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Master Program in Plant Protection

Factors Affecting Spore Germination and infection of *Botrytis cinerea*

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This thesis

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Dedicated to my Parents...

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List of Abbreviations

BCAs	Biological Control Agents
DPI	Days Post Inoculation
Fru.	Fructose
GB5	Gamborg's B5-basic Salt Mixture
Glu.	Glucose
hpi	Hours Post Inoculation
LSD	Least Significant Difference
mg	Milligram
ml	Milliliter
mm	Millimeter
mM	Millimolar
NA	Nicotinic Acid
PDA	Potato Dextrose Agar
Prol	Proline
rpm	Round Per Minute
Conidia/ml	Number of conidia per milliliter
SDW	Sterile Distilled Water
Suc.	Sucrose
Tryp.	Tryptophan
µM	Micromolar
CRD	Completely randomized design
MGR	Mycelium growth rate
ALGR	Average linear growth rate

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Abstract

Botrytis cinerea is a necrotrophic fungal plant pathogen distributed worldwide. The early stages of epidemiology namely spore germination is a topic of great interest among researchers. The objective of this study was to investigate the effect of various factors affecting germination of *B. cinerea* conidia *in vitro*. Potato dextrose agar amended with bean leaves extract improved significantly growth and spore production of *B. cinerea* without significant variation between isolates. Results indicated that there was no particular influence of spore age (5-14 days) on germination in 10mM fructose. In addition, germination-self-inhibition was found to be associated with increased spore concentrations (above 4.5×10^5 conidia/ml) without significant differences between isolates. Furthermore, the effect of temperature (5, 10, 15, 20, 25 and 30 °C) on conidial germination was studied *in vitro*; germination started at 10°C, peaked at 20 °C (93%), and completely inhibited at 30 °C. Disease severity followed the same trend were the highest disease was also obtained at 20-25 ºC. In the absence of external source of nutrients, conidial germination was efficiently induced (88-96%) by contact with rigid hydrophobic surfaces. In addition, germination was impaired by extreme pH values (below 6 and above 8). Germination of *Botrytis cinerea* was strongly induced (>90% after 24hours) in the presence of sugars at concentrations above 100mM while the cations (Ca^{2+}, Mg^{2+}, K^+, and Fe^{2+}) had no influence on conidial germination at a wide range of concentrations (0.001-1mM). In the presence of inorganic nitrogen forms, conidial germination responded similarly with no particular influence on germination, whilst germ tube

growth and elongation responded positively with increasing concentrations of both N-forms.

Chapter 1. Introduction

1.1 The pathogen *B. cinerea*

Botrytis cinerea Pers. ex. Fr. is the causal agent of gray mold. The name of the asexual stage or anamorph is derived from the Greek name ' βοιρυς' which means a bunch of grapes. The name of the sexual stage or teleomorph is *Botryotinia fuckeliana* (de Bary) Whetzel, but the ascocarps are rarely observed under field conditions (Polach and Abawi, 1975).

De Bary (1886) observed that carrot cells were killed in advance of invading hyphae of the soft rot fungus *Sclerotinia*. He also noted that fluid from rotten tissue could degrade healthy host tissue, while boiled fluid had no effect. This led to his conclusion that the fungus produced heat-labile enzymes and toxins that kill and degrade plant cells. The same is true for *Botrytis* species. They are equipped with a set of enzymes and/or metabolites that enable the pathogen to invade host tissue and kill host cells and eventually convert host tissue into fungal biomass. (Kars, 2007).

The fungus grows as mycelium; conidia are regarded as short lived propagules in the field and their survival is largely determined by temperature extremes, moisture availability, microbial activity and sunlight exposure. Generally, all species of *Botrytis* form sclerotia which are the most important survival structure of the fungus (Coley-Smith *et al*.,1980).

1.2 Taxonomy and nomenclature

Botrytis cinerea Pers. Fr. (teleomorph *Botryotinia fuckeliana* [de Bary] Whetzel) is currently classified as:

Lineage:

Cellular organisms

› Eukaryota

› Fungi/Metazoa group

› Fungi

› Dikarya

› Ascomycota

› Pezizomycotina

› Leotiomycetes

› Helotiales

› Sclerotiniaceae

› Botryotinia

› Botryotinia fuckeliana

(http://www.ncbi.nlm.nih.gov)

1.3 Genetic variation within the genus *Botrytis*

B. cinerea is a filamentous, heterothallic Ascomycete exhibiting great morphological variability such as in mycelial growth rate, conidia germination, pathogenicity, incidence of sporulation structures, production of sclerotia, and fungicide resistance (Grindle, 1979; Lorbeer, 1980; Di Lenna *et al.*,,1981; Kerssies *et al.*,1997).

Several molecular-based studies on the genetic structure of field populations of *B. cinerea* showed a high degree of genetic diversity in *B. cinerea* and generally, no differentiation between the isolates could be

attributed to parameters such as type of colonized plant tissue, geographic origin or sampling date (Van der *et al.*,1993; Giraud *et al.*,1997; Kerssies *et al.*,1997; Moyano *et al.*,2003).

Phenotypic and genetic variability in *B. cinerea* has often been attributed to the multinucleate and heterokaryotic nature of conidia and hyphae, and to aneuploidy (Van der *et al.*,1993; Buttner *et al.*,1994). Giraud *et al.*, (1997) found that populations of *B. cinerea* were divided into subpopulations, *Transposa* and *Vacuma*, that showed some degree of host specialization in grapes and other plant species in a vineyard in the Champagne region in France. This evidence contradicts the classical view of *B. cinerea* as a population with a high genetic diversity and without host specialization (Giraud *et al.*, 1999). Recent advances in sequencing and genomic techniques have made it possible to monitor gene expression changes at the whole-genome level.

1.4 Host range

The pathogen occurs worldwide, it attacks at least 235 plant species and a wide range of different plant tissues (MacFarlane, 1968). The host range includes many economically important crops such as fruits and berries (grape, strawberry and raspberry), vegetables (tomato and cucumber), ornamentals (rose and gerbera), bulbs (onions) and forest tree seedlings (Jarvis, 1977).

The fact that *B. cinerea* apparently does not display host specificity is in contrast with other species within the genus of *Botrytis*, for which the host range is considerably narrower. For example, *B. aclada* is found only on *Allium* spp., *B. tulipae* on *Tulipa* sp. and *Lilium regale*, *B. fabae* on Leguminosae, and *B. pelargonii* on *Geranium* spp. (Jarvis, 1977).

1.5 Disease symptoms and damage

As mentioned in the previous section, *B. cinerea* is responsible for a very wide range of symptoms and these cannot easily be generalized across plant organs and tissues. Soft rots, accompanied by collapse and water-soaking of parenchymatous tissues, followed by a rapid appearance of gray masses of conidia are perhaps the most typical symptoms on leaves and soft fruits (Figure 1).

In thick-skinned fruits, such as kiwifruits, the dark water-soaking symptom is evident only after cutting. On many fruits and vegetables the infection commonly begins on attached senescent flowers and then as a soft rot it spreads to affect the adjacent developing fruit (blossom-end rot), as in zucchini, cucumbers, French beans, strawberries and apples.

In greenhouse-grown tomato, the greatest damage occurs on stems at pruning wounds where the fungus can rot through the entire stem. Soft rotting of mature tomato fruits occurs mainly post-harvest; an unusual ‘ghost spot’ symptom in unripe tomato is associated with a successful host defense, but the symptom renders fruits unmarketable. (Mittal *et al.*,1987).

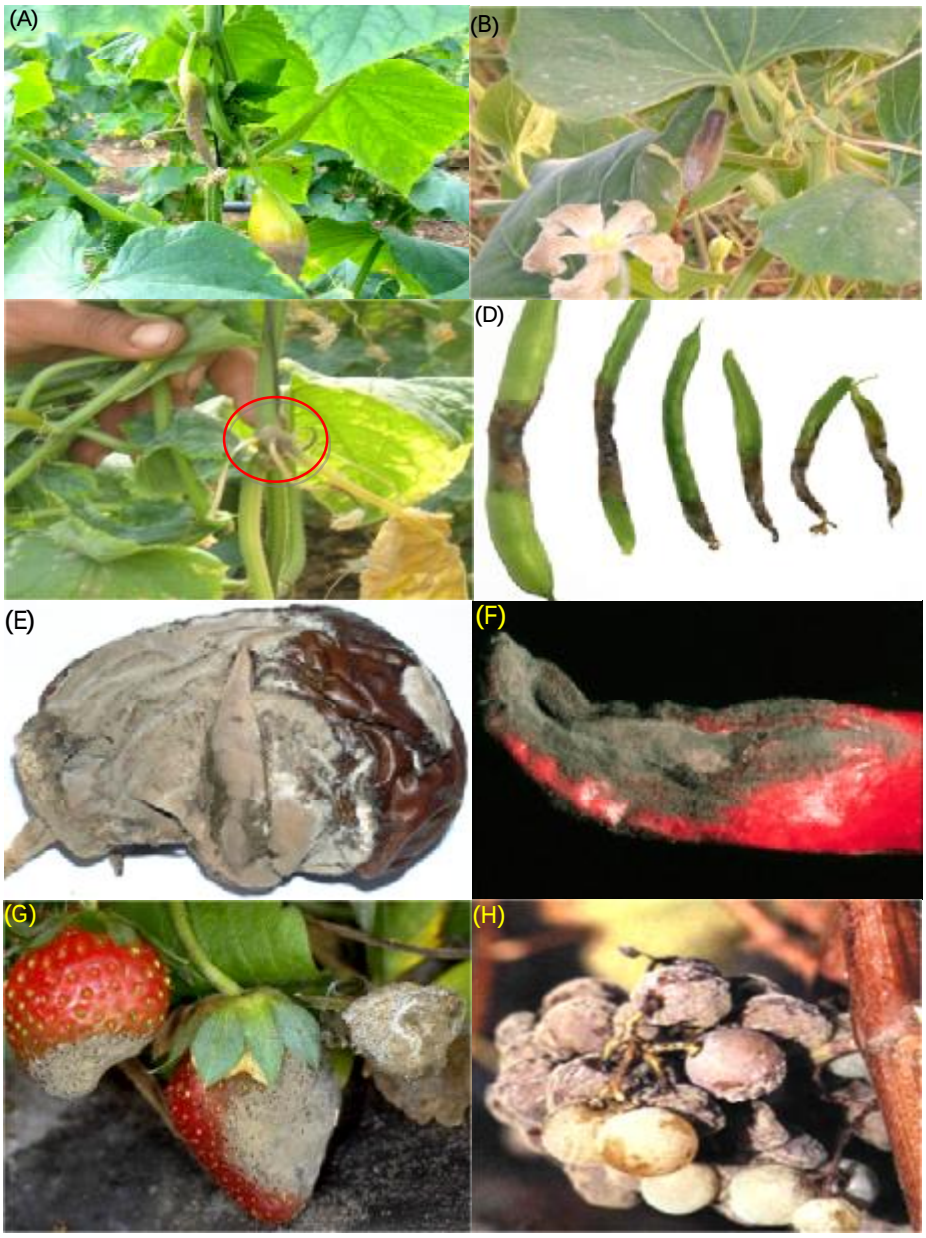


Figure 1. Symptoms of infection by *B. cinerea*.

