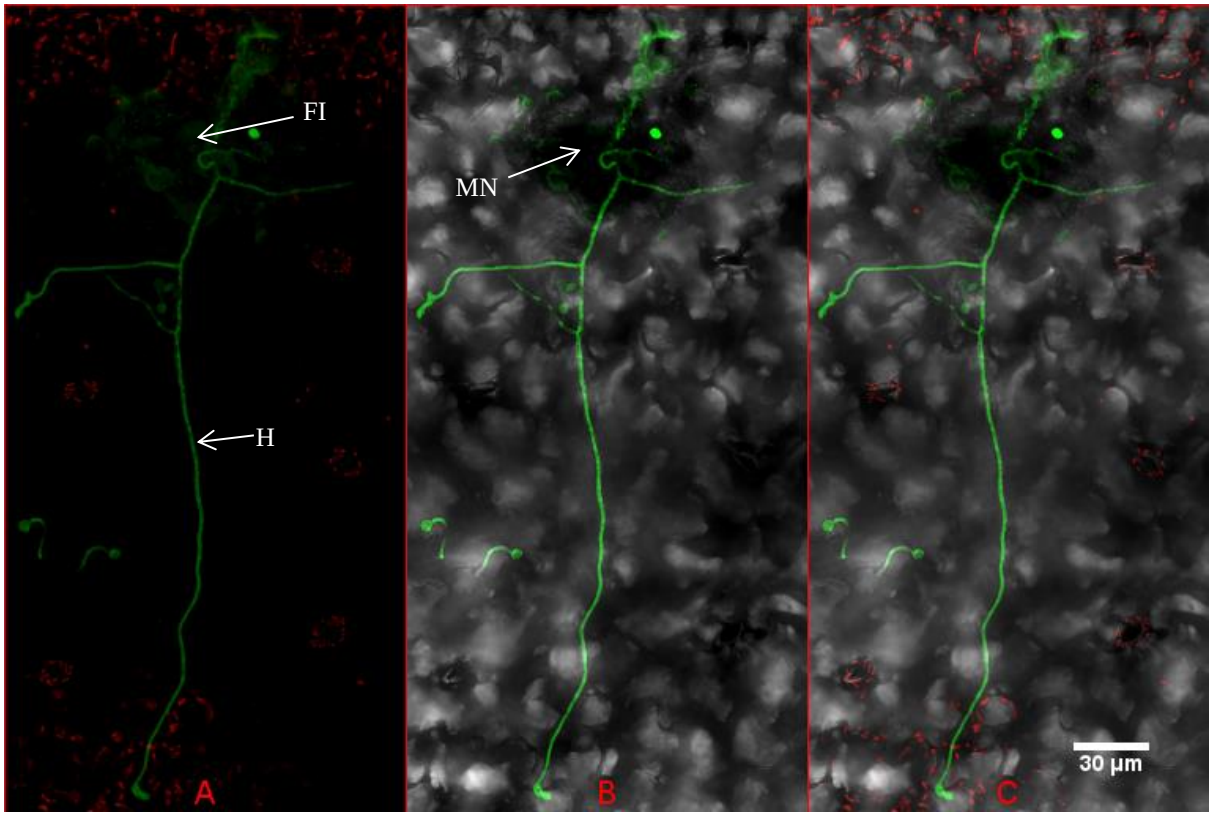


Figure 5-12: Long branched hyphae produced by the fungal inclusion in symptomless lettuce leaves 12 days after inoculation. The images (A, B and C) were obtained from lettuce leaves which were inoculated with GFP labelled *B. cinerea.* The images are combination of Z sections of different channels (green, red and bright field) of an image. The surface of the necrotic cells is damaged and the cells are filled with fungal inclusions or vesicles. The fungal vesicle produces long, branched hyphae which spread on the leaf surface. The branches of the hypha produce broad penetration structures at terminal ends. **FI**- Fungal inclusion, **H**- Hyphae, **MN**- Microscopic Necrotic lesion.

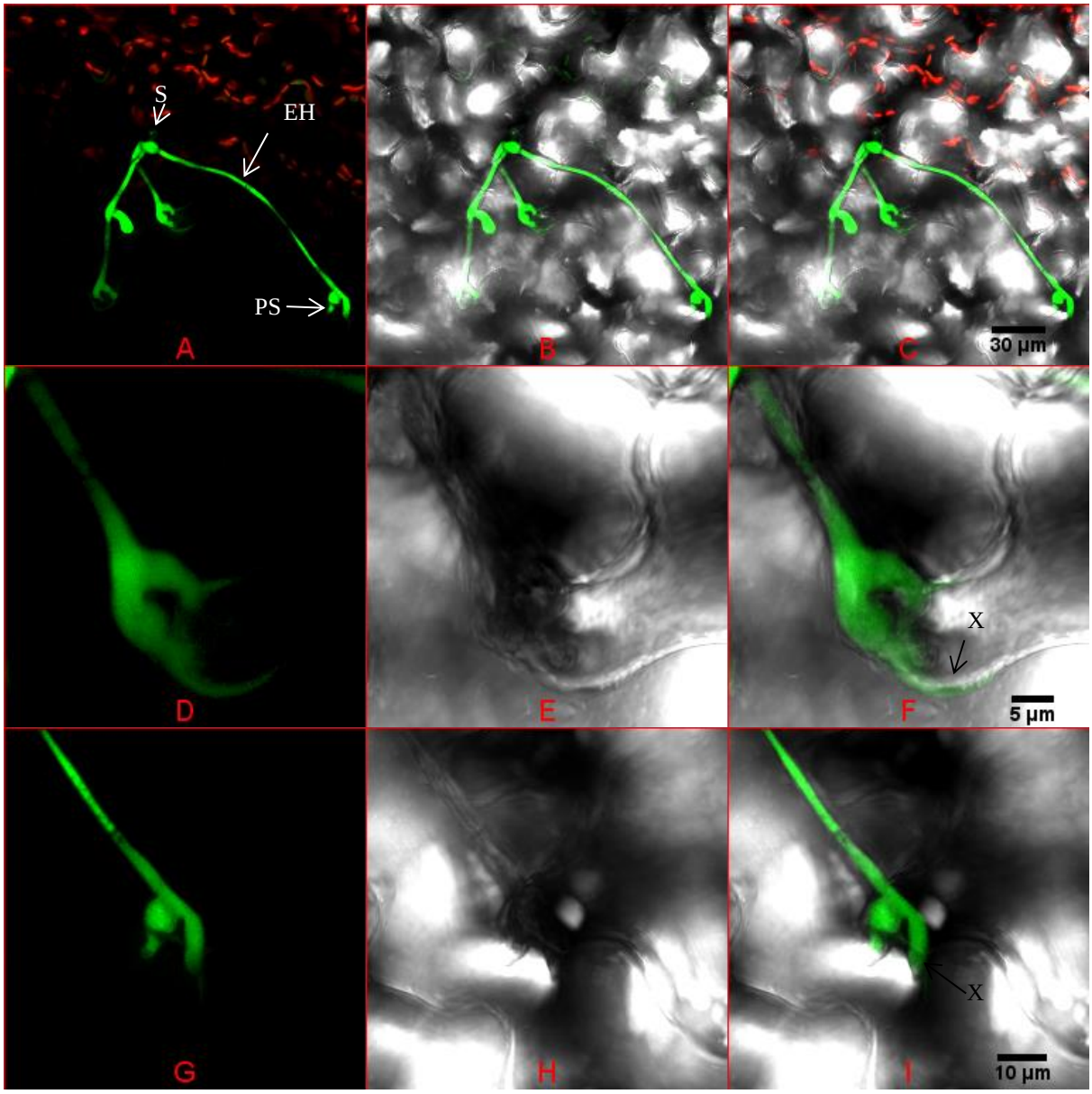


Figure 5-13: Hyphal tips of *B. cinerea* producing penetration structures 10 days after inoculation. The images are from a lettuce leaf sample having symptomless *B. cinerea* infection. **A-C** Spore (**S**) produces hyphae with penetration structures (**PS**). **D-F** and **G-I** are terminal ends of two hyphae with broad penetration structure. The finger like structures grows inside the cuticle (**X**). **EH**- external hyphae.

The fungal hyphae were also detected inside the leaf tissues, below the epidermal layers; the internal hyphal growth was detected when they were below the level of cells containing chloroplasts. However, they were not growing completely inside the plant tissues; the hyphae mainly moved along the cell junctions parallel to the leaf surface and occasionally penetrated the plant cells to grow inside (Figure 5-14 to 5-16).

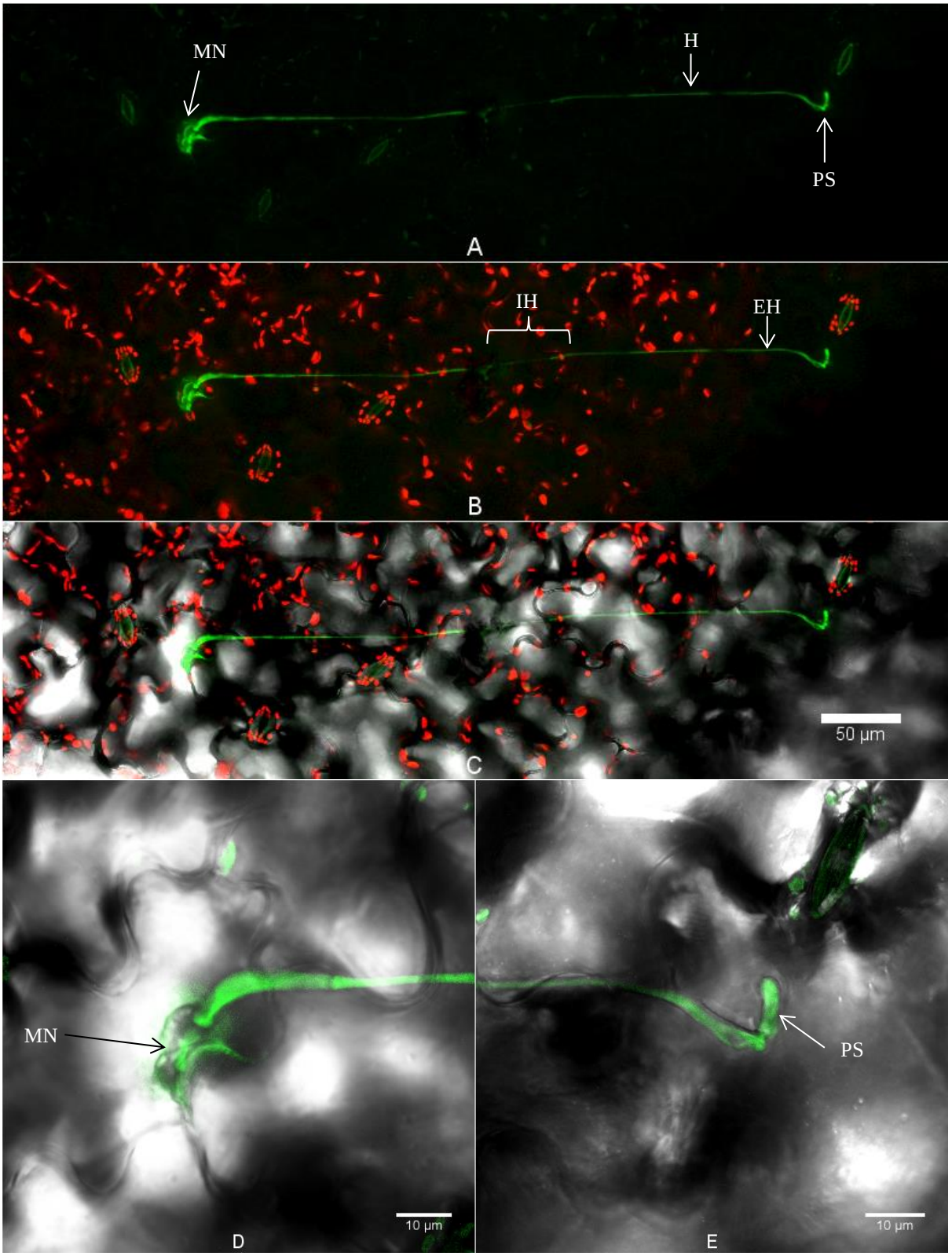


Figure 5-14: Hyphae growing inside the plant tissue. Symptomless lettuce leaf tissue with GFP labelled *B. cinerea* infection 13 days after inoculation. The images **A-C** are different combinations of Z sections of red, green and grey channels. A microscopic necrotic lesion (**MN**) produces long unbranched hyphae on the leaf surface. In certain regions the hypha grows below the level of chloroplasts as internal hypha (**IH**). The images **D** and **E** enlarged view of the two terminal ends of the above image. The terminal end of the hypha produces penetration structures (**PS**). **EH**- external hyphae.

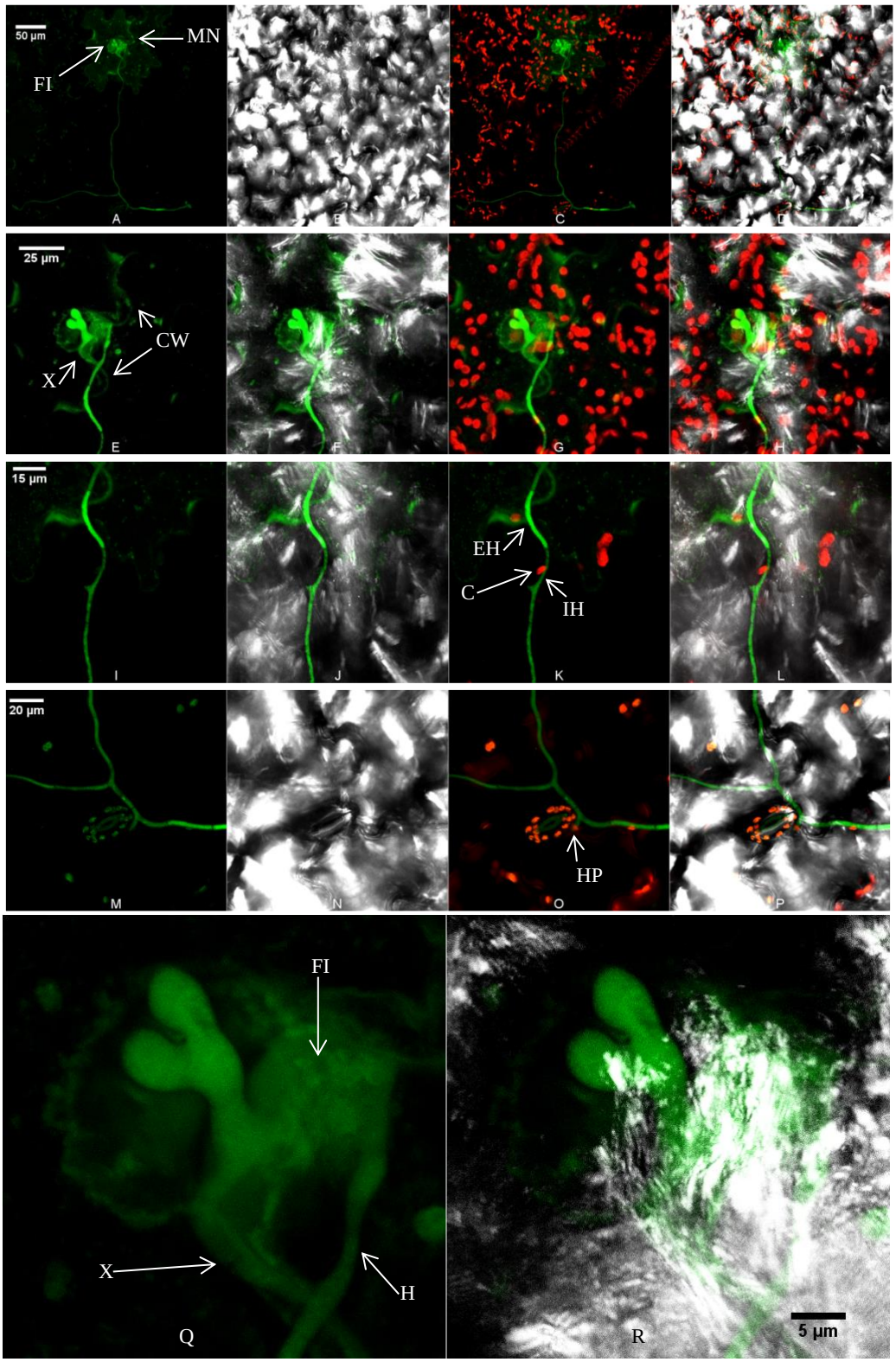


Figure 5-15: Detailed view of a microscopic necrotic lesion, fungal inclusion and spreading hyphae, 12 days after inoculation. Images were obtained from a lettuce leaf sample which had symptomless infection with *B. cinerea*. **A-D,** the whole view of fungal spread from the initial site of origin to other tissues. The images are combination of Z sections of different channels. The fungal inclusion (**FI**) produces about 500 µm long a hypha (**H**). Part of the inclusion is firmly attached to the plant cell wall (**CW**), and the fungus hypha is growing along the cell wall (**X**). The inclusion is below the cell surface, and it produces an appendage like structure which is exposed on the outer surface (**E-H**). The hypha grows in (**EH** and **IH**) and on the leaf tissue (**I-L**), dimmer

regions of the hypha and the presence of hyphae below the chloroplast (**C**) confirm their internal growth. The newly developed branches (**HP**) attempt to penetrate the leaf tissues (**M-P**). Magnified view of fungal inclusion is in **Q** and **R**.