(A): Gray mold on fruits of cucumber; *Cucumis sativus L*. grown under greenhouse conditions in northern Palestine, (http://www.apsnet.org/online/Archive/2007/iw000077.asp) (B): Gray mold on fruits of Pumpkin *Cucurbita pepo L*. grown under greenhouse conditions in North Palestine (C): The same crop and site as in (A) infection is clear on cucumber stem; (D): Gray mold on fruits of Beans: (*Phaseolus vulgaris*) grown under greenhouse in Hebron; (E): Gray mold on a mummified eggplant fruit grown in open field in Jericho; (F): Gray mold on fruits of Pepper *Capsicum annum L.*; (G): Gray mold on fruits of Strawberry; (H): *Botrytis* bunch rot on grapes *Vitis vinefera* L.

1.6 Epidemiology

The epidemiology of *B. cinerea* and control options are very different in greenhouse crops compared to field crops. Compared to the open field, epidemiology is more predictable and less weather sensitive in greenhouses. In addition, greenhouse cultivation especially vegetables, requires intensive labor; tying, pruning and frequent harvesting provide more entry sites for the pathogen and enhance *B. cinerea* conidia dispersal. The fungus exists in different habitats as mycelia, micro- and macro conidia, chlamydospores, sclerotia, apothecia and ascospores and these are dispersed by diverse means (Jarvis, 1980). Figure 2 shows the life cycle; conidia produced in winter and early spring on over-wintering mycelium and/or sclerotia on host tissues and sclerotia on the surface of the soil are considered the most important infective units of *B. cinerea* for infection in the spring. (Philip *et al.*,2004).

The disease cycle starts with a conidium landing on the host surface. Upon attachment, it germinates on the host surface and produces a germ tube that develops into an appressorium to facilitate penetration of the host surface. Invasion of host tissue can be achieved by active or passive penetration. (Williamson *et al.*,1995). *B. cinerea* is an opportunist that can initiate infection at wound sites, or at sites previously infected by other pathogens. Nevertheless, *Botrytis* species are perfectly able to penetrate intact host surfaces. The first barrier to breach is the host cuticle covering all aerial parts of the plant. The cuticle consists of cutin, and in many cases covered with a hydrophobic wax layer consisting of fatty alcohols. Physical damage or brute mechanical penetration of the cuticle by *B. cinerea* is not usually observed (Cole *et al.*,1996; Williamson *et al.*,1995) indicating that enzymatic activity is involved in penetrating intact host surfaces (Salinas and Verhoeff, 1995).

B. cinerea is also able to infect wide spectrum of host plant species, whereas other *Botrytis* species are confined to a single host species. All *Botrytis* species, whether specific or not, are necrotrophs implying they are able to kill host cells during the infection process.

The risk for *B. cinerea* infections is high when the climatic conditions are favorable (Butin, 1995). In contrast, gray mold is rarely severe in bare-root seedlings produced in outdoor beds (Mittal *et al*.,1987). Furthermore, *B. cinerea* may also infect and remain latent within plant tissues until conditions become favourable for further development of disease (Coley-Smith *et al*.,1980). The process of infection by *B. cinerea* is often associated with rapid colonization of wounded, predisposed, senescing or dead plant material as a nutrient-providing saprophytic base (Jarvis, 1977). In addition, *B. cinerea* is an unspecialized necrotroph that attacks above-ground portions of host plants, and relies on a saprotrophic phase to complete its pathogenic life cycle (Belanger and Avis, 2002).

Droby and Litcher 2004 provided a comprehensive list of post harvest rots caused by *B. cinerea*; these range from gray mold on different plant organs, including flowers, fruits, leaves, shoots and soil storage organs (i.e. carrot, sweet potato), although the fungus is not regarded as a true root pathogen or one causing soil-borne diseases. Vegetables (i.e. cabbage, lettuce, broccoli, beans) and small fruit crops (grape, strawberry, raspberry and blackberry) are most severely affected. (Gossen and Platford, 1999).

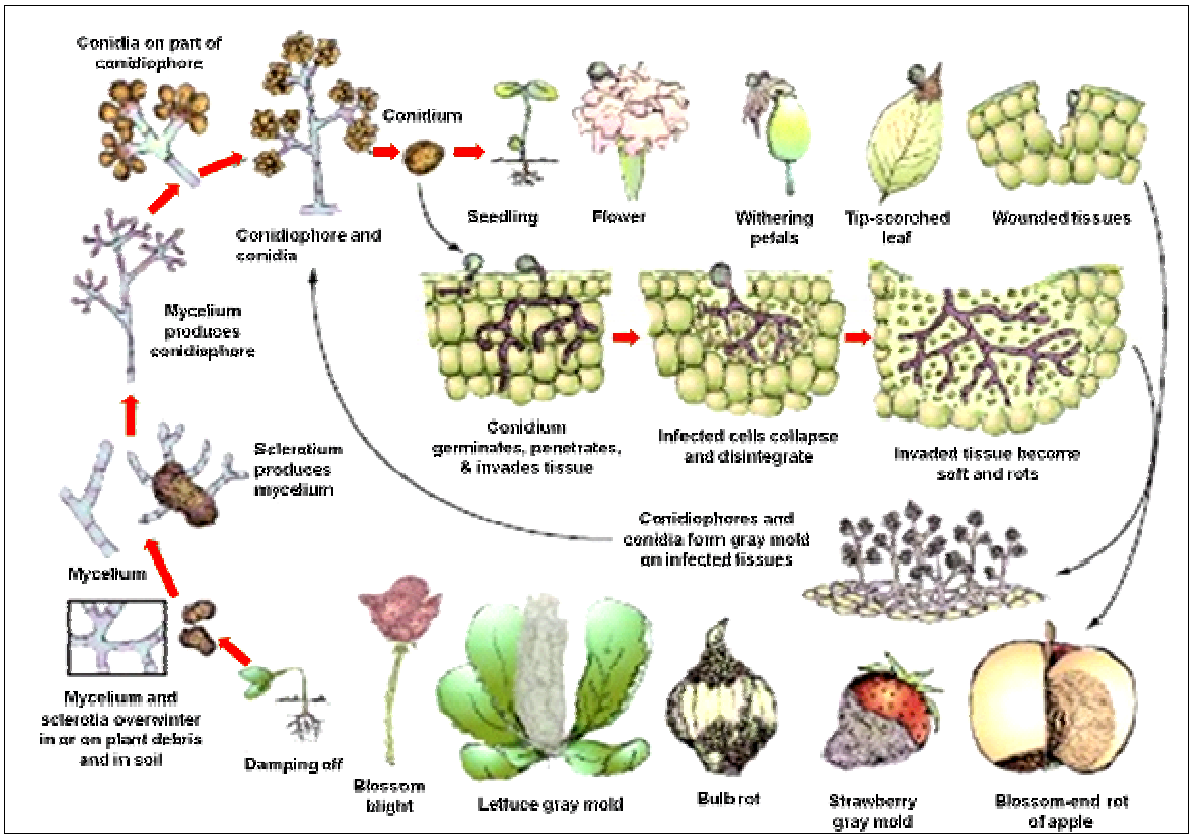


Figure 2. Infection cycle of *B. cinerea*. (Agrios, 2005)

After breaching the host cuticle, *B. cinerea* kills underlying epidermal cells before they are invaded by hyphae (Clark and Lorbeer, 1976). Invasion of plant tissue by *B. cinerea* triggers processes indicative of programmed cell death at a distance from the hyphae, implying that diffusible factors have a direct or indirect phytotoxic activity. (Kars and Van Kan, 2004).

B. cinerea is a facultative parasite; it colonizes wounded, decaying or dead plant material, but it can also attack living plant tissue under certain conditions. (Gossen *et al*., 1999).

1.7 *Botrytis* - Host interactions, early stages of pathogenesis

The early events of plants infection by plant pathogenic fungi are essential for disease initiation and progress. Such early events (adhesion, conidial germination, and formation of external infection structures) were intensively studied lately on *B. cinerea* by many researchers (Doehlemann *et al.*, 2006; Klimple *et al.*, 2002; Schumacher *et al.*, 2008). Electron microscopy has been largely used since years to aid in fine-tuning of *Botrytis* infection mechanisms. However, electron micrographs of the early stages of infection are notably rare in literature. Nevertheless, considerable progress has been made in understanding the ultra-structural and functional response of penetrating hyphae resulting in *Botrytis* as a model in phytopathology and mycology. (Tenberge, 2004).

1.7.1 Spore germination

The mechanics of conidial germination are as diverse and variable as the number of species that produce conidia. Nevertheless, several events generally occur in most instances: when supplied with the appropriate nutrients in the presence of water and air, conidia swell (hydrate) rapidly and undergo a change in surface properties, as evidenced by increased adhesion to one another and to the substrate. The nucleus reorganizes, and hyphal growth begins several hours later (Blakeman, 1975).

Conidial germination of *B. cinerea* is induced by different physical and chemical signals, including the presence and quality of nutrients in particular sugars such as fructose (Kosuge and Hewitt, 1964; Blakeman, 1975). Conidial germination in most filamentous fungi requires the presence of low-molecular-mass nutrients such as sugars, amino acids and inorganic salts (Carlile and Watkinson, 1994). Along with germination and after conidia

adhesion, different mucilages are secreted and assist in anchoring of the germ tube and appressoria to the host surface. In addition, several groups of proteins have been suggested to assist in germ tube and appressorium attachment and to mediate the exchange of early signaling between the fungus and the plant (Prins *et al.*, 2000). Furthermore, evidence exist for involvement of cutinases, hydrophobins, lectins, and integrins in these processes. Cuticle degrading enzymes are embedded in the conidial matrix or secreted by conidia upon contact with the host surface (Deising *et al.*, 2002). It has been suggested that by degrading the cuticular waxes, these enzymes help in removing the lypophylic waxes that coat plant organs thereby making them more receptive to water-coated fungal organs. Several lines of evidence have shown that cutinase activity is essential for conidial attachment and pathogenicity.

Conidia of *B. cinerea* are typically nutrient-dependent; they do not readily germinate in sterile water, and they usually require an exogenous input of nutrients for germination. In addition, it has been proposed that conidia of nutrient-dependent phytopathogenic fungi may use germination-stimulating compounds from a host plant as an alternative chemical cue when nutrient concentrations are too low for conidial germination (Filonow, 2002). In addition, diverse carbon sources (mono- and disaccharides, acetate) are effective at low concentrations (10 mM) to induce germination in *B. cinerea*. Rich media such as malt extract induced rapid germination and early germ tube branching. Induction of conidial germination by nutrients, in particular sugars, is well known in saprotrophic fungi (Osherov and May, 2000). The mechanism of nutrient sensing by *B. cinerea* conidia is unknown. As diverse sugars and acetate induce germination with similar efficiency, it appears

unlikely that nutrient sensing occurs by plasma membrane proteins (Forsberg and Ljungdahl, 2001).

Conidia are also able to germinate on inert artificial surfaces; various amino acids plus sugars efficiently induced germination of conidia, while mineral salts such as ammonium and phosphate were effective only in the presence of low concentrations of sugars (Blakeman, 1975). On cuticular surfaces, however, dry-inoculated conidia can germinate at high humidity in the absence of liquid water (Prins *et al.*, 2000).

In addition, surface hydrophobicity, together with surface hardness, is well known to induce germination of *B. cinerea* conidia in the absence of nutrients (Osherove and May, 2000). Initial adhesion of *B. cinerea* conidia mediated by hydrophobic interactions with the substratum is relatively weak (Doss *et al.*, 1993).

Induction of germination by hard, hydrophobic surfaces has also been shown for pycnidiospores of *Penicillium ampelicida* and conidia of *Colletotricum graminicola* (Kuo and Hoch, 1996; Chaky *et al.*, 2001), whereas conidia of *Colletotrichum lagenarium* germinated equally well on hydrophilic glass surfaces and on host cuticles (Takano *et al.*, 2000). Efficient germination in *Penecillium ampelicida* and *Colletotrichum graminicola* requires strong attachment of the conidia to the surface, which is achieved by the release of extracellular material (Shaw and Hoch, 2000; Chaky *et al.*, 2001).

1.7.2 Adhesion

Germinating conidia undergo a marked increase in their adhesive properties. This is usually a two-step process: initial adhesion that results from a pre-existing glycoprotein layer or from a component of the conidia cell wall,

and later, tighter adhesion that results from metabolic activation and protein synthesis. (Nicholson, 1992)

The most extensive studies of fungal adhesion have been carried out in the pathogenic dimorphic yeast *Candida albicans*, where multiple surface proteins called adhesins contribute to adherence of the yeast form of *Candida albicans* (Staab *et al.*, 1999). Furthermore, adhesion of conidia to surfaces that supply chemical resources for fungal developmental is an important phase in the life cycle of many fungi (Jones 1994; Epstein and Nicholson, 1997). It is generally accepted that for foliar- and fruit-infecting fungi, conidia must first adhere to host surfaces, thereby reducing dislodgement by rain or wind.

Adhesion may also signal metabolic events required for conidial germination (Shaw and Hoch, 1999). Numerous volatile, organic compounds produced by plants are mycoactive, influencing conidial germination, hyphal growth, sporulation, and pathogenicity of fungi (Eckert and Ratnayake, 1994; Filonow 1999, 2001).

Until recently, minimal research data were available on the effect of plant-derived, volatile, organic compounds on the adhesion of fungal conidia to surfaces (Cotoras and Silva, 2005). Acetate esters found in apple fruit stimulated the adhesion of *B. cinerea* conidia on polycarbonate membranes or on the skin of apple slices when conidia on these substrata were exposed to acetate esters in sealed glass bottles (Filonow 2001, 2002).

1.7.3 Penetration into host surfaces

Botrytis cinerea penetrates through the host cuticle, kills underlying epidermal cells before they are invaded by hyphae. (Clark and Lobreer, 1976).

Invasion of plant tissues by *B. cinerea* triggers processes indicative of programmed cell death at a distance from the hyphae. (Govrine and Lovine, 2000).

Furthermore, *Botrytis* produce toxic compounds during penetration of host tissues. The culture filtrates of *B. cinerea* may be phytotoxic when applied to plant tissues. (Rebordinos *et al.*, 1996). The chemical identities for compounds with phytotoxic potential were identified by some researchers as botcinolide (Cutler *et al.*, 1993) and botrydial (Colmenares *et al.*, 2002). Once it has penetrated the epidermal cell wall, *Botrytis* grows through the middle lamella and produces a range of cell wall degrading enzymes (CWDEs). Cell wall degradation by *Botrytis* is mediated by pectinases, cellulases and hemicellulases. (Kars and Van Kan, 2004)

1.8 Disease management

Adequate management of plant diseases is a prerequisite for stable and profitable production. Control of *B. cinerea* is extremely difficult because of the ability of this fungus to attack crops at almost any stage of growth, and to affect all the plant parts, including cotyledons, leaves, stems, flowers and fruits. (Gullino, 1992)

For the fact that it can survive as mycelia and/or conidia or for extended periods as sclerotia in crop debris, the use of single measure to manage *B. cinerea* is unlikely to succeed. (Shteinberge, 2004). Therefore, a deeper understanding of the host-pathogen interaction, the microenvironment in which the fungus operates and its microbial competitors on the host are essential.

1.8.1 Cultural control

Cultural practices that alleviate the effects of gray mold are diverse and often specific to particular species and cropping systems. In perennial woody plants, such as grapevines, pruning to reduce excessive vegetative growth of the plant has been shown to be beneficial (Gubler *et al.*, 1987).

Excessive use of nitrogen fertilizers encourages rapid vegetative growth and increases the risk of gray mold and other diseases. (West *et al.*, 2002). Some of the problems in soft fruit production caused by rainfall during the blossom period have been overcome by plastic rain shelters and tunnels, and facilitated a massive expansion in crop area for strawberries and raspberries. For example, 90% disease reductions in strawberries grown under plastic have been reported, compared with field-grown plants (Xiao *et al.*, 2001).

However, it is still important to encourage ventilation to reduce high relative humidity inside these structures and minimize wetting of foliage. When the plastic covers are removed in late summer there is still infection of leaves and stems, leading to over-wintering mycelium and sclerotia. Spectral modification of daylight by near-UV filters incorporated into plastic covers has been useful to reduce sporulation and infection in a number of crops (Reuveni and Raviv, 2002; Reuveni *et al.*,1989; West *et al.*, 2002).

In unheated greenhouses, the night temperature of plants can be lower than the air temperature due to irradiative cooling. Heating briefly before sunrise to raise plant temperature above the ambient air temperature reduces dew formation on leaves and can control gray mold (Dik and Wubben, 2004). Post-harvest management of fresh products relies extensively on ‘cold-chain-marketing’ of fruits harvested slightly under-ripe and with minimal wounding.

Disease forecasting, especially when combined with accurate local weather data, has been successful in reducing serious crop damage by

specifying timely treatment in grape (Broome *et al.*, 1995) or strawberry (Berrie *et al.*, 2002). In the context of integrated pest management (IPM) there is a great merit in using the maximum effort to reduce pesticide residues by minimal chemical treatment including:

- ⇒ Alternating chemical groups to reduce resistance build-up.
- ⇒ Application of biological control agent(s) appropriate for the temperature regime and humidity.
- ⇒ Continuous removal of dead crop material to remove inoculums.
- ⇒ Use of mulches to bury leaf litter and assist microbial breakdown of inoculum and conserve moisture.
- ⇒ Adequate plant spacing.
- ⇒ Effective pruning and good control of weeds to create open well-ventilated canopy.
- ⇒ Management of insect pests that wound the plant and act as vectors.

1.8.2 Biological control

At least, seven products have now been approved for use on food and non-food plants in greenhouses, under plastic tunnels or in the field in different countries (Elad and Stewart, 2004), Table 1. They have achieved niche markets in situations where heavy use of conventional fungicides has been restricted because of residues accumulating, or because of the restrictions imposed by importing countries.

Table 1. List of commercial biopesticides used against *B. cinerea*.

Product	Organism	Producer	Target crops
Binab®	*Trichoderma harzianum+Trichoderma polysporum*	Binab Bio-innovation, Sweden	Strawberries (Elad and Stewart , 2004)
Mycostop®	*Streptomyces griseoviridis*	Kemera Gro Oy, Finland	Cucumbers, Tomatoes, Lettuce, Peppers, and Ornamentals (White *et al.*, 1990)
Plantsheild®	*Trichoderma harzianum (T22)*	Bioworks Inc., USA	General (Utkhede and Mathur, 2002)
Serenade®	*Bacillus Subtilis (QST713)*	Agra Quest, USA	General
Trichodex®	*Trichoderma harzianum (T39)*	Makhteshim, Israel	Grapes, greenhouse crops (Barakat and Al-Masri, 2005; Elad, 2000; Elad and Steinberg, 1994 ; Elad *et al.*, 1999; O'Neill *et al.*, 1996)
Botry-Zen®	*Ulocladium oudemansii*	Botry-Zen Ltd, New Zealand	General (Elad and Stewart, 2004)
Aspire®	*Candida oleophila (I-182)*	Ecogen, USA	Fruits (Droby *et al.*, 1991)
Bio-save®	*Pseudomonas syringae*	Eco Science Corp., USA	Fruits (Elad and Stewart, 2004)

Biological control agents (BCA) formulations may include filamentous fungi such as *Trichoderma harzianum*, *Clonostachys rosea* (*Gliocladium roseum*) and *Ulocladium oudemansii*, the yeast *Candida oleophila*, or bacteria including *Streptomyces griseoviridis*, *Bacillus subtilis* and *Pseudomonas syringae*. Most BCAs are sprayed on the crop plant, but there has been some success in strawberries. (Kovach *et al.*, 2000; Bilu *et al.*, 2003; Bilu *et al.*, 2004).

Compared with fungicides, BCAs often have restricted ranges of temperature or humidity for maximum microbial action, and they may be influenced by fluctuations in natural populations of phylloplane microbes responding to changes in plant exudates and the environment. Mixed microbial BCAs have been evaluated. (Bilu *et al.*, 2003)

1.8.3 Chemical control

Control of gray mold disease often depends on frequent fungicide applications (Rosslenbroich and Stuebler, 2000). Unfortunately, *B. cinerea* is a classical 'high-risk' pathogen (Brent and Hollomon, 1998). Farmers rely on a wide range of fungicides to control *Botrytis* incited diseases worldwide. (Elad and Stewart, 2004).