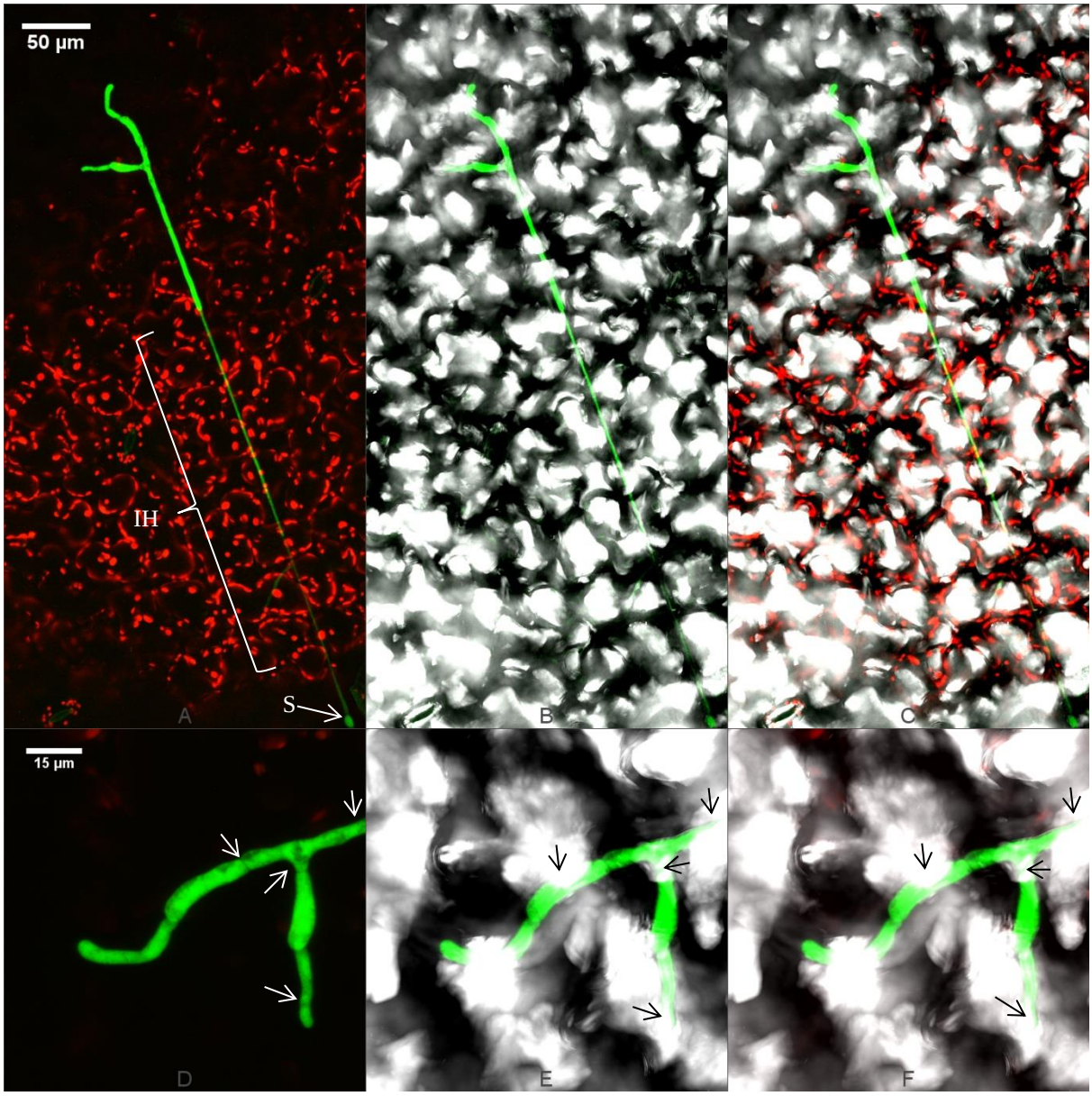


Figure 5-16: GFP labelled *B. cinerea* growing symptomless inside the plant tissue, 15 days after inoculation. A macro conidium produces long hypha which penetrates the leaf tissue at about 50 µm from spore. The hypha grows inside the tissue for further about 300 µm, during this the main hypha and its branch being below the level of chloroplast (**A-C**). The thickness of hyphae is less when they grow inside the tissue. The 1/3 terminal end of the hypha seems to be on the outer surface of the leaf tissues, but the detailed view of the terminal end (**D-F**) shows some regions of hyphae inside the cuticle (arrows).

Long and continuously growing hyphae were observed near to the lettuce leaf margin. They grew both inside and outside the lettuce leaf. These hyphae arose from microscopic necrotic lesions with fungal inclusions. These fungal inclusions produced a number of hyphae and form a fungal network on and in the leaf. However, apart from these microscopic necrotic lesions all other plant cells in the leaf tissue remained apparently healthy (Figure 5-17 and 5-18).

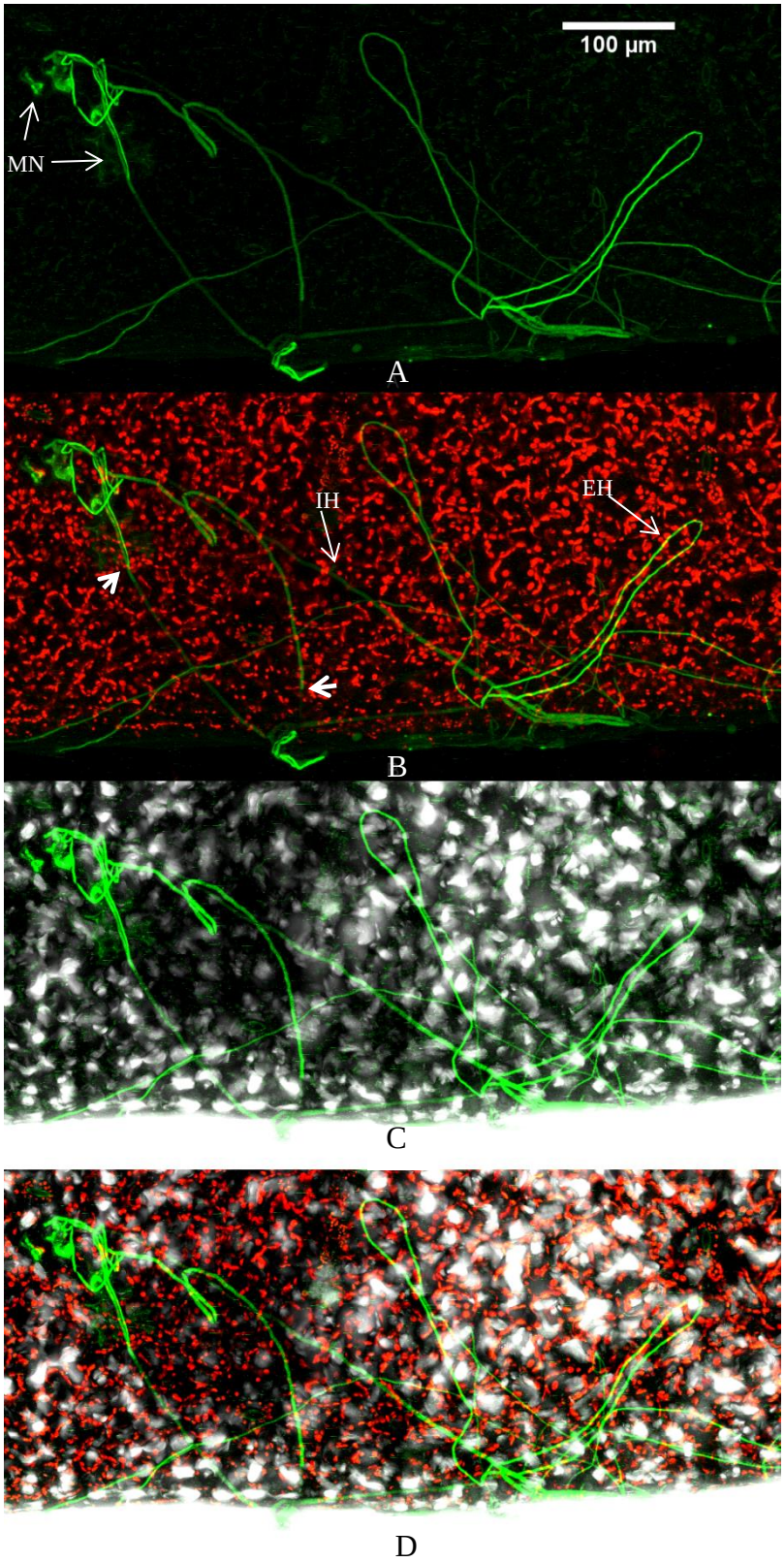


Figure 5-17: GFP labelled *B. cinerea* spreading in and on the apparently healthy lettuce leaf tissue, 30 days after inoculation. The fungal hyphae originate from fungal inclusions in microscopic necrotic lesions and then spread outside the cuticle, and inside the leaf tissues of lettuce leaf. The internal growth is confirmed by the reduced fluorescence intensity and the presence of hypha below the level of cells containing chloroplasts (**D**). **MN**- microscopic necrotic lesions, arrows shows penetration sites. **IH**- internal hyphae, **EH**- external hyphae, arrows- site of penetration of hyphae.

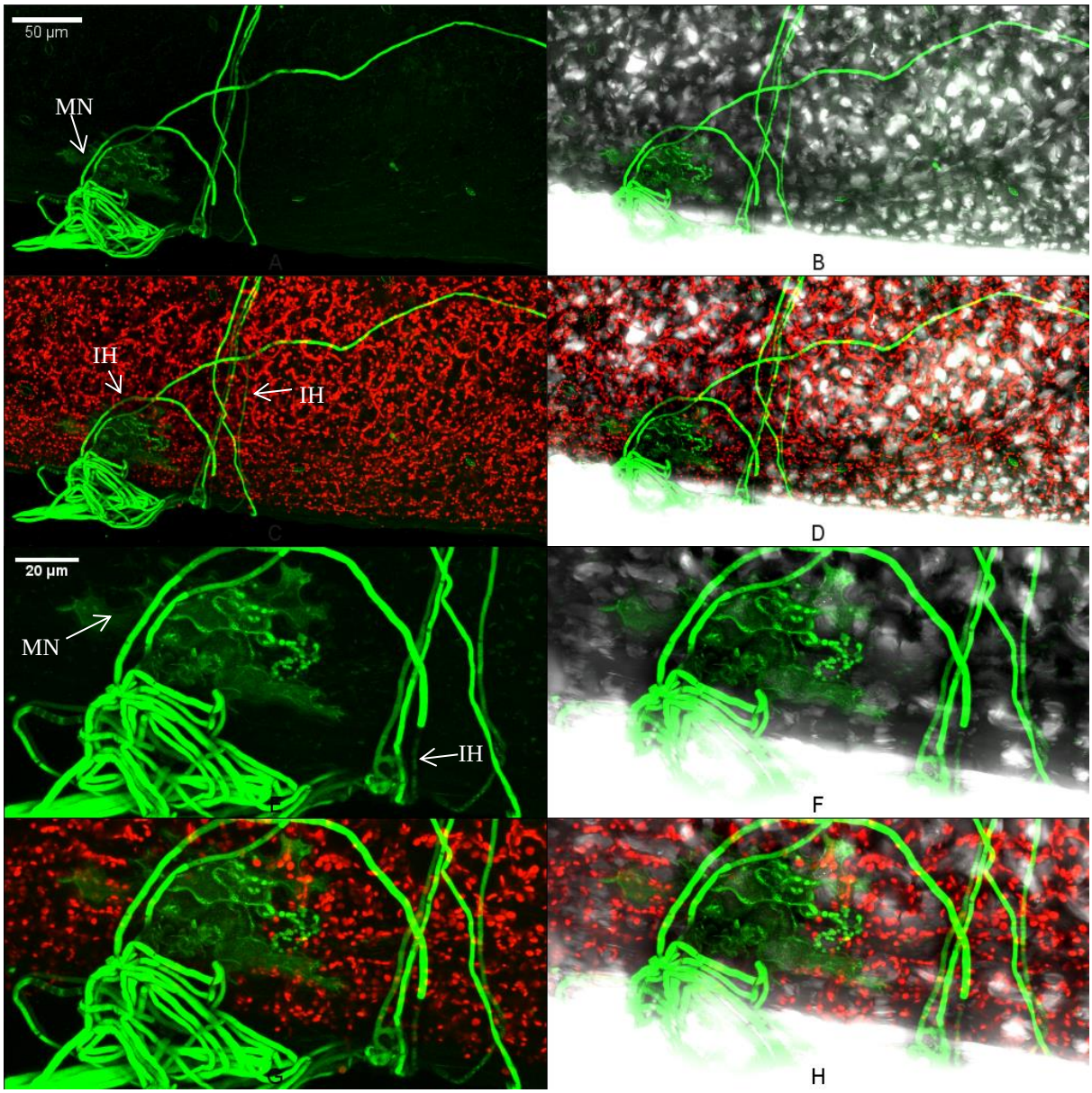


Figure 5-18: Mycelial mat developing from microscopic necrotic lesions 30 days after inoculation. The images were obtained from an apparently healthy lettuce leaf which was inoculated with GFP labelled *B. cinerea*. (**A-D)** Growth of mycelial network from a microscopic necrotic lesion (**MN**). Long hyphae arising from these networks grow on and in the healthy leaf tissues. (**E-H)** Detail view of microscopic necrotic lesion and mycelial mat. **IH** - internal hyphae.

5.3.2 In *A. thaliana*

A. thaliana had symptomless infection of both *B. cinerea* isolate B05 and GFP labelled *B. cinerea*. The fungus showed direct tissue penetration; the germ tubes penetrated epidermal cells and guard cell walls. But microscopic necrotic lesions were detected rarely in *A. thaliana* tissues (Figure 5-19 and 5-20). The fungal hyphae were often associated with trichomes in leaf, stem and stem-leaves (Figure 5-21 to 5-25); the micro conidia germinated on the trichome and it curled round it and moved down to the leaf epidermis. In some other observations a spore germinated at the base of the trichome and the germ-tube climbed to the tip of the trichome. Hyphae also connected adjacent trichomes. On the leaf surface fungus was detected as long branched hyphae which ran parallel to the leaf surface. The terminal end of some hyphae produced a structure similar to an infection cushion. Certain regions of fungal hyphae were also detected inside the plant tissue. The fungus was also growing on the root samples, mainly at the outer surface of the root (Figure 5-26).

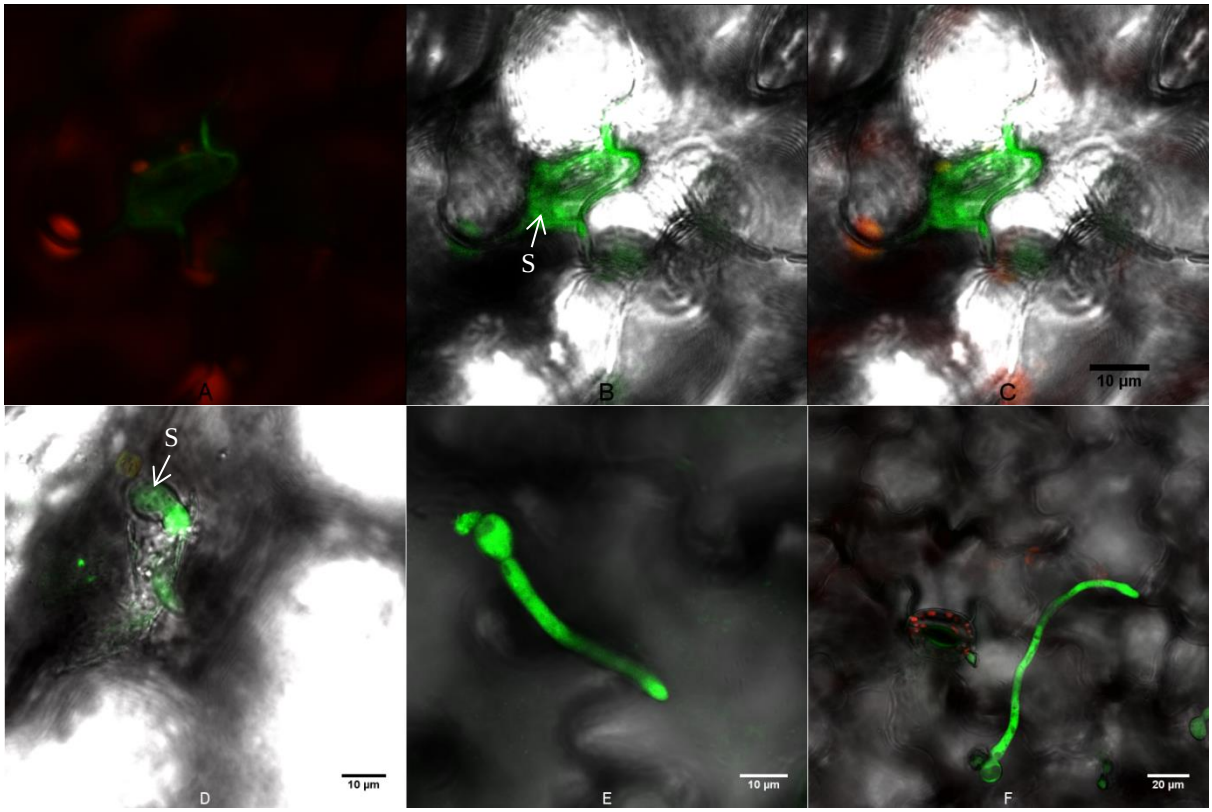


Figure 5-19: Spore germination and germ tube development by GFP labelled *B. cinerea* on *A. thaliana* leaf. (**A-C**) spore germinated and the hypha has spread around a guard cell, 4 days after inoculation. (**D**) Germ tubes developing from a spore directly penetrate and grow inside the leaf tissue or (**E** and **F**) spread parallel to the leaf surface, 9 days after inoculation.

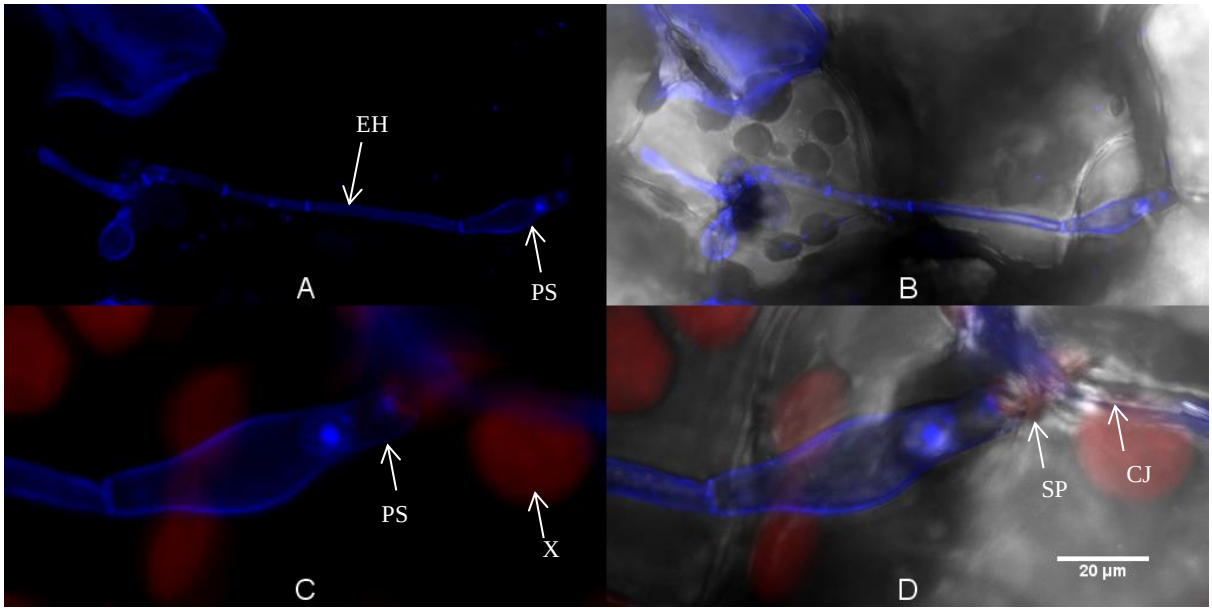


Figure 5-20: Penetration structures produced by *B. cinerea* isolate B05.10 on *A. thaliana* rosette leaves, 15 days after inoculation. The tissue sample was stained with calcofluor. The images were obtained by the combination of Z sections of different channels (red, blue and grey). (**A** and **B**) Germinated spore produces a short hypha (**EH**) and the terminal end of the hypha is modified as a penetration structure (**PS**). (**C** and **D**)The penetration structure is broader than the hypha and it appears to try to penetrate the plant tissue at the cell junction. **CJ**- plant cell junction, **SP**- site of penetration, **X**- chloroplast.

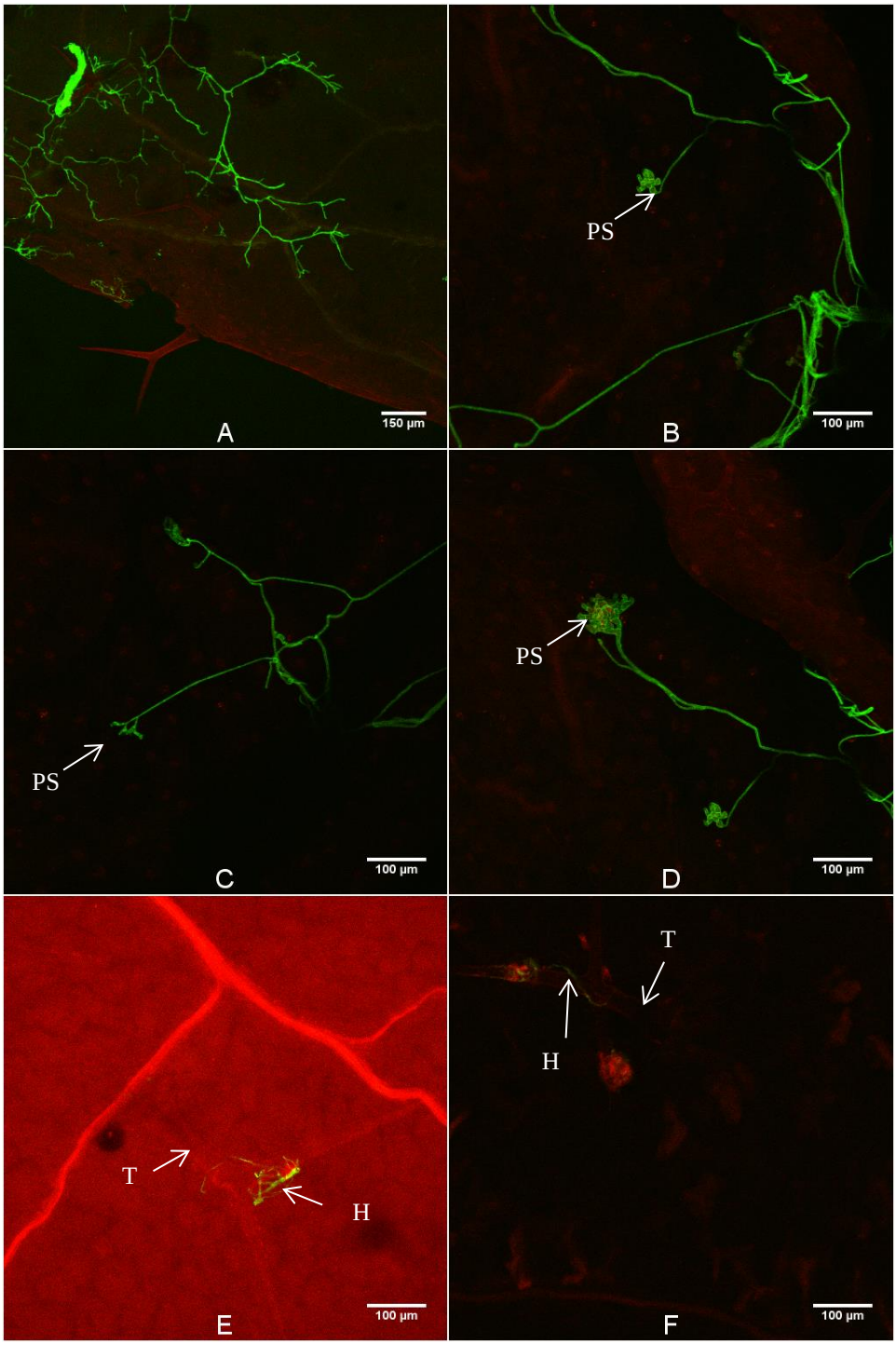


Figure 5-21: *B. cinerea* isolate B05.10 growing on the rosette leaves of *A. thaliana* 10 days after inoculation. Leaf samples were stained with WGA-FITC and Propidium iodide. The fungus produces a branched mycelial network on leaves near to the leaf margin (**A-D**). The terminal end of some hyphae produces special penetration structures (**PS**). Fungal mycelium was also detected on the trichomes of the leaves (**E** and **F**). **H**- hypha, **T**- trichome.

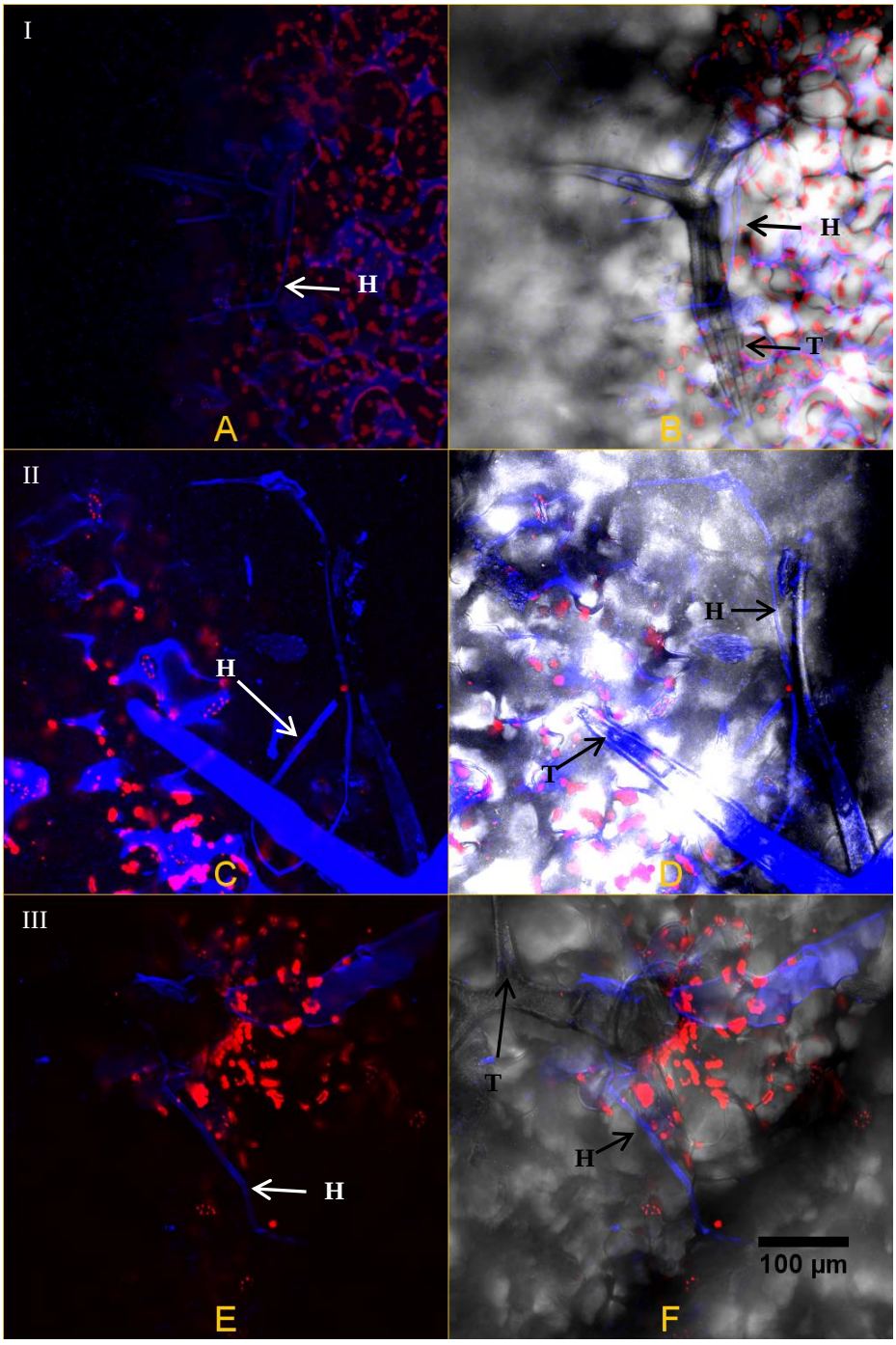


Figure 5-22: The *B. cinerea* isolate B05.10 growing on trichomes of rosette leaves of *A. thaliana*, 20 days after inoculation. The tissue samples were stained with calcofluor. Images in each row (**I-III**) were obtained from different leaves, and the images are combinations of Z sections of different channels (red, blue and grey). Spores germinate near the base of the trichome and the resulting hypha grows on the trichome. **H**- hypha, **T**- trichome.