During 35 years and since the first commercial use of methyl benzimidazole carbamate (MBC)-generating fungicides, acceptance has grown that for each new chemical the risk of resistance arising in *B. cinerea* is strong if the product is applied repeatedly. (Leroux, 2004). Consequently, mixed spray programmes have been devised, ideally with each spray chosen from a different fungicide group, to reduce the risk of substantial field resistance arising and to keep below the permitted maximum residue level for each active ingredient. The problem arises, however, when some horticultural crops need protection over extended periods because of sequential flowering and fruiting. The chemicals used for control of *B. cinerea* have recently been reviewed by (Leroux, 2004).

Five categories of fungicides are recognized, namely those affecting respiration, microtubule assembly, osmoregulation, sterol biosynthesis inhibitors and those whose toxicity is reversed by amino acids (Rosslenbroich and Stuebler, 2000).

Several multi-site toxicants affecting fungal respiration have been used against *B. cinerea* over a long period without substantial resistance developing in field populations (e.g. thiram, mancozeb, captan, dichlofluanid, tolylfluanid). Cross-resistance to various dithiocarbamates has been identified among captan-resistant isolates (Leroux, 2004).

Dicarboximides have been used extensively as botryticides although their primary target site is not known. They show activity against both conidia and mycelium by affecting sensitivity to osmotic stress (Faretra and Pollastro, 1993).

The anilinopyrimidines are useful botryticides that are antagonized by methionine and some other amino acids. These fungicides can prexvent secretion of hydrolytic enzymes that play a role in pathogenesis, such as cutinases, lipases, cellulases and proteases (Miura *et al*., 1994).

Fenhexamid, a sterol biosynthesis-inhibiting fungicide, is the most recent and effective fungicide against *B. cinerea* (Rosslenbroich and Stuebler, 2000). Certain isolates from a defined *B. cinerea* subpopulation differ in their resistance to this fungicide *in vitro* (Albertini *et al*., 2002; Fournier *et al*., 2003).

Evolution of resistance to different classes of fungicides has been frequently reported in Europe and China (Katan, 1983; Noethover and Matteoni, 1986; Beever *et al*., 1989; Latorre *et al*., 1994; Zhou *et al*., 1994; Pappas 1997; Leroux *et al*., 1999; Kang *et al*., 2000; Dianez *et al*., 2002; Baroffio *et al*., 2003; Zhang *et al*., 2003). Therefore, assessment of new chemical classes of fungicides, which have no cross-resistance with botryticides already in use, is a preferred strategy to adopt (Rosslenbroich and Stuebler, 2000; Leroux *et al*., 2002; O'Neill *et al*., 2004).

Boscalid is a completely new active ingredient belonging to the anilid group of fungicides via a completely novel mode of action that interferes with the enzyme succinate ubiquinone reductase (complex II) in the mitochondrial electron transport chain (Australian Pesticides and Veterinary Medicines Authority, 2004).

1.9 Study objectives

The current study was conducted to:

- Evaluate
  1. various growth parameters of *B. cinerea* isolates on culture media.
  2. The influence of various factors on conidial germination of *B. cinerea* including:

- ü *The effect of conidial age and concentration.*
- ü *The effect of time*.
- ü *The effect of temperature on conidial germination and pathogenesis*.
- ü *The influence of surface properties.*
- ü *The influence of pH.*
- ü *The effect of sugars and amino acids - as organic carbon sources.*
- ü *The role of inorganic nitrogen forms including* $NO3^-$, $NH4^+$.
- ü *The role of* K^+, Mg^{2+}, Ca^{2+} *and* Fe^{2+} *cations.*

Chapter 2. Materials and Methods:

2.1 Fungal isolates

Botrytis cinerea wild type isolates used throughout this study were provided by the research lab. of Prof. Radwan Barakat (Fungal collection). The first isolate, (**PBC1**) was isolated from infected Beans (*Phaseulous vulgaris*) growing under green house in Hebron. The second isolate, (**PBC3**) was isolated from infected grape berries (*Vitis vinefera L.*) growing in an open field in Hebron. Following isolation, the two isolates were grown on PDA medium and kept at 20±1 ºC under continuous light.

After 12 days, and when cultures sporulated, 5mm mycelium plug from each isolate culture was taken and placed in a fresh PDA culture plate; 24 hours later, one freely emerging conidium was transferred into another plate to get monosporic cultures. The monosporic cultures were grown on PDA medium amended with 10% homogenized bean leaves. Plates were then kept under continuous light in an incubator at 20±1°C for the different future tests and experiments.

Another isolate, B05.10 is a universal isolate. It was derived from the wild-type isolate SAS56 by treatment with benomyl for haploidization (Quidde *et al.*, 1999). This putative haploid wild type isolate B05.10 was provided by Prof. P. Tudzynski (University of Munster, Germany).

2.2 Growth parameters of *B. cinerea* isolates on culture media

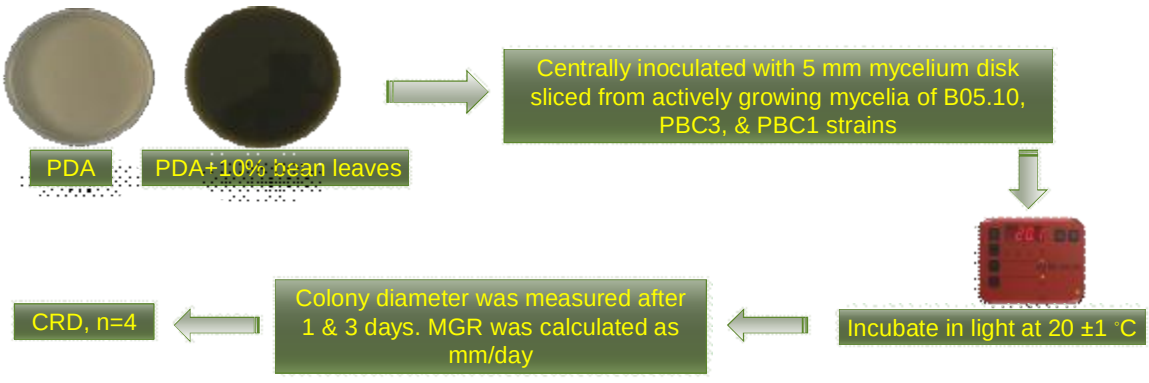
2.2.1 The effect of growth media on mycelial growth rate of *B. cinerea*

Mycelial growth rates of *B. cinerea* isolates were assessed on plates containing two types of media: potato dextrose agar (PDA) medium [(39%) to which 1.5% Streptomycin sulphate was added to eliminate bacterial contaminations] and potato dextrose agar (PDA+10% homogenized `bean leaves+1.5% Streptomycin sulphate) according to the following procedure:

Mycelial disks (5mm) were sliced from two days old cultures of each *B. cinerea* isolate. Four Petri dishes (90mm diameter) from each of the previously described media were centrally inoculated with the mycelial disks (5mm). Plates were then incubated at 21±1 °C under continuous light and inspected daily for three consecutive days. The colony diameter was measured as the mean of two perpendicular diameters measured at the third day minus the diameter at the first day. Average linear growth rates (ALGR) were calculated according to a formula from (Elad *et al.*, 1981).

The experimental design used was completely randomized with four replicates (plates) for each treatment.

Figure 3. Schematic representation for measuring the mycelium growth rates of *B. cinerea* isolates grown on two types of media.



PDA: potato dextrose agar; CRD: completely randomized design.

2.2.2 The effect of growth media on sporulation of *B. cinerea*

Sporulation of *B. cinerea* isolates was assessed on plates containing potato dextrose agar (PDA+1.5% Streptomycin sulphate) and potato dextrose agar (PDA+10% homogenized bean leaves+1.5% Streptomycin sulphate). Four plates of each media were inoculated with 5 mm mycelial disks sliced from a newly growing mycelium (two days old) from PBC3, PBC1 and B05.10 isolates, and incubated at 21±1 °C and continuous light. After twelve days and when plates were seen as well sporulated, four circular mycelium discs (10mm each) were sliced from *B. cinerea* cultures growing on the two types of media and placed in glass vials amended with 10ml 90% ethyl alcohol; vials were then vortexed vigorously.

Spore's production (number of conidia per cm^2) were then determined with the aid of a haemocytometer [Tiefe Depth Protondeur 0.200 mm]. A completely randomized design was used with 12 replicates for each treatment.

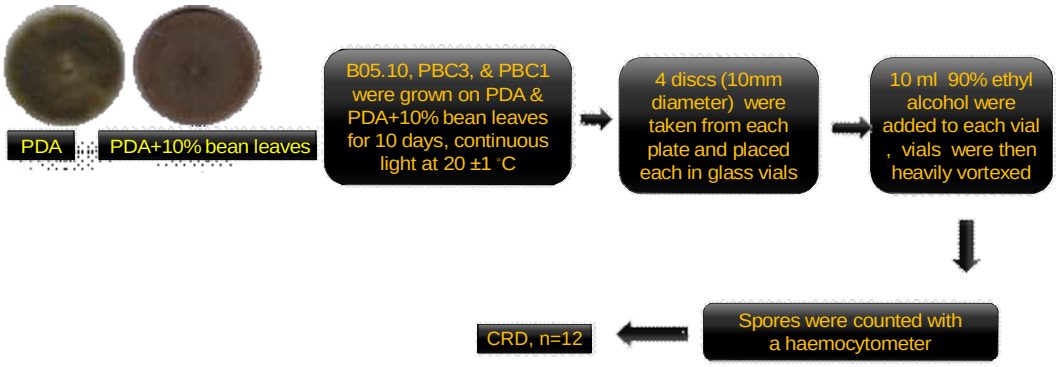


Figure 4. Schematic representation for measuring the spore production capacity of *B.cinerea* isolates grown on two types of media.

PDA: potato dextrose agar; CRD: completely randomized design.

2.3 Influence of incubation time on germination *B. cinerea* conidia

The influence of incubation time on germination of *B. cinerea*, isolates was determined in 10mM sterilized fructose solution. Conidia of *B. cinerea* isolates B05.10, PBC1 and PBC3 were harvested from 10 days old sporulating cultures grown previously on (PDA+10% bean leaves) medium with SDW. Spore concentration was set to 2.5×10^4 conidia/ml. Spherical glass coverslips were placed in the bottom of each of the 24 well of the Sarstedt microtitre plate. After that, 25 µl spore suspension was placed in the middle of each well, and 475 µl of Fructose solution were added to reach a final volume of 0.5 ml. A completely randomized design was used with 3 replicates for each treatment. Germination counts were done after 2, 5, 8, 10 and 24 hours.