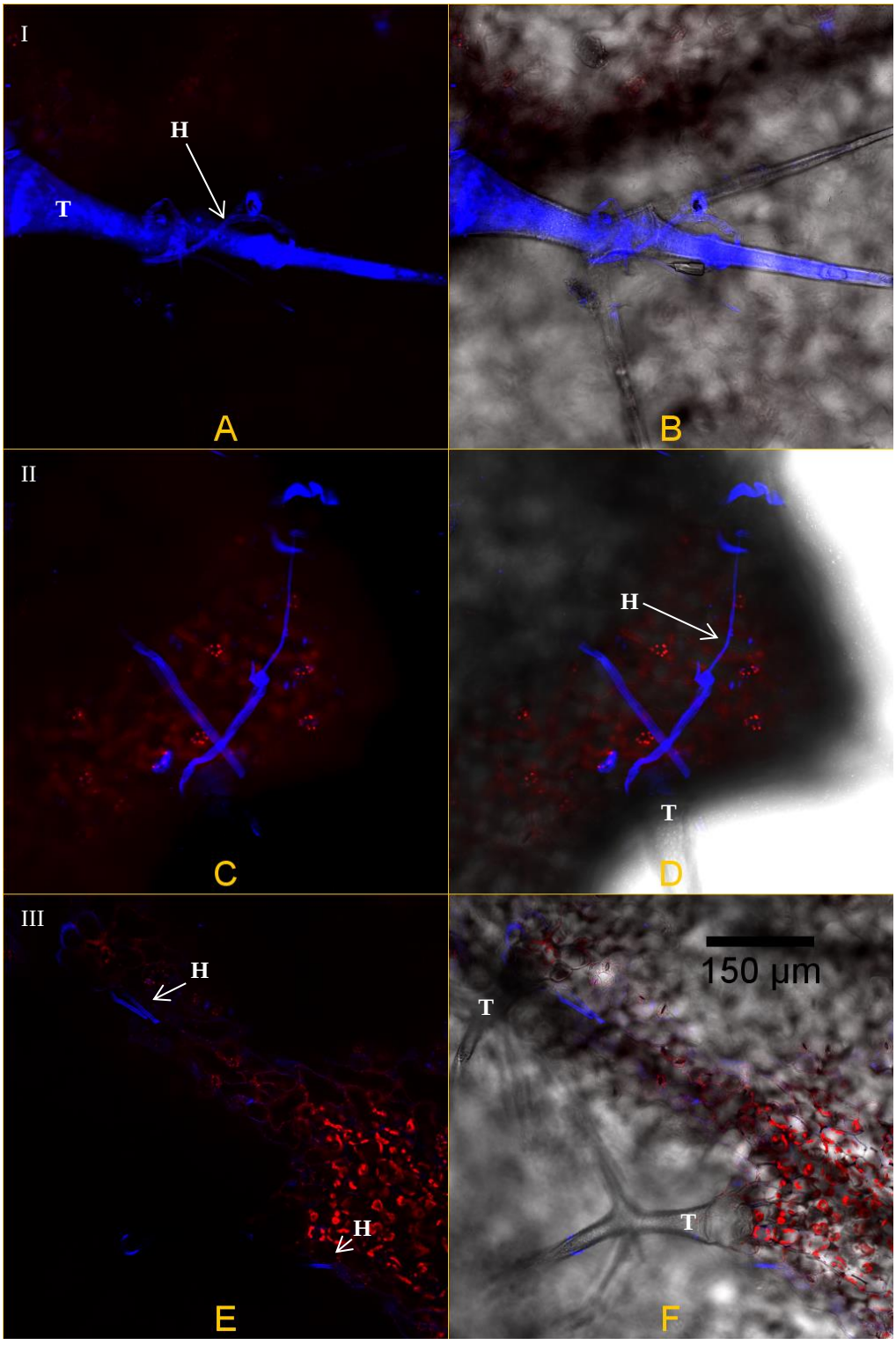


Figure 5-23: *B. cinerea* isolate B05.10 growing on the trichome of stem leaves of *A. thaliana*, 20 days after inoculation. The tissue samples were stained with calcofluor. Images in each row (**I-III**) were obtained from different leaves and the images are combination of Z sections of different channels (red, blue and grey). Images **AB**, **CD** and **EF** show the fungal hyphae growing on and at the base of the trichomes.

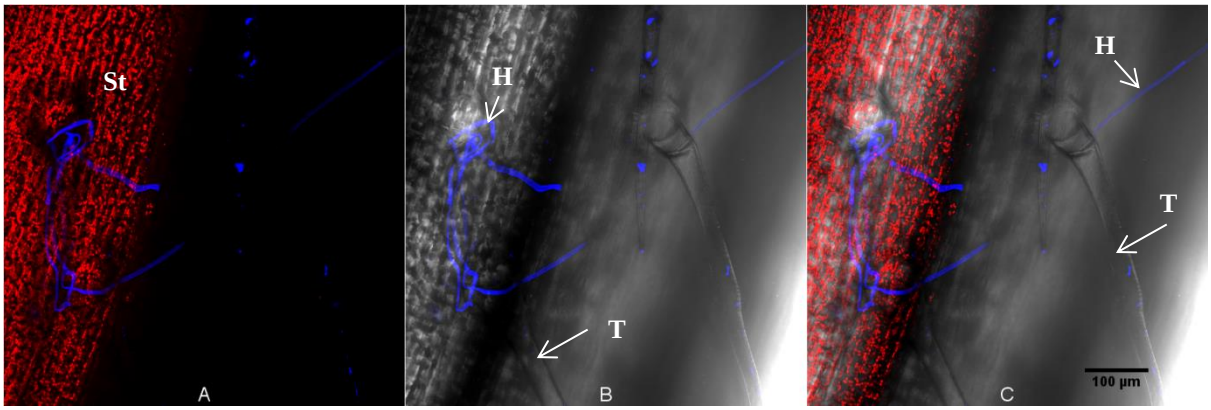


Figure 5-24: *B. cinerea* isolate B05.10 growing on flowering stem of *A. thaliana*, 20 days after inoculation. The tissue sample was stained with calcofluor and the images were obtained from different leaves and the images are combination of Z sections of different channels (red, blue and grey). The hypha curls around the base of the trichome and connects adjacent trichomes. **St**-stem, **H**-hypha, **T**- trichome.

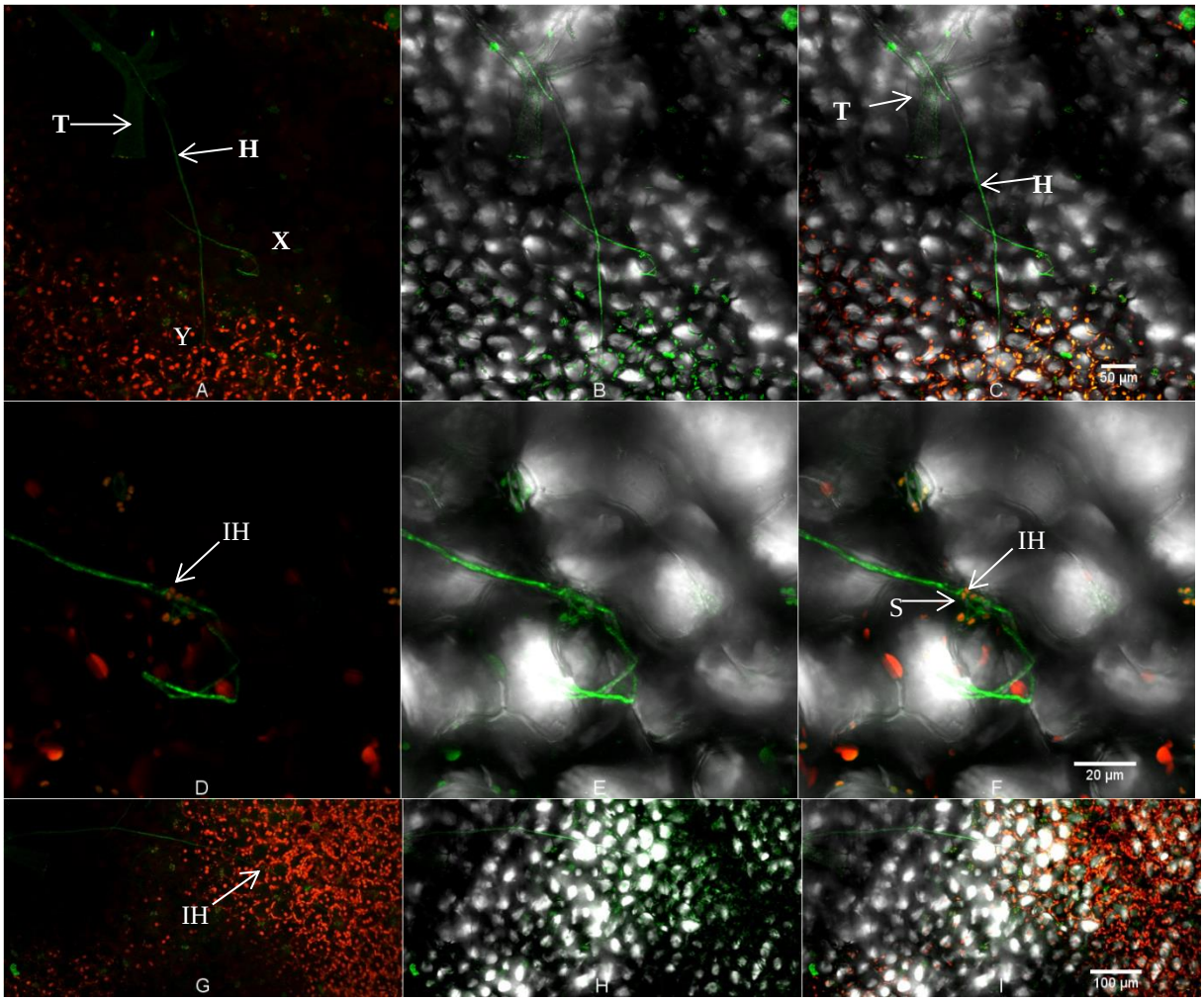


Figure 5-25: GFP labelled *B. cinerea* growing on stem leaves of *A. thaliana*, 20 days after inoculation. Images were obtained from different leaves and the images are combination of Z sections of different channels (red, blue and grey). Images **A-C** show spreading of fungal hyphae (**H**) from trichome (**H**) to the leaf surface. Images **D-F** shows the magnified region **X**; one hyphal end penetrates one side of a guard cell wall (**S**) and spreads to other side. Images **G-I** are magnified images of region **Y**: a hypha originating from the trichome penetrates the leaf tissue and grows inside the leaf tissue. **IH**- internal hypha.

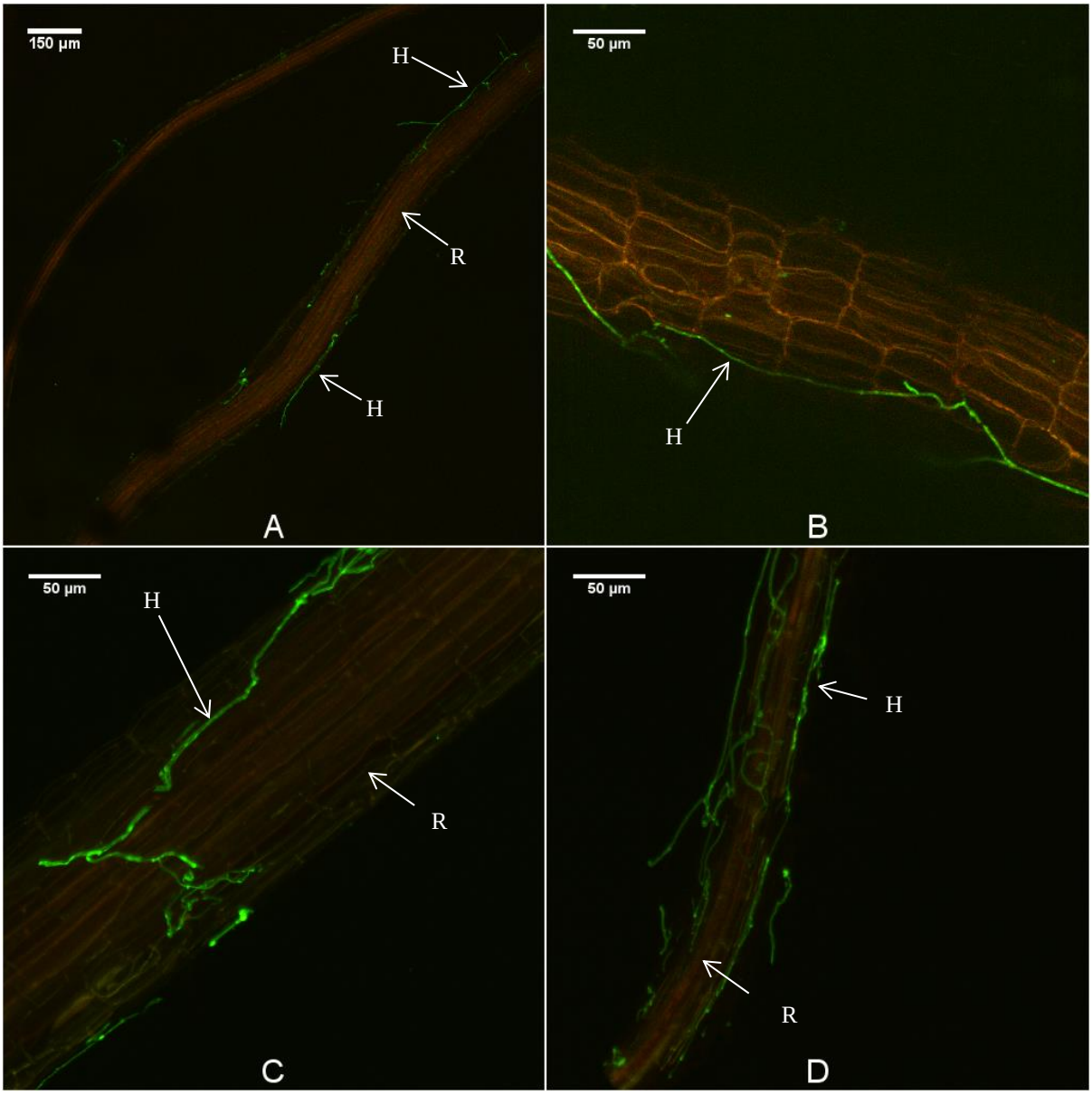


Figure 5-26: *B. cinerea* isolate B05.10 grows on the outer surface of *A. thaliana* root, 10 days after inoculation. Root samples (**A**-**D**) were stained with WGA-FITC. The fungus grows as long mycelium or mycelial network on the external surface of the root.

5.4 Discussion

The fungus *B. cinerea* is generally considered as a necrotrophic plant pathogen, and most of laboratory based experiments on establishment of infection and host-pathogen signalling have been done with protocols which facilitate the necrotrophic growth rather than allow the fungus to cause natural infection. In general, laboratory based inoculations are done with droplets having hundreds of spores in sugar and phosphate suspension (Holz *et al.*, 2007). The sugars in the droplet provide nutrients for the rapid germination and growth of the fungus, and also provide reactive oxygen species to fungus to cause oxidative burst on the plant surface (Holz *et al.*, 2007). The sum of the pathogenicity of the hundreds of spores is concentrated at the leaf area which has direct contact with the droplet. But in spore dust inoculation, there are no additional nutrition supplies and also the spores are sparsely scattered on the plant surface (Figure 5-1). Therefore, conditions in spore dust inoculation are more similar to natural infections by airborne spores. In the present study, lettuce and *Arabidopsis thaliana* were inoculated with spore dust of GFP labelled *B. cinerea*, high humidity, but not wetness, was provided at the beginning of the infection process, and the infection process was monitored under confocal microscopy at different time points.

In optimal condition, a necrotrophic infection cycle of *B. cinerea* may be completed with visible symptoms in as little as 3-4 days. A conidium germinates and produces germ tubes that develop into appressoria, and host surface penetration is aided by an appressorium (van Kan, 2005). Underlying cells are killed, and the fungus establishes a primary lesion. If the conditions favour further disease development fungus starts a vigorous outgrowth, resulting in rapid maceration of plant tissue, on which the fungus sporulates to produce the next generation (van Kan, 2005). But in spore dust inoculated plants it takes few weeks to months to produce visible necrotic symptoms.

In the present study lettuce and *A. thaliana* showed symptoms respectively at about 3 months and 1 month after inoculation. During that period fungus remained alive, but in a leaf out of

hundreds of inoculated spores only less than 10 spores produced long spreading mycelium. This mycelium spread from the site of inoculation to other parts of the plant as a combination of external mycelium and subcuticular mycelium (Figure 5-17) with occasional growth in internal regions of the host.