2.4 The effect of conidial concentration on germination of *B. cinerea* conidia

The influence of conidial concentration on germination assays of *B. cinerea* isolates was assessed on a 24 well Sarstedt microtitre plate (Sarstedt, Newton. USA), (Figure 18) according to (Doehlemann, 2006). Two plates of PDA medium [(39%) to which 1.5% Streptomycin sulphate were added to eliminate bacterial contaminations] amended with 10% homogenized bean leaves were inoculated with 100 µl of conidial suspension (1×10^6 conidia/ml) from PBC3, PBC1 and B05.10 isolates. The inoculum was spread over the surface of the medium with the aid of a glass rod. When plates were seen well sporulated, conidia were harvested from each plate by 10 ml of SDW. Conidia were then filtered through a Nytex membrane to remove traces of mycelia and placed in a sterile plastic vial for each isolate.

Spore suspension was then washed three times with 10 ml of SDW centrifuged (**IEC** Centra- CLD) for 3 minutes at 3000 rpm. The concentration

of the conidial suspension was determined by a haemocytometer and diluted to the final concentrations of 4×10^5, 2.5×10^4, 5×10^3 and 2.5×10^3 conidia/ml.

Spherical glass coverslips - 15mm (Roth, Karlsruhe, Germany) were placed in each well of the 24-welled microtitre plate. 25 µl of each concentration were placed in the bottom of the well to which 475 µl of 10mM D-Fructose solution were added to reach a final volume of 500 µl and according to (Doehlemann , 2006). Plates were then incubated in the dark at $20^oC\pm1$ and conidial germination counted after 5 hours of incubation. Each treatment consisted of 4 replicates (wells) and 100 randomly selected conidia were counted in each of the 4 wells under an inverted microscope, Figure 19. A conidium was considered as germinated when the germ tube length was less, equal and/or exceeding the conidial diameter, Figure 20.

2.5 The effect of the age of conidia on germination of *B. cinerea* conidia

The influence of conidial age on germination of *B. cinerea*-isolate B05.10 conidia was assessed. The isolate B05.10 was grown on plates containing potato dextrose agar (PDA) amended with 10% homogenized bean leaves. Four plates of PDA medium were inoculated with 5 mm mycelium plug sliced from a newly growing mycelium (two days old), and incubated at 21 $^\circ$C and continuous light. Conidia were then harvested after 7, 9, 10, 12, and 14 days with 10 ml of SDW. Conidia were then filtered through a Nytex membrane to remove traces of mycelia and placed in a sterile plastic vial.
Spore suspension was then washed three times with 10 ml of SDW and centrifuged (**IEC** Centra- CLD) for 3 minutes at 3000 rpm; supernatant was discarded each time. Conidial concentrations were then determined with the aid of a haemocytometer [Tiefe Depth Protondeur 0.200 mm] and fixed at 2.5×10^4 Conidia/ml. Spherical glass coverslips (15mm, Roth, Karlsruhe.

Germany) were placed on each well of the 24-welled microtitre plate. Conidia (25 µl of each age) were placed in the bottom of the well. Fructose was prepared and suspended in liquid Gamborg B5 basal salt mixture (GB5) (Duchefa Biochem. BV, Haarlem, The Netherlands; Art: G0209.0050) to reach a final concentration of 10 mM. After that, 475 µl of the 10mM fructose+GB5 solution were added to reach a final volume of 500 µl. Sarstedt plates were then incubated in the dark at 20±1 ºC.

Using the same selected conidial ages, germination was monitored on a hydrophobic surface; polypropylene film was stacked at the surface of a glass slide. Slides were then placed on a moist filter paper inside closed sterile petri dishes. Conidial suspension was prepared from the isolate B05.10 and fixed at a concentration of 1×10^5 Conidia/ml. The surfaces were then inoculated with 4 separate droplets of conidial suspension 25 µl each and then placed in an incubator.

Each treatment consisted of 4 replicates (wells) and germinated spores were counted out of 100 randomly selected spores under an inverted microscope. A completely randomized design was used with 4 replicates for each treatment.

2.6 Temperature

2.6.1 The effect of temperature on germination *B. cinerea* conidia

B. cinerea (B05.10) was grown on PDA medium amended with 10% homogenized bean leaves. Conidia from ten days old cultures were harvested with 10 ml SDW/each plate. The suspension was then filtered through a Nytex membrane and poured into 50 ml sterile plastic vials. Conidia were then washed 3 times and centrifuged for 3 minutes at 3000 rpm. The conidial

concentration was fixed at 2.5×10^4 Conidia/ml. One spherical glass coverslip was placed on each Sarstedt microtitre plate. In the middle of the glass coverslip, 25 µl of the conidia suspension were placed. Procedure for testing influence of temperature on conidial germination of *B. cinerea* is summarized in Figure 5.

The effect of fructose on conidial germination was found to be correlated with its concentration. Therefore, fructose was prepared into five concentrations: 1µM, 10µM, 100µM, 1mM, and 10mM. (Doehlemann, 2006). Fructose solution (475 µl of each concentration) were suspended carefully into the middle of a 25 µl conidia in each of the 4 wells. Conidial germination was then determined after 8 hours of incubation under the temperatures of 5, 10, 15, 20, 25 and 30 °C. Each treatment consisted of 4 replicates (wells) and the number of germinated conidia out of 100 randomly selected conidia was determined under an inverted microscope. A completely randomized design was used.

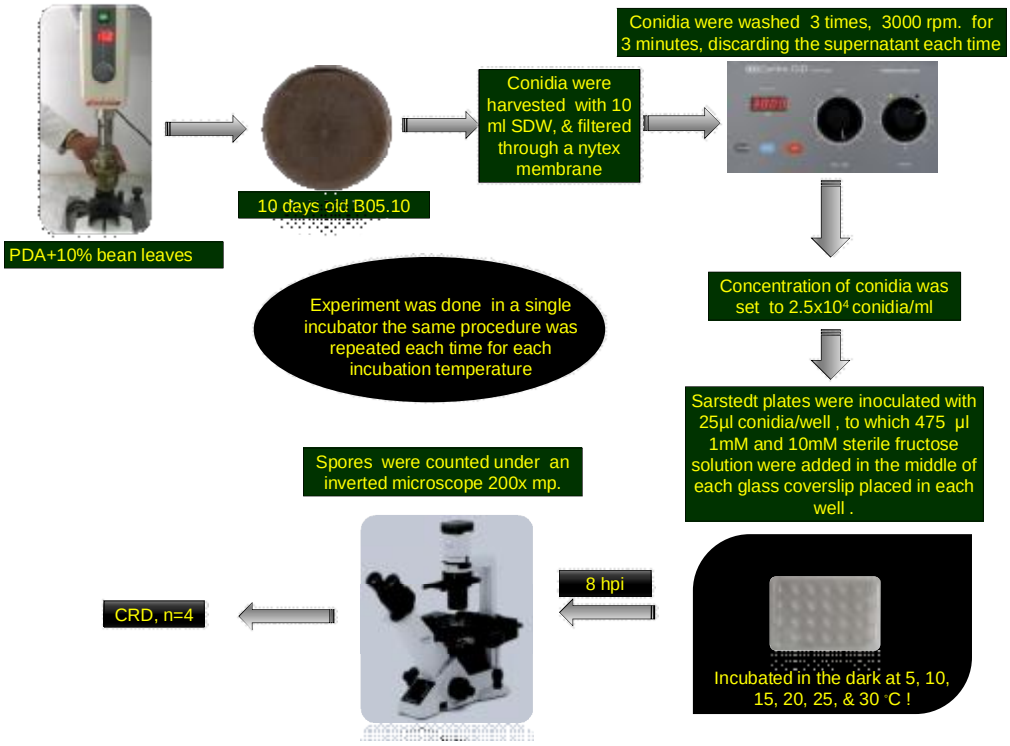


Figure 5. Schematic representation for testing the effect of temperature on conidial germination of *B. cinerea* - Isolate B05.10 using Sarstedt plates.

PDA: potato dextrose agar; rpm: round per minute; SDW: sterile distilled water; hpi: hours post inoculation; mp: magnification power; CRD: completely randomized design.

2.6.2 The effect of temperature on pathogenicity of *B. cinerea*

B. cinerea isolates, (PBC3, PBC1, and B05.10) were grown on PDA medium amended with 10% homogenized bean leaves. Beans (*Phaseoulus vulgaris*) were planted in peat moss until the age of 4 weeks. Plastic boxes were obtained and washed with ethyl alcohol. In the bottom of each box, a plastic mesh was placed over a towel paper to absorb moisture and keep the leaves a way from direct contact with the bottom of the box to avoid contamination. Healthy bean leaves were detached and placed in the moist plastic boxes. The boxes were then covered with transparent polyethylene bags and kept in a humid growth chamber. Mycelium circular discs (5mm diameter) from 2 days

old culture were sliced and placed in the middle of the primary leaf midrib; six bean leaves were used as replicates for each treatment.

Sufficient amount of water was added to the bottom of the boxes to maintain high humidity in order to support infection. The experiment was done under 5, 10, 15, 20, 25, and 30 ºC and continuous light. Disease severity was measured 4 days after inoculation as mean lesion diameter. Procedure for testing the influence of temperature on disease severity induced by *B. cinerea* isolates is presented in Figure 6.