Reduced spore density and absence of external nutrition supply might be the reason for the slow disease development, however, still there is a great unanswered question; how this fungus escaped from plant defence machinery even though it was present in very low density?

The mode of invasion of *B. cinerea* in plant tissues is diverse in necrotic infection; it can be an active penetration of intact host tissue or passive ingress through wounds, sites previously infected by other pathogens or via stomatal opening (van Kan, 2005). In the present experiment, hyphal invasions were noted in the leaf tissues. None of these invasions entered through the stomata and no any other microbial infections were observed in the leaf tissue.

Cole *et al.* (1996) studied the infection process of *B. fabae* on bean (*Vicia faba*) leaves. Low-temperature scanning microscopy showed successful penetration of the leaf surface by a single germ tube often leads to the collapse of the infected and adjacent epidermal cells that leads to the development of necrotic lesion. Ultra-thin sections of infected bean leaves were examined by transmission electron microscope to investigate the infection mechanism of wet (suspended in aqueous glucose) and dry conidia. The wet conidia produced long germ-tubes and cuticular material was often absent from the surface of the host epidermis at regions close to or beneath sporelings on the leaf surface. However, dry conidia produced short germ-tube and the cuticular material at the surface of the plant epidermis appeared unaltered except at the site of penetration. In present experiment, plant cell wall or cell membrane were not visualized at the site of infection with specific stains. Therefore, the structural change that occurs at the site of penetration was not clear. However, images with bright field combination provided certain level of information. There is a clear difference between the internal and

external hyphae at the point of penetration; the former one is thinner and less fluorescent (Figure 5-14 and 5-16).

It has been reported that *B. cinerea* could produce appressoria-like structures during penetration of plant tissue. However, it is not well developed as in plant pathogenic fungi that have typical appressorium formation (Tenberge, 2004). In the infection of bean (*Vicia faba*) leaves by dry conidia of *B. fabae*, Cole *et al.* (1996) found a distinct pad of amorphous material that encircles the short germ-tube at the site of penetration. They suggested that this matrix material acts as an adhesive pad and thus serves to secure the position of the germ-tube at the site of penetration. In the present study, most often swelling of hyphal tips was noted in the germ tube or tip of the hyphae which were growing on the outer surface of leaf tissue (Figure 5-5, 5-11, 5-12, and 5-13). The broad swollen tips mostly penetrated the cuticle layer and lay inside the tissue below the level of the rest of the hypha. The site of the penetration was usually at cell junctions.

Various pathogens invade hosts in different ways and to different extents. Some fungi, such as that causing apple scab, produced mycelium which grow only in the area between the cuticle and epidermis, others such as those causing powdery mildew, produce mycelium only on the surface of the plant but send haustoria into the epidermal cells. Most fungi spread in to the internal tissues, either by growing as intracellular mycelium or by growing as intercellular mycelium. Some fungi, primarily among those causing downy mildews, smuts and rusts invade their hosts systemically (Agrios, 2005). In the present study, fungal mycelium was detected both inside and outside the plant tissues and spread from the site of inoculation to other parts of the plant and other tissues. In lettuce, fungus spread to large areas of the leaf tissues. Hyphae grew on the outer surface to a certain length, then penetrated it to grow inside the tissue. But in *A. thaliana*, the fungal mycelium was frequently detected on the trichomes

of rosette leaves, and also observed on stem and stem leaves, the plant parts which were developed several days after inoculation (Figure 5-21 to 5-25**)**. Most of the images show that in *A. thaliana* the fungus greatly preferred to grow on the outer surface of the plant body, but in lettuce mycelium was more frequently detected inside the leaf tissues.

At the beginning of a *B. cinerea* infection cycle, free surface water or high relative humidity (>93%) is essential to germinate and penetrate the host epidermis (Williamson *et al.*, 1995). In the present study, after spore dust inoculation the plant trays were tightly covered with lids to provide high humidity. Plants treated with higher humidity for 24 h still had few germinated spores, but prolonging humidity treatment to 48 h caused the majority of spores to germinate. The same observation was made by Rajaguru (2008) in symptomless interaction of *B. cinerea* with *Primula*.

Previous studies on dry and wet conidia inoculation provide evidence that the mode of inoculation may not only influence conidial growth on plant surfaces, but also subsequent symptom expression. On gerbera flowers, dry and wet *B. cinerea* spore inoculations produced different symptoms; dry spores produced typical necrotic symptoms, on the other hand wet inoculation with conidial suspension produced different types of symptoms ranging from large necrotic lesions to non-symptoms (Holz *et al.*, 2007). In the present experiment, lettuce and *A. thaliana* were inoculated in two different ways which produced different results: drop inoculation of spores with potato dextrose broth (PDB) produced visible necrotic lesions within 48 h, but it took several weeks to show visible symptoms in spore dust inoculation.

There is little information about the behaviour of microconidia and microsclerotia of *B. cinerea in planta* (Holz *et al.* 2004). In the present experiments, on lettuce, germinated microconidia with long, thin and less branched hyphae were observed on the leaf surface (Figure 5-6, 5-7 and 5-16**)**. These hyphae also penetrated the leaf surface and grew in and out of the leaf tissue. Germinated micro-conidia were also observed in *A. thaliana* leaves;

microconidia attached to the trichome and germinated on it and the resulting hyphae curled round the trichome and grew down towards the epidermal cells (Figure 5-22 and 5-23**)**. The infected lettuce leaves also had subcuticular vesicles: a green fluorescing, solid mass that developed within the epidermis cell, and which was a source of fungal hyphae (Figure 5-9, 5-10 and 5-15**)**. These subcuticular vesicles might be microsclerotia or similar structure, however, further studies are needed to confirm this finding.

In some symptomless infected lettuce leaf samples apoptotic activity of fungal hyphae was observed: in the long hyphae some regions produced very much less autofluorescence. Shlezinger *et al.* (2011) reported that *B. cinerea* can show apoptotic-like programmed cell death (PCD) following germination on host plant. Plant defence molecules target the fungal PCD machinery and trigger massive cell death in the pathogens. Conversely, the fungal anti-apoptotic machinery, mediated by the IAP like protein BcBir1, protects the fungus from host induced PCD, thereby allowing establishment of small infection zones, from which spreading lesions will be produced at the second infection stage. They conclude that weakening or strengthening of *B. cinerea* anti-apoptotic machinery results in reduced or enhanced disease symptoms. Therefore, some regions in the growing mycelium might result from localized fungal apoptotic-like programmed cell death (Figure 5-11) triggered by plant defences. However, this was observed only in few infection sites.

Rust fungi are obligate biotrophic plant pathogens. They produce sub-stomatal vesicles during their infection process. Later, infection hyphae arise from these vesicles and ramify through the apoplastic space, inserting haustoria into adjacent plant cells to acquire nutrition (Bettgenhaeuser *et al.*, 2014). In the present study, the sub-cuticular vesicles produced by the *B. cinerea* might be similar in function as sub-stomatal vesicles in rust fungus. The differences are that in *B. cinerea* infections the vesicles were always inside a plant cell, probably a dead cell, and also hyphae which arise from these vesicles do not produce

haustoria. Further detailed studies need to be done to confirm the actual role of vesicles in *B. cinerea*.

In some plant tissues *B. cinerea* causes long-lasting, often are several weeks, quiescent infections. In quiescent infection, due to the active plant defence, fungal inoculum remains dormant within the primary lesion. Further development of infection process depends on the strength of the plant defence (Prusky, 1996). The symptomless infection described in this study differs from quiescent infection by showing continuous growth and development.

The observations of this experiment agree with the infection process of some other plant pathogenic fungi. The barley leaf blotch fungus, *Rhynchosporium secalis*, causes symptomless infection in the early stages of infection process, extending until the early stages of sporulation (Fountaine *et al.*, 2010). On the barley leaf, conidial germ tubes directly penetrate the cuticle, and the hyphae form inter-connected network, frequently following the outline of the anticlinal walls of epidermal cells. During this phase of growth the fungus has been described as biotrophic and it causes localized degradation of cell wall. The nutrients from leaky epidermal cells might provide nourishment for the fungus. In the late stages of infection process the fungus becomes necrotrophic (Thirugnanasambandam *et al.*, 2011). More or less similar features are expressed by the apple scab fungus *Venturia inaequalis*; the fungus shows biotrophic growth at the beginning of infection process, grows symptomlessly in subcuticular region, later in the season the mycelium invades the mesophyll cells and growth becomes necrotrophic (Vanderplank, 1982). *B. cinerea* also behave in a similar manner when spore dust inoculated; there is a long symptomless growth phase before the development of necrotic infection. During that growth phase fungal hyphae penetrate the cuticle and spreads on the cell anticlinal wall and on the cell junctions. Infected tissues remain apparently healthy, but there are microscopic necrotic lesions or cell damages in one or few cells, and those cells always have fungal vesicles or microsclerotia. However, *B. cinerea*

shows crucial difference from *R. secalis* and *V. inaequalis* by spreading from the initial site of infection to newly developing plant parts.

In leaf samples with symptomless *B. cinerea* infection, at the site of infection, plant cell wall thickening and autofluorescence of cell wall was noted (Figure 5-8). In many plant-pathogen interactions deposition of callose on the cell wall has been reported, particularly, during the defence reaction of tolerant hosts to fungal pathogens. In the interaction of the non-fungal pathogen *Plasmodiophora brassicae* with cabbage and the oomycete *Bremia lactucae* with lettuce, callose and cellulose are synthesized after induction by fungal penetration and layered on to the inside of the cell wall and outside of the cytoplasm (Aist, 1976). Similarly, *A. thaliana* deposits callose in epidermal cell and underlying mesophyll cells to prevent the invasion of powdery mildew fungus, *Golovinomyces cichoracearum* (Ellinger *et al.*, 2013). In the present study, cell wall thickening and autofluorescence were observed only in some sites of inoculation and were localized to only a few epidermal cells. Some of these cells contained fungal inclusions, and fungal mycelium arising from these cells spread to other leaf area on the leaf surface, Therefore, the fungus must be able to breach this barrier eventually.

The present microscopic study has confirmed the symptomless infection caused by *B. cinerea*. The fungus grows on apparently healthy tissues without producing symptoms, the spreading mycelium travels widely both in and on the plant tissues.

Chapter 6 Extent of non-symptomatic infection of *Botrytis cinerea* in wild growing *Arabidopsis thaliana*

6.1 Introduction

The term endophyte has been defined differently by different authors based on their research view. Literally, the word endophyte means 'in the plant' (*endon* Gr., within; *phyton*, plant). Researchers who have a primary interest in ecology or biodiversity studies broadly include all microbes found inhabiting host tissue, including ruderal saprobes, latent pathogens or opportunistic pathogens (Stone *et al.*, 2004). In contrast, many mycologists use this term only for those fungi that colonize a plant without causing visible disease symptoms at any specific moment (Schulz & Boyle, 2005).

Plants growing in natural environments harbour symbiotic fungal endophytes, and these fungi can have significant impacts on plant growth. The plant fitness can be increased through the endophyte conferring abiotic and biotic stress tolerance, increasing biomass and decreasing water use efficiency. Plant fitness can alternatively be decreased by altered resource allocation (Rodriguez, 2009).

The endophytes are classified into two major groups, Balansiaceous and non-Balansiaceous. The members of Balansiaceous, *Epichloe* and *Balansia*, grow systemically, rarely epicuticularly, and intercellularly within the above ground plant organs of grasses. Non-Balansiaceous group consist of a heterogeneous range of fungal endophytes including latent pathogens. These fungi spread inter- or intra-cellularly and cause localized or systemic infection, the localized infections restricted to particular plant parts such as photosynthetic and herbaceous tissues (Schulz & Boyle 2005; Rodriguez *et al.* 2009).

Commonly endophytes are isolated by initial surface sterilization of plant tissues followed by plating out the plant tissues on appropriate culture medium (Nair & Padmavathy, 2014). The

fungus recovered from a surface disinfected tissue sample is generally considered as an endophyte. The effectiveness of surface disinfection is therefore a key factor in determining endophyte status. The commonly used surface sterilization chemical agents are sodium hypochlorite (NaOCl) and ethanol (Reissinger *et al.*, 2001). The effective concentration of the chemical agents and the duration of the treatment vary with type of plant tissue. The effectiveness of surface sterilization can be tested by imprinting the sterilized tissue on appropriate growth media (Schulz *et al.*, 1993).

The number of fungal endophytic genera and species also vary with the age of the plant tissues. Mature leaves of *Tectona grandis* and *Samanea saman* had greater diversity than that in the young leaves (Chareprasert *et al.*, 2006).

Botrytis species such as *B. allii* and *B. narcissicola* have been reported to have symptomless spread on apparently healthy plants. The mycelium of *B. allii* infects the growing leaves of onion plants and continues to spreads towards the bulb (Tichelaar, 1967). In *B. narcissicola*, mycelium spreads from initial infections above ground, through the flower stalk towards the bulb (Verhoeff, 1974).

Recently, Grant-Downton *et al.* (2014) identified a novel *Botrytis* species, *B. deweyae*, which normally grows as an endophyte in ornamental daylilies (*Hemerocallis*) but displays facultative pathogenic behaviour. Although *B. deweyae* could produce macro-conidia in culture medium, conidiation never occurred on infected plants.

van Kan *et al.* (2014) reviewed the recent works which illustrated that *B. cinerea* is capable of colonizing plants internally, presumably as an endophyte without causing any disease or stress symptoms. There are several studies that have revealed that *B. cinerea* can cause symptomless infection on various plant species in laboratory conditions. Spore dust inoculation of *B. cinerea* produced symptomless infection in lettuce and *Primula* plants. Barnes (2002) isolated symptomless *B. cinerea* from 60% of *Primula* plants when they were

plated on selective medium. Symptomless infection was detected both in plants that had seed-borne infection and in plants which were inoculated with spore dust. Sowley (2006) reported symptomless infection in lettuce plants when they were growing in either indoor or outdoor conditions. Systemic symptomless infection was found in multiple varieties of lettuce and the fungus was isolated from surface disinfected stems, roots and leaves of lettuce plants.

A few studies have been done on wild plants, plants which are growing in natural environmental conditions, to show the symptomless infection caused by *B. cinerea*. Rajaguru & Shaw (2010) carried out an experiment to genetically characterize the *B. cinerea* isolates causing symptomless infection in fruits of blackberry (*Rubus fruticosus*) and strawberry (*Fragaria* × *ananassa*), and wild growing *Primula vulgaris* and dandelion (*Taraxacum agg*), in the years 2005 and 2006. Isolations were only found from fruit or flowers in blackberry and strawberry. The incidence of non–symptomatic infection in *Primula* and dandelion (*Taraxacum agg*) was very variable both geographically and between years. Shafia (2009) screened several wild plants for the symptomless *B. cinerea* infection. Nine different host species harboured the fungus symptomlessly. The incidence of symptomless infection was moderate in some species (*Achillea millefolium*, *Arabidopsis thaliana*, *Centaurea nigra*, *Cirsium vulgare*, *Senecio jacobaea*, *Senecio vulgaris* and *Taraxacum* agg.) and rare or absent in others (*Tussilago farfara* and *Bellis perennis*). The highest percentage of *B. cinerea* (30%) was recovered from surface disinfected *A. thaliana* tissue samples. García *et al.* (2013) analysed the culturable endophytic mycobiota associated in the wild growing *A. thaliana* in different geographical location in central Spain. However, none of the plant out of sampled 208 had *B. cinerea* infection.

A. thaliana is a model plant in plant science studies and several laboratory based works have been done to understand its interaction with *B. cinerea*. This chapter describes an experiment carried out to determine the degree of non-symptomatic infection of *B. cinerea* in wild growing *A. thaliana* in years 2013 and 2014.