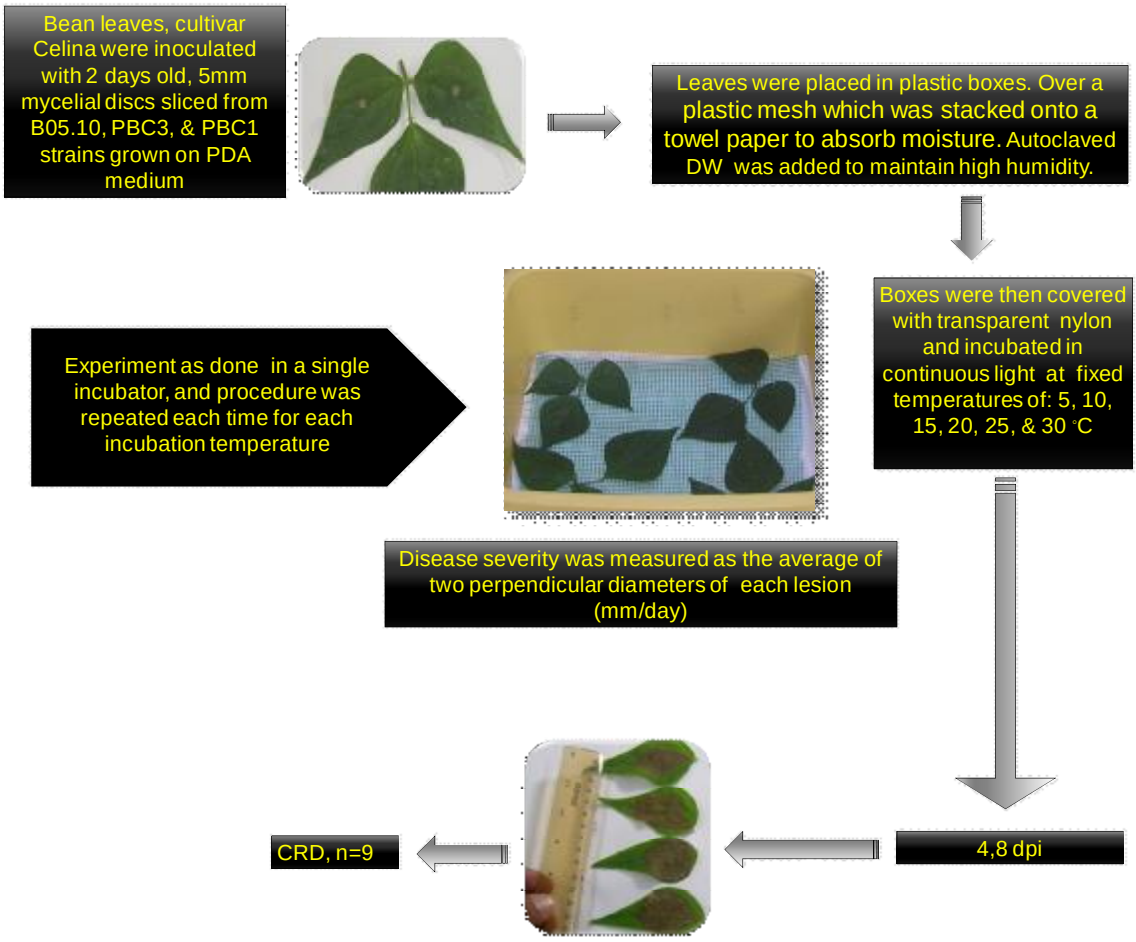


Figure 6. Schematic representation for testing the effect of temperature on pathogenicity of *B. cinerea* - isolate B05.10 on bean detached leaves**.**

PDA: potato dextrose agar; rpm: round per minute; DW: distilled water; dpi: days post inoculation; CRD: completely randomized design.

2.7 The effect of surface properties on germination of *B. cinerea* conidia

For monitoring conidial germination in the absence of exogenously applied nutrients, 5 different surfaces were tested; the inner onion epidermis surface, the surface of the (well) in the Sarstedt plate (Figure 18), the Spherical glass coverslip surface (15mm Roth, Karlsruhe. Germany), the Polypropylene film surface, the Polypropylene film surface coated with vacuum grease and the surface of the glass slides (76×26mm, Knittel Glaser-Germany) coated with the same vaccum grease. Onion inner epidermis was obtained by pealing off the inner layer of onion and carefully laying it on a glass slide; for the preparation of the polypropylene surfaces, a thin layer of polypropylene film was cut and placed onto the surface of glass slides.

All surfaces were placed on moist filter papers inside closed sterile petri dishes. Conidial suspension was prepared from the isolate B05.10 and fixed at a concentration of 1×10^5 conidia/ml. The surfaces were then inoculated with 4 separate droplets of conidial suspension (10 µl each) and then placed in an incubator. Germination counts were done after 19 hours of incubation by counting germinated spores out of 100 randomly selected spores in each droplet. A detailed procedure is presented in Figure 7.

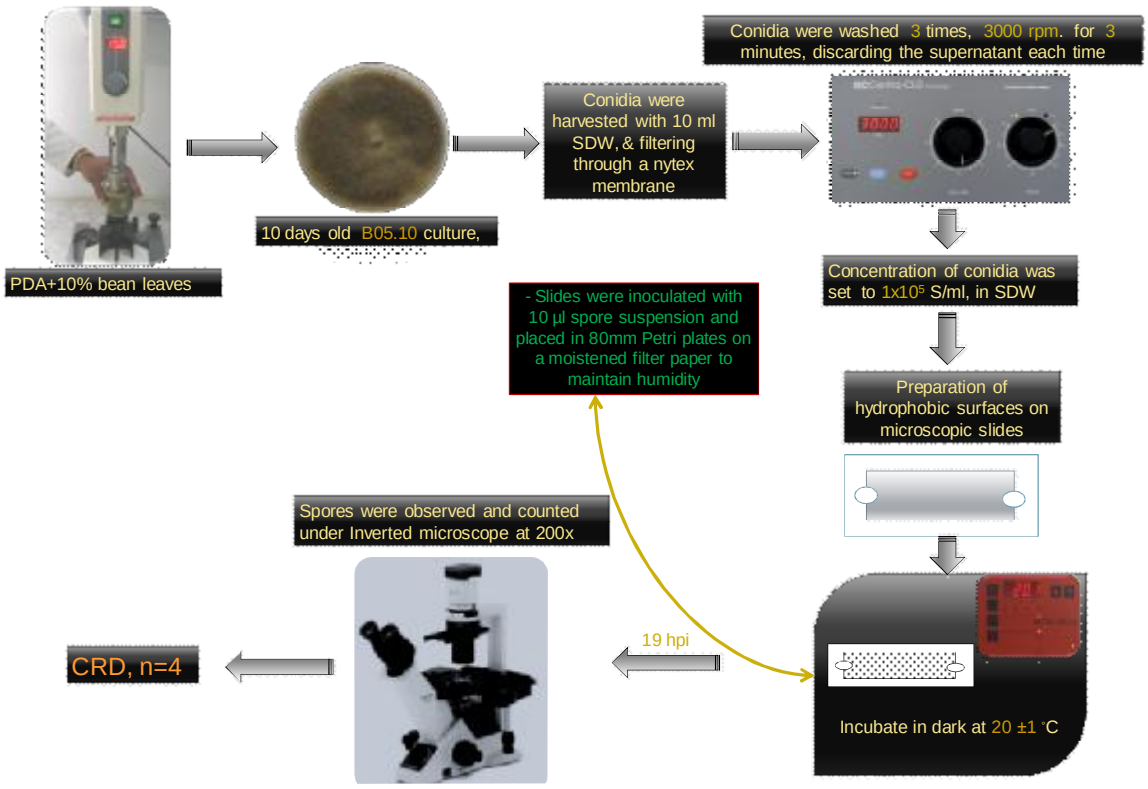


Figure 7. Schematic representation for testing the influence of surface hydrophobicity on conidial germination of *B. cinerea*.

PDA: potato dextrose agar; rpm: round per minute; SDW: sterile distilled water; hpi: hours post inoculation; CRD: completely randomized design.

2.8 The effect of Microclimate pH on germination of *B. cinerea* conidia

The influence of microclimate pH on germination of *B. cinerea*, isolate B05.10 was determined in 1mM fructose solution. Fructose solutions were prepared and adjusted to pH ranges starting from 3, 4, 5, 6, 7, 8, 9 and up to 10 using 1M NaOH and 1M HCl. Conidia of *B. cinerea* (B05.10) were harvested from 10 days old sporulating cultures grown previously on (PDA+beans) medium with SDW and conidial concentration was fixed at 2.5×10^4 conidia/ml. Spherical glass coverslips were placed in the bottom of each of the 24 well of the Sarstedt microtitre plate. After that, 25 µl spore suspension was placed in the middle of each well and 475 µl of Fructose solution were added to reach a final volume of 0.5 ml. A completely randomized design was used with 3 replicates for each treatment. Numbers of germinated conidia were recorded after 5 hours.

2.9 The effect of sugars on germination of *B. cinerea* conidia

The role of carbon sources in conidial germination of *Botrytis cinerea* was investigated using three sugars: Fructose, Glucose and Sucrose in 5 molar concentrations 1µM, 10 µM, 100 µM, 1mM and 10mM. Sugar solutions were prepared in DW and sterilized in the autoclave for 30 minutes at 127°C. *B. cinerea* was grown on (PDA+10% beans) and incubated at 21°C and continuous light for ten days. Spore suspensions from the isolates B05.10, PBC3 and PBC1 were prepared using SDW and adjusted to a final concentration of 2.5×10^4 conidia/ml. Spherical glass coverslips were placed in the bottom of each of the 24 wells of the Sarstedt microtitre plate. Spore suspension (25µl) was placed in the middle of each well and 475 µl of each sugar treatment were added to reach a final volume of 0.5 ml. A completely

randomized design was used with 4 replicates for each treatment. Numbers of germinated conidia were recorded after 5 and 25 hours.

2.10 The effect of amino acids on germination of *B. cinerea* conidia

The role of amino acids in conidial germination of *Botrytis cinerea* was investigated using 5 amino acids: Alanine, Proline, Nicotinic Acid and Tryptophane. Amino acids were used in 5 molar concentrations (1µM, 10 µM, 100 µM, 1mM and 10mM). Solutions were prepared in SDW and sterilized in the autoclave for 30 minutes at 127ºC. *B. cinerea* was grown on (PDA+10% beans) and incubated at 21°C and continuous light for ten days. Spore suspensions from 10 days old cultures of the isolates B05.10, PBC3 and PBC1 were prepared using SDW and fixed at 2.5×10^4 conidia/ml. Spherical glass coverslips were placed in the bottom of each of the 24 wells of the Sarstedt plate. Spore suspension (25 µl) was placed in the middle of each well and 475 µl of each amino acid treatment were added to reach a final volume of 0.5 ml. A completely randomized design was used with 4 replicates for each treatment. Numbers of germinated conidia were recorded after 5 and 25 hours.

2.11 The effect of cations on germination of *B. cinerea* conidia

The role of the cations, Ca^{2+}, Mg^{2+}, and Fe^{2+} in conidial germination of *Botrytis cinerea* was investigated. Ca ($CaCl_2$), Mg ($MgCl_2$), and Fe (FeSO4.7H2O) were prepared into 6 concentrations (0.001M, 0.01M, 0.1M, 100mM, and 1M). Solutions were prepared in distilled water and sterilized in the autoclave for 30 minutes at 127ºC. *B. cinerea* was grown on (PDA+10% beans) and incubated at 21°C and continuous light for ten days. Conidial suspensions from the isolates B05.10 and PBC3 were harvested by SDW. Conidia were then filtered through Nytex membrane and washed three times

for 3 minutes/each to remove traces of mycelium. The concentration was adjusted to a final concentration of 1×10^3 conidia/ml. Spherical glass coverslips were placed in the bottom of each of the 24 wells of the Sarstedt microtitre plate. Spore suspension (25 µl) was placed in the middle of each well and 475 µl of each treatment were added to reach a final volume of 0.5 ml. A completely randomized design was used with 4 replicates for each treatment. Numbers of germinated conidia were recorded after 40 hours of incubation at 21°C. At the same time, the average germ tube length of 10 random germinated conidia (replicates) was recorded.

2.12 The effect of inorganic nitrogen forms on germination of *B. cinerea* conidia

The effect of the nitrogen forms, NH_4^+ and NO_3^- on conidial germination of *Botrytis cinerea* was studied. The procedure is the same as that of section 2.4.4. A completely randomized design was used with 4 replicates for each treatment. Numbers of germinated conidia were recorded after 25 hours of incubation at 21°C. At the same time, the average germ tube length of 10 random germinated conidia (replicates) was recorded.

2.13 Statistical analysis

The data of all experiments were analyzed statistically using analysis of variance (one way ANOVA) and fisher least significant difference (LSD) test with the aid of SigmaStat 2.0 for Windows® statistical package.

Chapter 3. Results

3.1 Growth parameters of *B. cinerea* isolates

3.1.1 The effect of growth media on mycelial growth rate of *B. cinerea*

When the isolates were grown on PDA medium amended with 10% homogenized bean leaves, the means of mycelium growth rate of *B. cinerea* ranged from (32.8-35.1 mm/day) compared to (27.2 -27.8 mm/day) when isolates were grown on PDA medium alone. There was a level of variation between the three isolates in mycelium growth rates on both media but apparently not significant. The highest growth rate (27.8 mm/day) was recorded by the isolate PBC1 grown on PDA; B05.10 however, recorded the highest growth rate (35.1 mm/day) PDA medium amended with 10% bean leaves. Results of the mycelium growth rates of *B. cinerea* isolates grown on the two different types of media are presented in Figure 8.

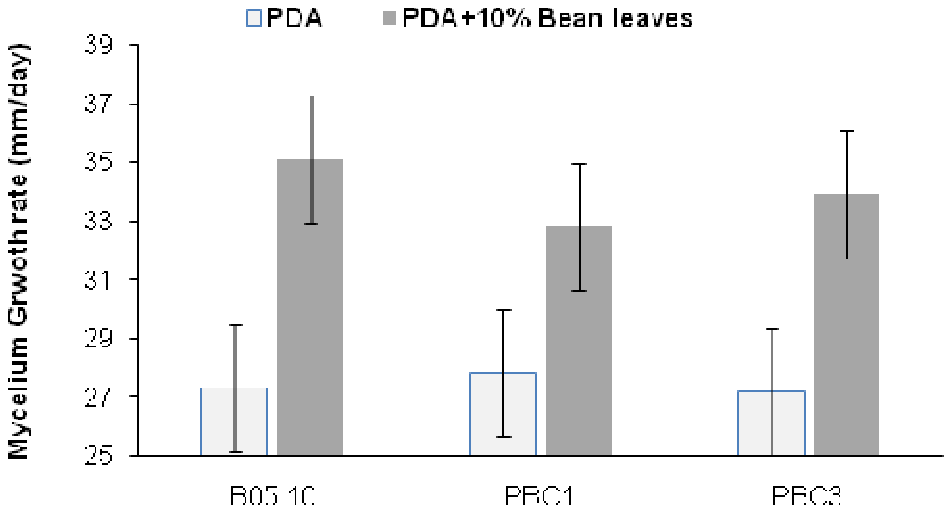


Figure 8. Average mycelial growth rates (mm/day) of *B. cinerea* isolates growing on PDA and PDA+10% Bean leaves at 20±1 ºC under continuous light.

Bars above columns represent the LSD value=2.167, n=4 and (P <0.001)

3.1.2 The effect of growth media on sporulation of *B. cinerea*

Production of conidia by *B. cinerea* isolates was quantified on two types of media (PDA and PDA+10% bean leaves). The logarithm number of conidia production capacity (presented as the log. conidia/cm^2) of the isolates B05.10, PBC1, and PBC3 ranged from 5.73 conidia /cm^2 for the isolate PBC1 and 5.92 conidia/cm^2 for the isolates B05.10 and PBC3 grown on PDA , and from 6.07 conidia/cm^2 for the isolate PBC1 to 6.35 conidia/cm^2 for the isolate PBC3 when grown on PDA+10% bean leaves.

The highest number of conidia was produced by the local isolate PBC3 when grown on PDA+10% Bean leaves medium. Results of the Sporulation of *B. cinerea* isolates grown on both types of media and incubated under continuous light at 20±1 ºC are presented in Figure 9.

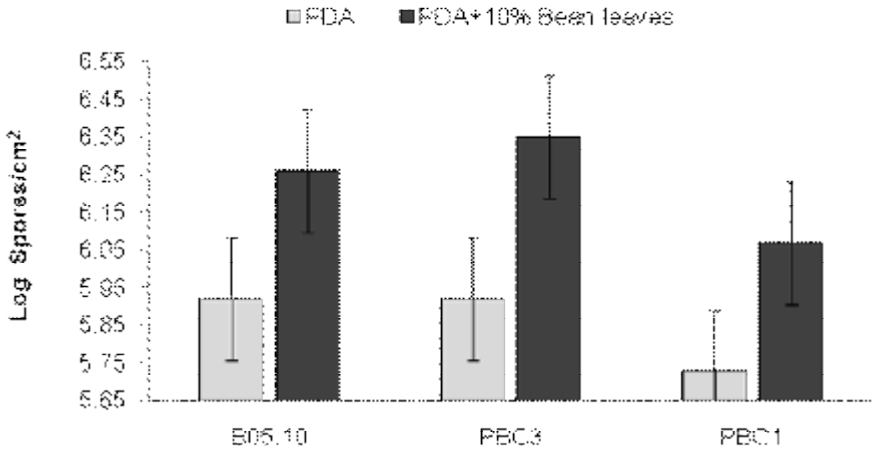


Figure 9. Sporulation of *B. cinerea* isolates on two types of media after 10 days of incubation under continuous light at 20±1 ºC.

Means followed by the same letters above columns are not statistically different at (P<0.001) according to Fishers Least Significance Difference test. (LSD=0.162, n=9).

3.2 The effect of Incubation time on germination of *B. cinerea* conidia

Germination of *B. cinerea* conidia with time was quantified in 10mM Fructose solution (Fig. 10). Conidial germination started after 2 hours of incubation . However, significant statistical differences in germination rates between isolates appeared only after 5 hours of incubation. Conidial germination rates increased sharply after 8 hours and statistical differences between *B. cinerea* isolates were not significant. The highest germination percentage (97%) was recorded by the local isolate PBC1 after 24 hours of incubation and no further statistical significances were found between isolates after 24 hours of incubation.

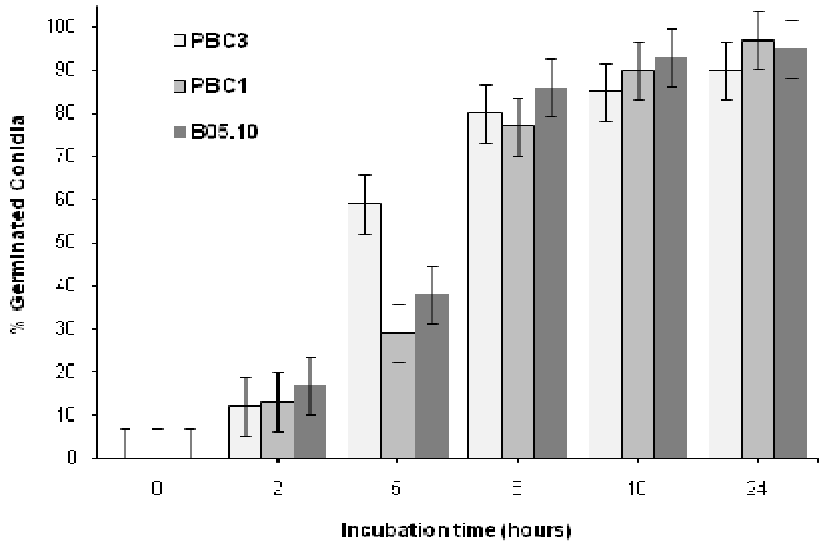


Figure 10. Conidial germination rates of *B. cinerea* isolates in 10mM Fructose solution at 20±1 ºC in the dark after 0, 2, 5, 8, 10 and 24 hours.

Vertical bars represent the value of Fishers Least Significance Difference at P<0.001. (LSD= 6.783, n=3).

3.3 The effect of concentration of conidia on germination of *B. cinerea* conidia

The influence of spore concentration of *B. cinerea*-isolates B05.10, PBC1 and PBC3 on conidial germination was determined in 10mM Fructose solution (Fig. 11). Results showed that conidial germination rates decreased with increasing spore concentrations. The highest germination rate was recorded at the spore concentration (2.5×10^3 conidia/ml) for all isolates. Generally, there was no significant difference in germination rates between the three *B. cinerea* isolates. It was evident that the three isolates responded similarly in which germination rates decreased with increasing spore concentrations (Figure 11 and 22: C, D, and E).