6.2 Methodology

6.2.1 In 2013

6.2.1.1 Collection of Arabidopsis plants

Healthy Arabidopsis plants were collected from six different locations in and close to Reading University. Sampling was done at one month intervals from March to June 2013. The locations are labelled on Figure 6-1. Usually they were incubated on Botrytis Selective Medium (BSM) immediately after collection, otherwise they were stored at 4 °C overnight.

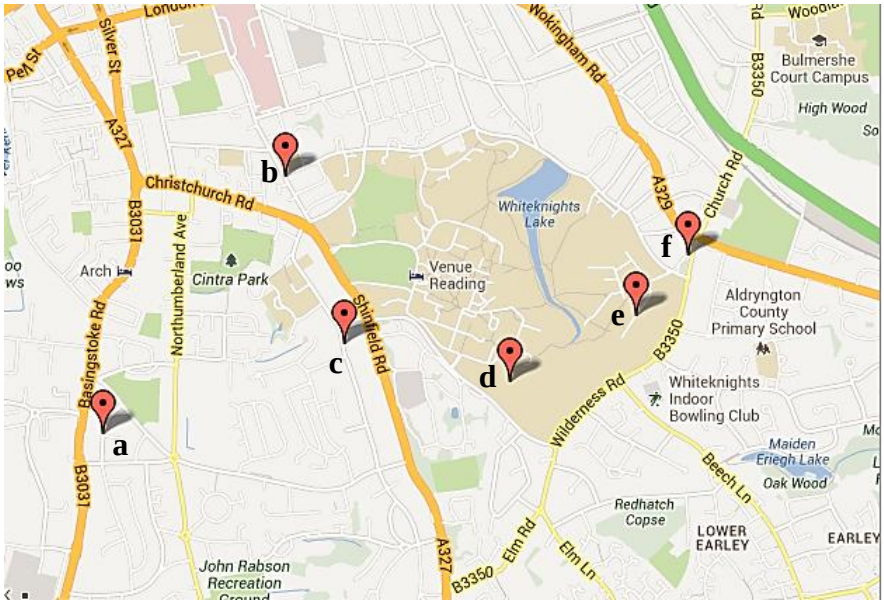


Figure 6-1: Arabidopsis plant collection sites. a-Tavistock road; b-Upper Redlands road; c-Northcourt Avenue; d-Experimental field; e-Agriculture; f-Whitekinghts road.

6.2.1.2 Sampling on Botrytis selective medium

In total, 76 plants were sampled (Table 6-1). Sampling was done as follows: the plants were washed in running tap water to remove soil and debris, and then dissected into root, stem, rosette leaves, stem leaves, inflorescences and fruits. They were divided in to two sets. One set (in total 500 tissues) was directly placed on BSM, the other portion (in total 499 tissues) was surface disinfected in 20% commercial bleach (1% NaOCl) for 2-3 minutes, rinsed in sterilized water three times, and blotted on paper towel prior to placement on BSM. Plates were incubated at 20 °C in 16 hours/day UV light and observed at 2 day intervals under a dissection microscope at for 15 to 20 days. Samples showing both brown colouration of the medium and characteristic spore producing structures were considered positive for *B. cinerea* infection.

Table 6-1: Sampling of wild growing *A. thaliana* in 2013

Date	Location	Total number of plants	Total number of plant parts	
			Non-surface disinfected	Surface disinfected
27th March	Upper Redlands Road	3	18	18
	Northcourt Avenue	3	18	18
	Tavistock Road	3	18	18
29th March	Agriculture	3	18	18
	Readlands Road	3	18	18
	Experimental Field	9	56	55
	Whiteknights Road	3	18	18
12th April	Upper Redlands Road	3	18	18
	Northcourt Avenue	3	18	18
	Tavistock Road	3	18	18
	Whiteknights Road	3	18	18
	Agriculture	3	18	18
	Experimental Field	3	18	18
16th May	Agriculture	10	68	68
17th May	Tavistock Road	6	39	39
21st May	Northcourt Avenue	6	49	49
11th June	Experimental Field	9	72	72
	Total	***76***	***500***	***499***

6.2.2 In 2014

A. thaliana samples were collected from same location as in 2013. In total, 21 apparently healthy plant samples were collected and dissected into root, stem, rosette leaves, stem leaves, inflorescences and fruits (Table 6-2). Both surface disinfected and non-surface disinfected tissue samples, each 184 in number, were plated on BSM and *B. cinerea* recovery was scored as described above.

Table 6-2: Sampling of wild growing *A. thaliana* in 2014

Date	**Location**	**Total number of plants**	**Total number of plant parts**	
			Non-surface disinfected	Surface disinfected
8th February	Experimental Field	5	38	38
16th March	Tavistock Road	10	92	92
18th April	Northcourt Avenue	6	54	54
	Total	***21***	***184***	***184***

6.3 Results

The results of *B. cinerea* recovery were analysed in different ways. First, the recovery percentage was determined based on number of plants having symptomless infection (Figure 6-2 and 6-4). Then the result was analysed as sum of symptomless plant parts (Table 6-3 and 6-4). Finally, the symptomless infection detected plants were individually illustrated to show their site of infection (Figure 6-3 and 6-5).

6.3.1 In 2013

40% (30/76; s.e. of estimate approximately 0.06) of plants had natural *B. cinerea* infection internally or externally (Figure 6-2). Most of these, 77% (23/30; s.e. of estimate approximately 0.08), had *B. cinerea* only externally (on their non-surface disinfected tissues). About 10% (7/76) of the total had internal infection (*B. cinerea* in surface disinfected tissues).

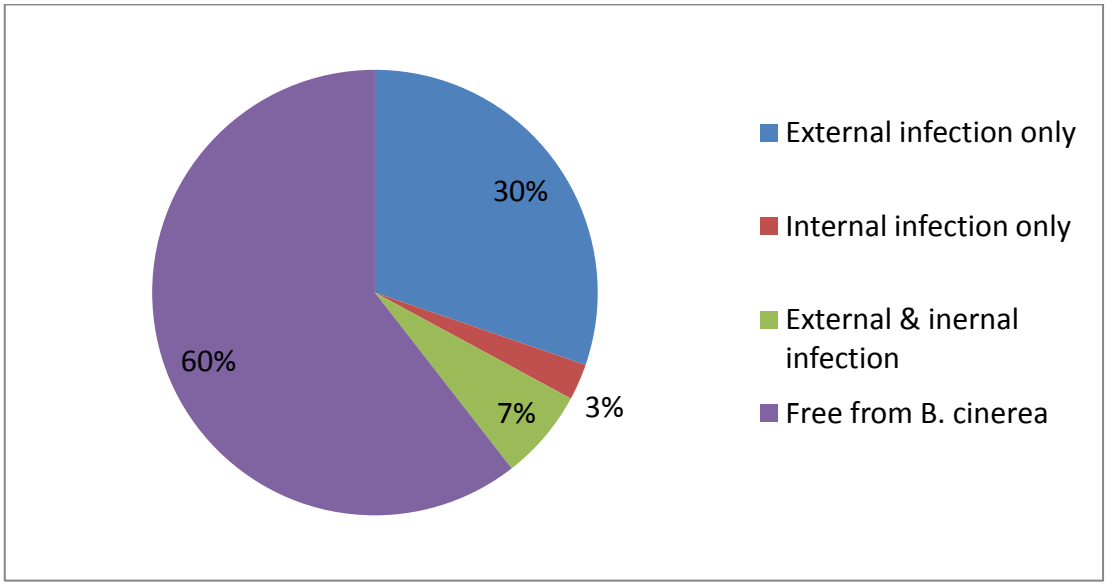


Figure 6-2: *B. cinerea* infection status of Arabidopsis plants collected from the wild, and plated on BSM in 2013. Isolations were classified as internal if they were from surface disinfected tissue; external otherwise.

9.4 % of non-surface disinfected tissue samples (47 tissue samples) showed *B. cinerea* growth. Only 1.8 % of sampled tissues had internal *B. cinerea* infection only (Table 6-3). Recovery of *B. cinerea* varied (chi-square =25.2, 5 d.f., P<0.001) among different types of non-surface disinfected samples: rosette leaves had most infection (22.2%), followed by inflorescence (9.5%) and stem leaves (8.7%). The surface disinfected organs had less than 5% infection; root had most, but differences were not significant (chi-square =6.13, 5 d.f., P=0.29) (Table 6-3).

Table 6-3: Incidence of *B. cinerea* isolation from different tissue samples of wild *A. thaliana* in 2013

Type of plant tissue	**Non-surface disinfected tissues**			**Surface disinfected tissues**		
	Sample size	Infected samples	%	Sample size	Infected samples	%
Rosette leaves	90	20	22.2	89	2	2.2
Stem	76	5	6.6	76	2	2.6
stem leaves	103	9	8.7	105	0	0.0
Inflorescence	74	7	9.5	75	2	2.7
Root	76	5	6.6	75	3	4.0
Fruit	81	1	1.2	79	0	0.0
Total	*500*	*47*	*9.4*	*499*	*9*	*1.8*

Of 30 individual infected plants only six had infection in more than one tissue. Two plants had *B. cinerea* on four different organs (root, rosette leaves, stem leaves and stem or inflorescence). Four plants showed *B. cinerea* growth from three different organs (Figure 6-3).

Fruit infection was rare; only one plant had infection on fruit. One plant (#9) had *B. cinerea* infection in more than one surface disinfected organ; non-surface disinfected samples from this plant also had *B. cinerea* infection (Figure 6-3). The likelihood of the observed number of multiple infections was estimated by Prof. Mike Shaw using randomization test (Barnes and Shaw 2003). The probability of the degree of clustering observed is judged by repeatedly randomly allocating the total number of positives seen among the total number of tissues (plants x tissues), grouping in sets of "tissues". The results confirmed that a single plant can have multiple infection in non-surface disinfected (NSD) tissues ($p<0.001$) and in surface disinfected (SD) tissues ($p=0.003$).

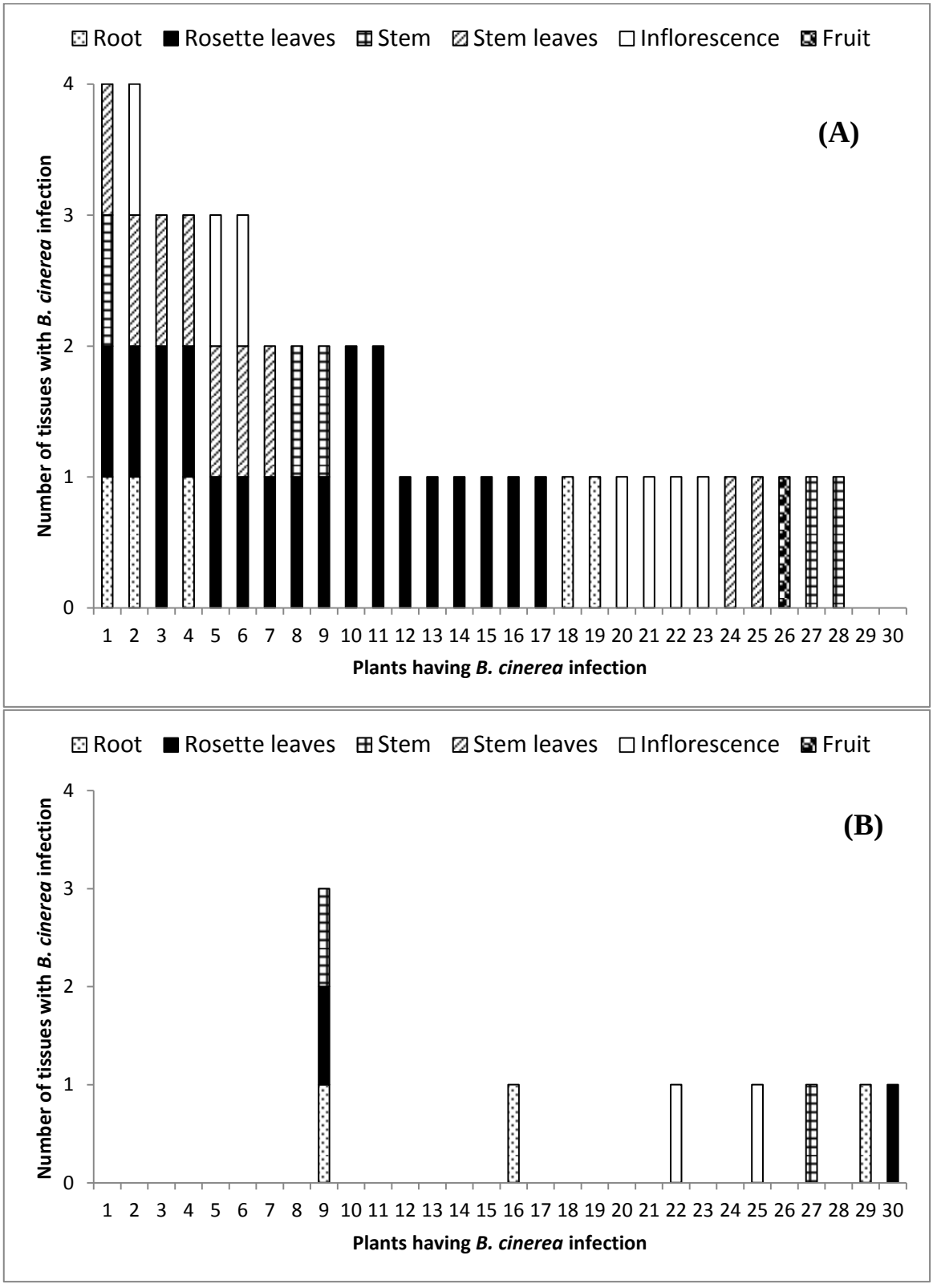


Figure 6-3: *B. cinerea* recovery from individual *A. thaliana* plants sampled from the wild in 2013. In both (A) non-surface disinfected tissues and (B) surface disinfected tissues, plants are arranged in same order.

6.3.2 In 2014

48 % (10/21; s.e. of estimate approximately 0.11) of *A. thaliana* plants had symptomless *B. cinerea* infection, and out of this about 90% (9/10) of the plants were infected only at the outer surface of plant parts (Figure 6-4).

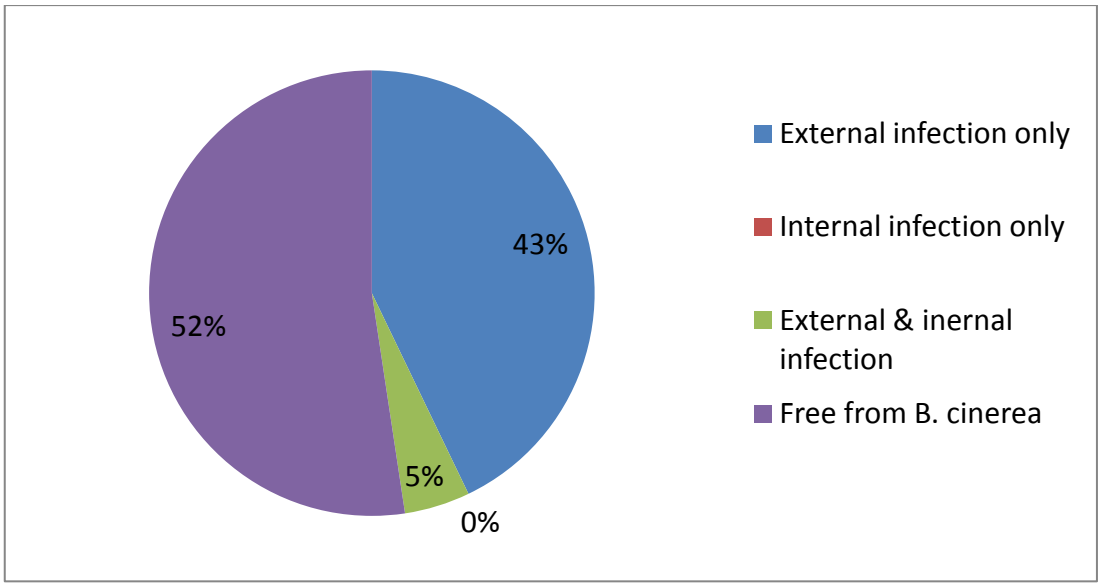


Figure 6-4: *B. cinerea* infection status of Arabidopsis plants collected from the wild and plated on BSM in 2014. Isolations were classified as internal if they were from surface disinfected tissue; external otherwise.

12% of non-surface disinfected tissue samples showed *B. cinerea* growth out of 184 plated tissue samples. Inflorescence had most infection, about 30%, followed by rosette leaves (18.8%) and stem (7.1%). Only one surface disinfected tissue sample showed *B. cinerea* growth (Table 6-4).

Table 6-4: Incidence of *B. cinerea* isolation from different tissue samples of wild *A. thaliana* in year 2014

Type of plant tissue	Non-surface disinfected tissues			Surface disinfected tissues		
	Sample size	Infected samples	%	Sample size	Infected samples	%
Rosette leaves	48	9	18.8	48	1	2.1
Stem	42	3	7.1	42	0	0.0
stem leaves	42	2	4.8	42	0	0.0
Inflorescence	26	7	26.9	26	0	0.0
Root	20	1	5.0	20	0	0.0
Fruit	6	0	0.0	6	0	0.0
Total	*184*	*22*	*12*	*184*	*1*	*0.5*

Only five plants had infection in more than one tissue per plant, the rest of the six plants showed infection only in one plant tissue. *B. cinerea* infection in more than one plant organ (rosette leaf, stem, root, inflorescence or stem leaf) was noticed only in four plants. The plant number six had both internal and external infection (Figure 6-5). The likelihood of the observed number of multiple infections was estimated by Prof. Mike Shaw using randomization test. The probability of the degree of clustering observed is judged by repeatedly randomly allocating the total number of positives seen among the total number of tissues (plants x tissues), grouping in sets of "tissues". The results confirmed that a single plant can have multiple infection in non-surface disinfected (NSD) tissues ($p<0.001$) and in surface disinfected (SD) tissues ($p<0.001$).

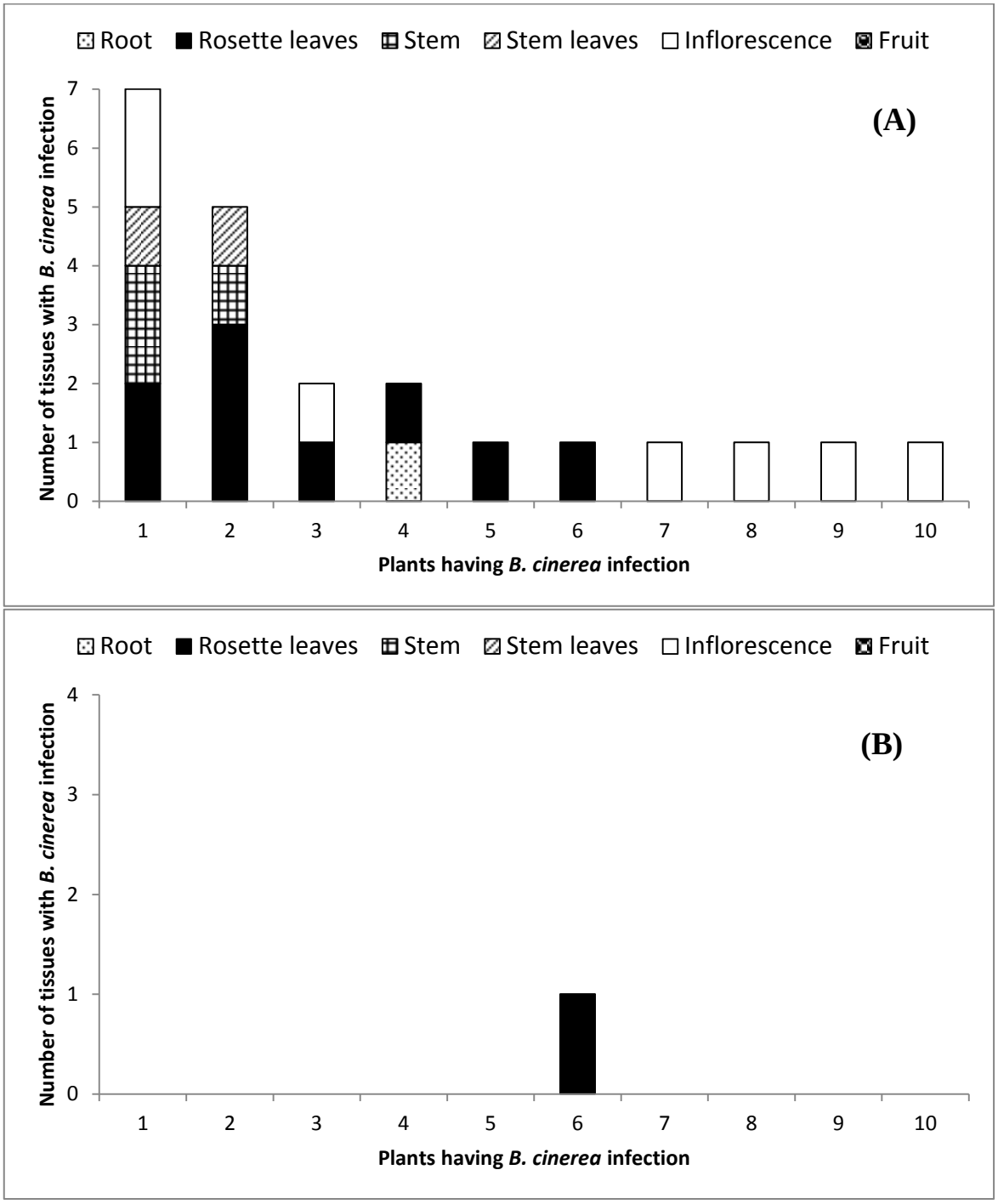


Figure 6-5: *B. cinerea* recovery from individual *A. thaliana* plants sampled from the wild in 2014. In both (A) non-surface disinfected tissues and (B) surface disinfected tissues, plants are arranged in same order.

6.4 Discussion

In the present experiment, recovery of *B. cinerea* from apparently healthy wild growing *A. thaliana* reveals that the fungus is producing symptomless infection on this plant from natural sources of inoculum and that the plant is a natural host for symptomless infection. This result correlates with some other researchers' findings. Shafia (2009) reported symptomless growth of *B. cinerea* in wild growing *A. thaliana*; she found *B. cinerea* in 30% of surface disinfected tissues out of 264 tissues sampled. Unpublished work by MW Shaw found two internally infected *A. thaliana* plants out of 20 sampled on selective medium, from plants sampled in early January. Junker *et al.* (2012) did endophyte screening in wild growing *A. thaliana* and they recovered *B. cinerea* from stem samples.

In general, surface disinfection removes epiphytic fungi and helps to detect slower-growing endophytic fungi when the tissues are plated out on nutrient medium. In this survey, both year 2013 and 2014, *B. cinerea* was recovered from surface disinfected and non-surface disinfected tissue samples of wild growing *A. thaliana* though much less commonly than in previous studies. Therefore, the fungus may grow inside the plant tissues as an endophyte. But the percentage of *B. cinerea* recovery in surface disinfected tissues (less than 2%) was less than that in non-surface disinfected tissue (about 10%). This suggests that the fungus tends to grow on the outer surface of the plant most often, only occasionally growing inside the plant. This has been supported by the laboratory based inoculation and microscopic observation with GFP-labelled *B. cinerea* (Chapter 5).

The amount of symptomless *B. cinerea* infection was similar (chi-square =0.45, 1 d.f., p=0.5) in this study between the years 2013 and 2014. The percentage of symptomless plants in 2013 (40%) increased by 10 percentage in year 2014. But in both years *B. cinerea* recovery from surface disinfected tissues was less than 2%. On the other hand, 9.4 % and 12% of non-

surface disinfected tissue samples had symptomless *B. cinerea* infection in year 2013 and 2014 respectively.

In a previous study, 30% of surface disinfected tissues revealed *B. cinerea* growth out of sampled 264 tissues (Shafia 2009). This clearly deviates from present finding, 1.8% in year 2013 and 0.5% in 2014. The findings of unpublished work by MW Shaw in year 2008 and 2009 were more similar to the present experiment. In 2008, none of the surface disinfected tissues of *A. thaliana* had infection, but *B. cinerea* was recovered from non-surface disinfected samples from two plants out of nine tested. In the next year, he found three internally infected plants out of 20 sampled, which is 10%. This is comparable to the data of present experiment; 10% and 5% of plants in year 2013 and 2014 respectively. Annual and geographic variation of symptomless *B. cinerea* infection has been also noted in *Primula veris* and *Taraxacum agg*. (Rajaguru 2008). The variation of results may be due to changes in spore density between seasons.

In Spain, García *et al.* (2013) studied the effect of biotic and abiotic factors on the frequency of fungal endophytes in plant specimens and species composition of the endophytic community in wild growing *A. thaliana*. The results of that study indicated that the frequency of *A. thaliana* plants hosting endophyes depends on the time of the year and the phenological stage of the plant.

In Germany, Junker *et al.* (2012) did a two year survey to find out the endophytic fungi in healthy *A. thaliana* growing in the natural environment; tissues were plated on selective medium to score fungus recovery. The isolates include several pathogenic fungi, the first year samples had symptomless endophytic *B. cinerea* infection in the stem. In this two year experiment, the fungi isolated varied in number and type; in 2007 the predominant isolate was *Leptosphaeria maculans* but in 2008 this fungus was not isolated, and the total isolate was

reduced from 229 to 71. The authors suggest that a greater amount of precipitation in the first year may be the reason for this variation.

As a conclusion, the grey mould fungus *B. cinerea* grows symptomlessly on apparently healthy wild growing *A. thaliana*. Aerial plant parts presumably receive natural spore inoculum and harbour the growing fungus. Even though about 50% of sampled plants had symptomless infection, internal infection was only detectable in less than 2% of plant issues. The data here suggest that the fungus mainly grows on the outer surface of *A. thaliana* with occasional internal infection, and this is consistent with the microscopy study (Chapter 5). However, the amount of symptomless infection and internal fungal growth may vary with seasonal variation, since Shafia (2008) recorded abundant internal infection, especially in the roots.

Chapter 7 General Discussion

Experiments were carried out to check whether there is any difference in *B. cinerea* gene expression between necrotic and symptomless infections in *A. thaliana* and lettuce. Selected pathogenicity and signalling gene expression of *B. cinerea* were compared at mRNA level. Clear differences were noticed in the pattern of mRNA transcript abundance. In particular, some pathogenicity genes were detected rarely or not at all in symptomless samples. Therefore, it is clear that *B. cinerea* behaves differently at the molecular level during symptomless infection than during aggressive necrotic infection. Finally, to find out the infection path of *B. cinerea* during symptomless infection, spore germination and mycelial spreading of GFP labelled *B. cinerea* was monitored on and in *A. thaliana* and lettuce using a confocal laser scanning microscope. Apparently symptomless plants showed mainly mycelial growth on the organ surface, but sub-cuticular and internal fungal structures were also noticed.

Quiescence defines an extended sequence of time during which pathogen activity appears to be suspended and almost no growth is apparent (Elad *et al.*, 2007). Several species of fungal pathogens, such as *Colletotrichum*, *Alternaria*, *Botrytis*, *Monilinia*, *Sclerotinia*, *Lasiodiplodia*, *Phomopsis*, and *Botryospheria*, have been reported to live quiescently in their hosts (Prusky *et al.*, 2013). Quiescent infection of *B. cinerea* has been reported in several host plants, where after germination, fungal hyphae extended to certain distance and then remained without growth until conditions become favourable for further growth and infection. In blackcurrant, conidia germinate in the stigmatic fluid, and hyphae spread endophytically throughout the stylet without eliciting visible symptoms and infect the pericarp and ovules (Prins *et al.*, 2000). In strawberry flowers, conidia germinated on the stigmas and their hyphae grew into the transmitting tissue of the styles, but so slowly that they sometimes took 4-6 wk to reach the style bases (Bristow *et al.*, 1986). There is evidence that shows that *B. cinerea*

conidia can survive on the surface of kiwifruit and remain viable and infectious throughout the season (Walter *et al.*, 1999). In strawberry (Sutton & Peng, 1993) and grapes (Holz *et al.*, 2003) newly emerged and partially expanded leaves are infected by *B. cinerea*, but the infection remains at a quiescent stage. These early quiescent infections provide little inoculum for flower infection unless the leaves undergo premature senescence. Grape berry infection takes place during bloom and fungus remains quiescent in immature berries. Nair & Parker (1985) found that *B. cinerea* invades the stigma and then becomes quiescent in necrotic style tissue at the stylar-end of the berry. The symptomless infection of *B. cinerea* investigated in this study shows remarkable difference from previously reported quiescent infection.

In this study, plating out experiments with symptomless plants, nucleic acid-based detection of *B. cinerea* in newly developed plant parts, and microscopic studies of infected but apparently healthy tissues revealed that fungus was spreading from initial site of infection to newly developing plant parts. Similar findings were made in symptomless infection of lettuce (Sowley, 2006) and *Primula* (Barnes, 2002). An argument that could be raised is that the recovery of *B. cinerea* from newly developed plant parts could be due to the newly arrived spores from the surrounding environment or spores produced from the initial inoculation. However, this is unlikely because in the experiment with *A. thaliana*, plants were grown in isolated propagators with a filtered air supply; spore entry through air circulation was strictly prevented. In isolated propagator, inoculated plants and the surface of compost in which the plants grew were daily checked for the absence *B. cinerea* spore bearing structures. Microscopic observation of symptomless samples showed no spore bearing structures on plant tissues. Seed borne infection could be a source of inoculum as reported by Sowley (2006) and Barnes (2002) in lettuce and primula respectively. But in *A. thaliana*, seed samples did not have *B. cinerea* infection whether plated surface disinfected and non-surface disinfected. Therefore, in the *A. thaliana* experiments reported here, symptomless infection was purely from the single initial inoculation. However, lettuce was grown in unfiltered air.

Plants, especially greenhouse grown, were freely exposed to airborne infection. But plant samples used in microscopic studies were grown in controlled environment cabinets where the chances to get airborne infection were very low. I used GFP labelled *B. cinerea* in microscopic experiments; the green fluorescent hyphae precisely represent only the initial deliberate inoculum.

Microscopic observation of symptomless tissues showed sub-cuticular vesicles of *B. cinerea*. From these, fungal hyphae arose and spread on and in leaves with little branching. The plant cells which had the fungal vesicles in, and cells around those infected cells, were damaged. These observations seem to be new in studies of *B. cinerea* infection. Therefore, further investigations are needed to explain the role of these vesicles.

The spreading, less branching, mycelium was noticed mainly on leaf surfaces but mycelium also spread below the cuticle and sometimes below the epidermal layer. In these microscopic experiments I failed to clearly differentiate plant cell boundaries with a specific stain. An attempt was made to stain plant cell wall with calcofluor white, but it was not successful because both plant cells and fungus were stained. However, auto-fluorescence of chloroplasts helped to localise hyphal positions. Combined Z-sections of bright field images also helped to decide whether the hyphae grow below the cuticle or not. Future studies with plant cell wall or cell membrane specific stains or fluorescence labelling would provide more insight at the cellular level. Another constraint in this experiment was the very small amount of fungus in symptomless samples; therefore, I had to spend several hours (2 h/single *A. thaliana* leaf!) with a microscope to find fungal structures, especially at the beginning of infection process. Longer exposure of tissue samples will cause damage, and longer use of confocal laser scanning microscope costs a lot. Therefore, in future, it would be important to enrich the amount of fungus in symptomless samples or devise methods to quickly find hyphae in sparsely infected tissue.

In present study, GFP labelled microscopic experiments were carried out in a different laboratory (the Biffen building, John Innes Centre (JIC), Norwich, UK) from the rest of the work. The growing conditions in the JIC were different from in the experiments carried out in University of Reading (UoR); especially the intensity of light in the growth chambers was much lower than in glasshouses and growth chambers in University of Reading (UoR). Under the JIC conditions, plants grew more slowly and the lettuce leaves were thin and slightly less greenish. In plant tissue plating experiment of lettuce, *B. cinerea* was abundantly detected in root samples in UoR, but GFP labelled fungus was not detected in root samples in JIC. Similarly, the presence of *B. cinerea* in very young leaves was detected in plating experiments and gene expression experiments in UoR, but not in microscopy experiments in JIC. This indicates that the intensity of light may play a significant role in the development of symptomless infection; plants which grow in lower light intensity may show less susceptible to symptomless infection. However, further studies are needed to confirm this finding.

In this study an attempt was made to compare the gene expression of *B. cinerea* in symptomless and necrotic infections. In general, expression of a gene occurs in two consecutive steps; firstly, the gene coding in DNA is transcribed as mRNA and then the mRNA coding is translated into respective protein molecules. In this study I quantified amount of mRNA of selected genes. There are several factors that can regulate the translation of mRNA into protein. Therefore, abundance of mRNA is not always an indication of the abundance or presence of the respective protein. However, mRNA based detection of gene expression has been widely used in molecular studies to provide an initial guide.

In symptomless lettuce, mRNA of selected genes was quantified from leaf samples collected from different leaf positions at different time intervals. The results were consistent between the biological replicates in leaf positions which were very close to the initial infection, but some replicates contained undetectable amounts of some mRNA when moving away from

initial site of infection. This was because of the reduced amount of fungus in newly developing leaves. Therefore, the leaf positions 5and 6 (nearest to the site of inoculation, 4th leaf) were selected for the comparison between symptomless and necrotic infections.

The mRNA of gene (*bcnep1*) coding for Nep1-like proteins (NLPs) was detected in relatively higher amounts in symptomless infection compared to in necrotic infection in lettuce leaves. NLPs induce necrosis and ethylene production in plants. Purified NLPs act differently in plant tissues: in low concentrations, they induce callose apposition, accumulation of reactive oxygen species and ethylene, and activation of genes involved in stress and defence responses; at higher concentrations, they induce cell death (Gijzen & Nurnberger, 2006). *B. cinerea* contains two genes encoding NLPs, named *bcnep1* and *bcnep2* (Arenas *et al.*, 2010). In symptomless samples of lettuce, the amount of *bcnep1* mRNA remained high throughout the symptomless phase, but later, when the infection became symptomatic (delayed necrotic infection) it greatly reduced. In direct necrotic infection the amount of mRNA declined with time. The differential expression of NLPs has been noticed in some previous experiments as well. Peak expression of MgNLP in *Mycosphaerella graminicola* was observed during the immediate pre-symptomatic phase of the colonization of susceptible wheat leaves, followed by a dramatic decrease during subsequent disease lesion development (Motteram *et al.*, 2009). In another hemibiotrophic pathogen, *Phytophthora sojae*, the highest level of Nep gene expression coincided with the transition from the biotrophic to necrotrophic stage when infecting soybean plant tissues (Qutob *et al.*, 2002). Therefore, in the present study, the presence of higher amount of mRNA of gene *bcnep1* in symptomless lettuce leaves perhaps indicates a greater attempt of the fungus to induce necrotic infection in host plant tissues than occurs in normal necrotic infection. However, in *A. thaliana* the observation was entirely different; the mRNA was not detected in any of the symptomless samples. This shows that *B. cinerea* expresses different colonisation strategies in different host plants.

The mRNA of the gene (*bcsod1*) coding for superoxide dismutase was detected in similar or sometimes slightly higher amounts in symptomless infection of both *A. thaliana* and lettuce than in their respective necrotic infections. A deletion mutant has proved the importance of expression of *bcsod1* in necrotic lesion development in *Phaseolus vulgaris* (Rolke *et al.*, 2004). Cytochemical analysis showed the presence of O_2^- in hyphal tips of *B. cinerea* and H_2O_2 generated in and around the penetrated cell wall (Tenberge *et al.*, 2002). The fungus *Neotyphodium lolii* grows as a symbiont in the intercellular spaces of the ryegrass *Lolium perenne* and so forms a useful contrast. Extracts from infected plants had a fungal Cu/Zn superoxide dismutase which was very abundant compared with other *N. lolii* proteins present in the symbiosis (Zhang *et al.*, 2011). The findings above show that in both biotrophic and necrotrophic interactions fungus secretes proteins which cause oxidative burst in host plants. In the present experiments mRNA coding for the gene *bcsod1* was detected in non-symptomatic infections in amounts similar to the necrotic infections, but in young leaves of lettuce and stem samples of *A. thaliana* it was detected slightly at higher concentrations than in necrotic infections. The presence of a higher level of this mRNA suggest that in a non-symptomatic infection the fungus expresses its potential to cause necrotic infection by inducing an oxidative burst as it does in necrotic infections.

The mRNA of the gene (*bcbot1*) coding for the non-specific phytotoxic metabolite botrydial was not detected in symptomless infection of lettuce and *A. thaliana*. In addition to phytotoxic effect, Rossi *et al.* (2011) found that botrydial is able to induce the hypersensitive reactions (HR) in *A. thaliana*. Botrydial induced the expression of the HR marker HSR3, callose deposition, and the accumulation of reactive oxygen species and phenolic compounds. Botrydial also induced the expression of PR1 and PDF1.2, two pathogenesis-related proteins involved in defense responses regulated by salicylic acid (SA) and jasmonic acid (JA), respectively (Rossi *et al.*, 2011). In present experiment mRNA of this gene was not detected in the symptomless samples but it was detected in necrotic samples. Therefore, absence or

very low levels of botrydial may be one of the factors allowing symptomless growth. However, botrydial is not expected to be the sole effector involved in triggering the HR on plant hosts, since some botrydial-deficient strains are still virulent (Siewers *et al.*, 2005). However, Siewers *et al.*(2005) did not mentioned whether botrydial-deficient *B. cinerea* strain B05.10 was virulent or not.

B. cinerea produces various endo-polygalacturonases during plant infection, and the expression of individual polygalacturonase genes depends on the host and the stage of infection (ten Have *et al.*, 2001). In my experiments, less mRNA of gene *bcpg1* was detected in symptomless infection of lettuce and *A. thaliana* leaves than in necrotic infection of leaves, but in *A. thaliana* stem samples it was similar to necrotic infection of leaves. However, the amount of mRNA of gene *bcpg2* in symptomless infection in lettuce was similar to that in necrotic infection. ten Have *et al.* (1998) found that expression of *bcpg1* is required for full virulence of *B. cinerea* on tomato and apple. A similar observation was reported by Akagi & Stotz (2007) for *B. cinerea* strain B05.10 infecting pear fruits. Vandelle *et al.* (2006) also proved that in addition to degradation of plant cell wall pectin, bcpg1 acts as a elicitor to provoke defence response in plants. Therefore, the reduced amounts of *bcpg1* in symptomless infection could be causally related to fungal growth without visible necrosis lesion in the host. Protein coded by the gene *bcpg2* is essential for penetration and primary lesion formation but contributes less to lesion expansion/colonization of fungus in plant tissue (Kars *et al.*, 2005a). In symptomless samples a slight increase was noticed with time. This may be due to the development of microscopic necrotic lesions or reflect primary lesion development not triggering necrosis.

In this study four different signalling genes were analysed for their expression. Expression of all of these genes has been proved essential for various cellular activities including regulation

of host pathogen interactions (Williamson et al 2007; Nakajima and Akutsu 2014). The mRNA of signalling genes *bcn* and *bcg1*were detected at similar levels in necrotic and non-symptomatic infection in lettuce and *A. thaliana*. However, in necrotic infections downstream signalling of these genes regulates expression of genes involved in botrydial synthesis (Schumacher *et al.*, 2008). But in present study the mRNA of *bcbot1* was not detected in symptomless samples. This observation shows that the signalling pathway involving *bcn* and *bcg1* might be altered in symptomless infections. The mRNA of another signalling gene, *bmp1,* was detected abundantly in symptomlessly infected lettuce and *A. thaliana* compared to their respective necrotically infected samples. This signalling gene regulates host surface recognition and penetration ability of germinated conidia (Doehlemann *et al.*, 2006). mRNA of the gene *bac* was detected in symptomless lettuce but not *A. thaliana*. The amount expressed in symptomless lettuce was similar to that expressed in necrotic infection.

In present study, I did not study the role of host plants in symptomless infections. However, in a plant-pathogen interaction, host plants play a significant role in disease development. A number of observations suggest that the plant regulates growth and metabolism of endophytes. The rate of hyphal extension of *Neotyphodium lolii* is synchronized with host plant *Lolium perenne* and extension ceases when the emerging leaves of the grass stop growing; cessation of hyphal growth occurs even though the endophytes remain metabolically active (Tan *et al.*, 2001). Zhang et al (2006) found that in incompatible associations, mycelial branching of *Neotyphodium lolii* increased and hyphal and plant growth and death of hyphae became unsynchronised. In the present study *B. cinerea* showed symptomless, to a certain extent endophytic, growth. During this symptomless infection, the growth rate of *B. cinerea* was less than in aggressive necrotic infection. This could be due to regulation by the plant or limited nutrient availability. Studies have also revealed that even in a mutualistic association a fungus grows under stress. Eaton *et al.* (2010) show an essential role for the fungal stress-activated and mitogen-activated protein kinase (*sakA*) in the establishment and maintenance

of mutualistic interaction between *Epichloë festucae* and perennial ryegrass (*Lolium perenne*). Deletion of *sakA* switches the fungal interaction with the host from mutualistic to pathogenic interaction; further high-throughput mRNA sequencing revealed dramatic changes in fungal gene expression. Tanaka *et al.* (2006) described a novel role for reactive oxygen species (ROS) in regulating the mutualistic interaction between *E. festucae* and *Lolium perenne*. A single-copy plasmid insertion was made in the coding region of a NADPH oxidase gene, *noxA*. Plants infected with the *noxA* mutant lose apical dominance, become severely stunted, show precocious senescence, and eventually die. The fungal biomass in these associations is increased dramatically, with hyphae showing increased vacuolation. ROS accumulation was detected cytochemically in the endophyte extracellular matrix and at the interface between the extracellular matrix and host cell walls of meristematic tissue in wild-type but not in *noxA* mutant associations. These results demonstrate that fungal ROS production is critical in maintaining a mutualistic fungus–plant interaction. Zhang *et al.* (2011) found that increased levels of a pathogenesis related class 10 (PR-10) protein in silver-stained two-dimensional gels of protein extracts from *Lolium perenne* infected with *Neotyphodium lolii*. Based on the above observations I conclude that symptomless infection arises as a balance of fungal virulence and plant defence. When this balance collapses symptomless infection may develop into aggressive necrotic infection.

In present study I only focused on a small selection of virulence and signalling genes to compare symptomless and necrotic infection *of B. cinerea*. However, *B. cinerea* is a well-established fungal pathogen and it has various adaptations to overcome plant defences. In order to find out the mechanism of symptomless growth it would be wise to do whole transcriptome analysis (Kong *et al.*, 2015) of samples having symptomless infection. This could reveal both fungal expression and defence mechanisms expressed by the host plant (Windram *et al.*, 2012). Comparison of whole genome expression of necrotic and symptomless infection could provide a clear picture of genes regulation and expression. In my

project one of the objectives was to carry out a transcriptome analysis of symptomless *A. thaliana* samples. However, due to the very small amounts of fungus in symptomless samples, many transcripts present in moderate or low amounts, which may play significant role in symptomless infection, might not be detected in a transcriptome analysis of reasonable size. Therefore, I stopped further work in that direction. The advantage of *A. thaliana-B. cinerea* system is that the genome of both organisms has been sequenced. In the *A. thaliana-B. cinerea* symptomless interaction more optimization is needed to enhance the amount of fungus in symptomless infection. Alternatively, well established systems (producing more symptomless infection) for the symptomless infection such as, lettuce- *B. cinerea* or primula-*B. cinerea* could be used in whole transcriptome studies.

The symptomless expression of *B. cinerea* is not strain specific; it is based on physiological differences. The host plant, fungal inoculum and environmental conditions where the host-pathogen interaction occurs are the factors which decide the mode of infection. In the present experiments I checked the ability to produce symptomless and necrotic infections of five different *B. cinerea* isolates, four from symptomless wild growing *A. thaliana* and an aggressive 'standard' necrotrophic isolate B05.10. All the isolates were able to produce both necrotic infection and symptomless infection, however, the degree of infection varied among the isolates. This finding agrees with the findings of Rajaguru and Shaw (2010).

Symptomless *B. cinerea* growth in apparently healthy plants has been identified in various wild growing plants. In America, Shipunov *et al.* (2008) identified symptomlessly growing several putative *Botrytis spp*. and *B. cinerea* in knapweed (*Centaurea stoebe*) based on sequence analysis. In Reading, United Kingdom, Shafia (2009) screened several wild plants for the symptomless *B. cinerea* infection. Nine different host species harboured the fungus symptomlessly. The incidence of symptomless infection was moderate in some species (*Achillea millefolium*, *Arabidopsis thaliana*, *Centaurea nigra*, *Cirsium vulgare*, *Senecio jacobaea*, *Senecio vulgaris* and *Taraxacum agg*.) and rare or absent in others (*Tussilago*

farfara and *Bellis perennis*). The highest percentage of *B. cinerea* (30%) was recovered from surface disinfected *A. thaliana* tissue samples. Shafia's finding in *A. thaliana* is much greater than my and Prof. Mike Shaw's (personal communication) observation; the percentage of recovery was much less in our observations. In Germany, Junker *et al.* (2012) did endophyte screening in wild growing *A. thaliana* and they recovered *B. cinerea* from stem samples.

Barnes (2002) isolated symptomless *B. cinerea* from 60% of primula plants when they were plated on selective medium. Symptomless infection was detected both in plants that had seed-borne infection and in plants which were inoculated with spore dust. Sowley (2006) reported symptomless infection in lettuce plants when they were growing in either indoor or outdoor conditions. Systemic symptomless infection was found in multiple varieties of lettuce and the fungus was isolated from surface disinfected stems, roots and leaves of lettuce plants. There is therefore growing evidence for symptomless infection of *B. cinerea* in wild growing and experimentally inoculated plants.

The findings of this study are important with regards to the control of *B. cinerea*. In this symptomless infection fungus spreads widely on and in the plant tissues, and when it switches to necrotrophic mode of infection several plant parts can be affected at the same time. The fungal hyphae also grow inside the tissue or subcuticularly, therefore it is less accessible to fungicides. Also, the results show that the disease management practices need to be initiated much earlier than the appearance of visible symptoms.

The major conclusions of this study are as follows.

1. *B. cinerea* can cause symptomless infection in *A. thaliana* and symptomless infection can be developed in control environmental conditions

2. Symptomless infection of *B. cinerea* in wild growing *A. thaliana* is not consistent over time

3. The expression (at mRNA level) of some genes significantly varied between symptomless infection and necrotrophic infection. mRNA of some pathogenicity related genes were not detected (*bcbot1*) or detected (*bcpg1*) in less amount in symptomless samples. The signalling gene *bmp1* was highly expressed in symptomless infection. Differences were also noticed between non-symptomatic infection of lettuce and *A. thaliana*; genes *bac* and *bcnep1* were detected in lettuce but not in *A. thaliana*.

4. Symptomless *B. cinerea* grows on and in the lettuce and *A. thaliana*. The fungus produces sub-cellular vesicles in plant tissue, from which less branched hyphae arise and spread on and in the plant tissue. Fungal hyphae were also noticed in the sub-cuticular region and below the epidermal layer. Hyphae spread from the site of initial infection to newly developing plant parts.

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