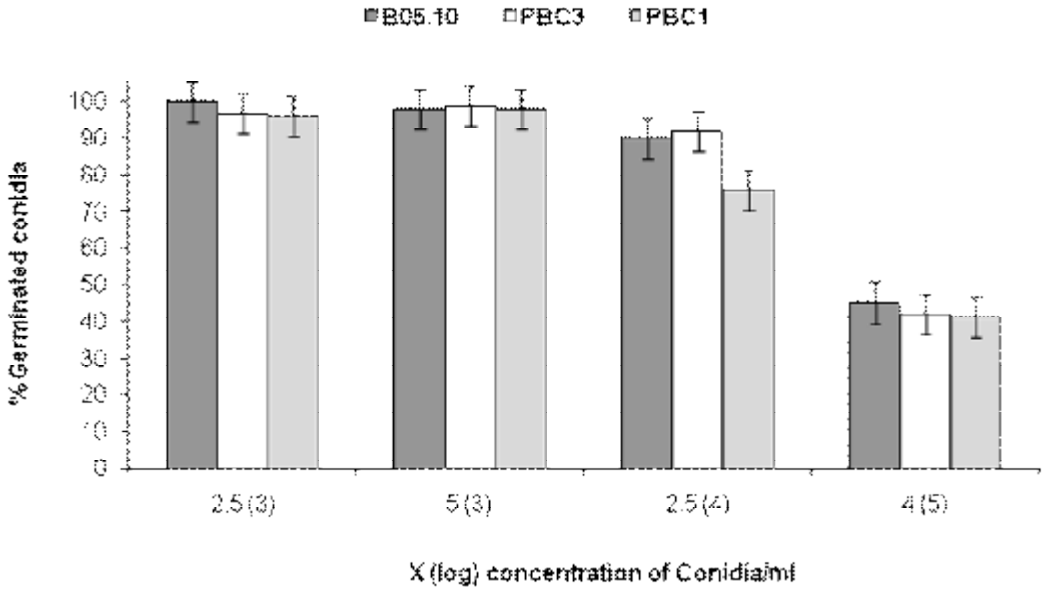


Figure 11. Effect of spore concentration on conidial germination rates of *B. cinerea* isolates grown on (PDA+10% bean leaves) medium and incubated in 10mM Fructose at 20±1 ºC under continuous light after 20 hours of incubation.

Means followed by the same letters above columns are not statistically different at P<0.001 according to Fishers Least Significance Difference test. (LSD= 5.49, n=3).

3.4 The effect of age of conidia on germination of *B. cinerea* conidia

Spore age could be another factor involved in early conidial germination in fungi. The influence of conidial age of *B. cinerea* (B05.10) on germination percentage was investigated. No significant differences in germination percentages were found between different conidial ages in sugar amended with Gamborg' B5- salt mixture (GB5). Conidial germination percentages, however, was significantly reduced in older conidia (67% at 14days) compared to younger conidia (91% at 5 days) when germination was tested on a hydrophobic surface (Polypropylene).

Table 2. Influence of conidial age on germination of *B. cinerea*-isolate B05.10 after 20 hours of incubation in 10 mM fructose solution+GB5

Age of B05.10 culture (days)	% Germination
5	97a
7	95a
10	96a
12	95a
14	93a

Means followed by the same letter in the same column are not significantly different (P = 0.064). GB5: Gamborgs B5-basic salt mixture.

Table 3. Influence of conidial age on germination of *B. cinerea* conidia isolate B05.10 after 20 hours of incubation on polypropylene surface.

Age of B05.10 culture (days)	% Germination
5	91 a
7	84 ab
10	92 a
12	78 bc
14	67 c

-Means followed by the same letter in the same column are not significantly different (LSD=11.309, n=4). GB5: Gamborgs B5-basic salt mixture.

3.5 Temperature

3.5.1 The effect of temperature on conidial germination of *B. cinerea*

The effect of temperature on germination of *B. cinerea* (B05.10) conidia was assessed under various temperatures (5-30 ºC) in several concentrations of fructose solution (Fig. 12). Results showed that conidial germination was significantly affected by the incubation temperature. The highest germination rate (93%) was obtained at 20 ºC; germination was almost zero at 5 and 30 ºC.

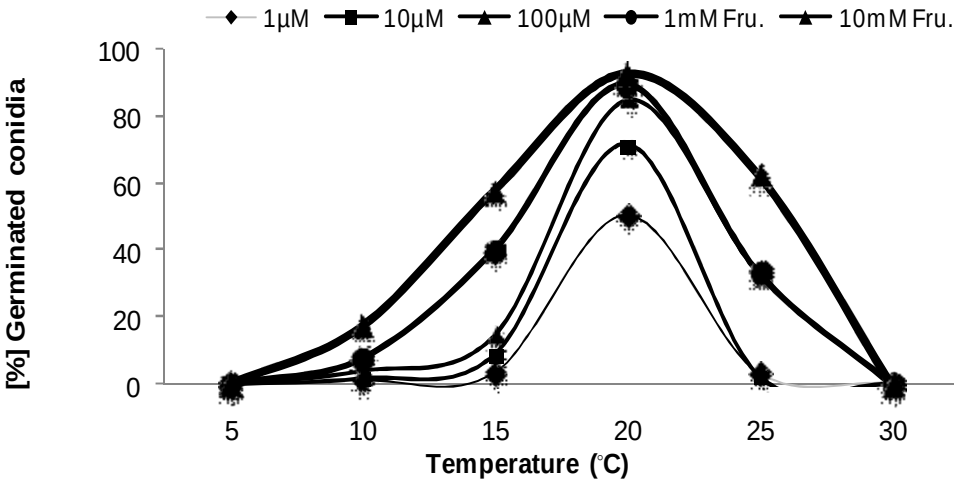


Figure 12. Influence of incubation temperature on germination of *B. cinerea*-isolate B05.10 conidia in different concentrations of fructose in the dark at 20±1 ºC after 8 hours of incubation. Statistical analysis of the data is presented in appendix 1.

3.5.2 The effect of temperature on pathogenicity of *B. cinerea*

In this experiment, the effect of temperature (5-30 ºC) on pathogenesis of *B. cinerea* mycelium was assessed. Disease severity expressed as gray mold lesion diameter was significantly affected by temperatures but to a lesser extent compared to conidial germination. The highest disease severity was obtained at 20-25 ºC. However, disease severity was almost zero at the lowest temperature (5 ºC) and the highest temperature tested (30 ºC). No significant

variations were found between the isolates. Data presenting disease severity in response to temperature is presented in Figure 13 and 23.

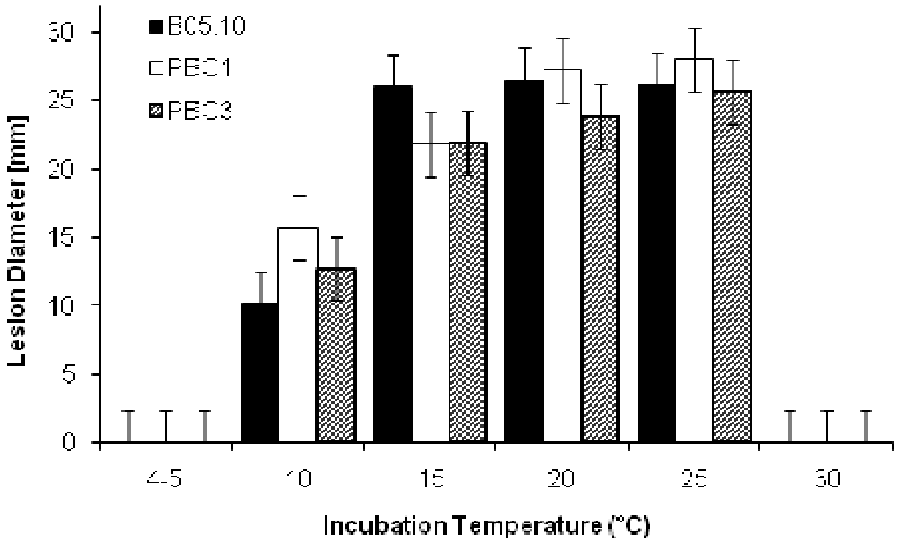


Figure 13. Pathogenicity of *B. cinerea* isolates (mycelial discs) on bean leaves in response to different incubation temperatures. Bars represent Fishers Least Significance Difference test at P<0.001. (LSD=2.350, n=9)

3.6 The effect of surface properties on germination of *B. cinerea* conidia

Surface hardness is known to affect fungal spore germination. In this experiment, the influence of different surfaces on conidial germination of *B. cinerea* was evaluated. In the absence of externally added nutrients, surface modification with the grease induced almost complete germination of B05.10 conidia on polypropylene (94%) and on glass (96%) after 18 hours. Statistical differences between treatment were minor. The lowest germination rate however, was recorded on the polypropylene surface. Data on average germination rates of B05.10 conidia on hydrophobic surfaces is presented in Table 4 and figures 27 and 28.

Table 4. Influence of surface hardness and hydrophobicity on germination of *B. cinerea*-isolate B05.10 after 18 hours post inoculation.

Surface	% Germinated conidia
Sarstedt surface	88 cd
Glass coverslips	91 bc
Polypropylene film	85 d
Polypropylene film+Grease	94 ab
Glass slides + Grease	96 a

- Means followed by the same letter are not significantly different (LSD= 4.049, n=4)

3.7 The effect of microclimate pH on germination of *B. cinerea* conidia

The influence of microclimate pH on germination of *B. cinerea* conidia was assessed on Sarstedt plates. *B. cinerea* conidia were able to germinate well at pH ranging from 6-8; the highest germination rate was obtained at pH 7. However, B05.10 conidia germinated poorly at pH= 3 and 10. The experiment was repeated twice. Data on the average germination rates in different microclimate pH is presented in Figure 14 and 26.

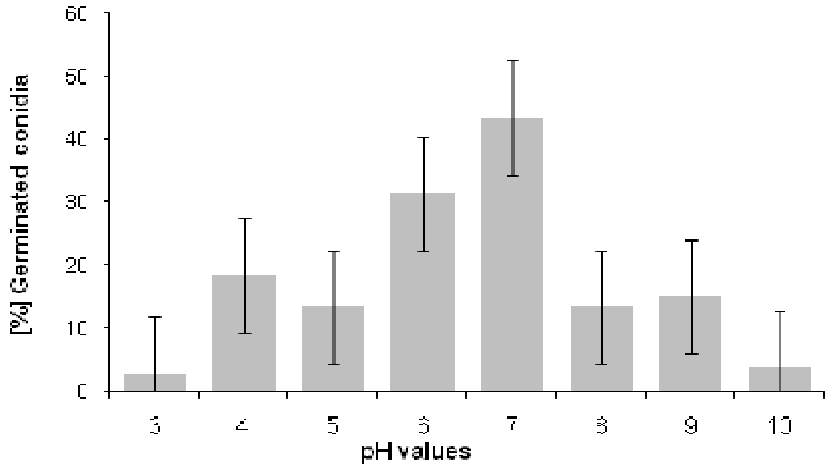


Figure 14. Influence of microclimate pH on conidial germination of *B. cinerea*-isolate B05.10 in 1mM fructose solution after 5 hours of incubation. (LSD = 9.020, n=3).

3.8 The effect of sugars on germination of *B. cinerea* conidia

The influence of the sugars (Fructose, Sucrose and Glucose) on conidial germination of *B. cinerea* was tested in various concentrations. Results showed that germination of conidia was stimulated in sugars in various proportions according to various concentrations compared to SDW. Sucrose was the best in inducing conidial germination even after 5 hpi only recording 87% compared to glucose 18% and fructose 59%. Almost all sugars have induced full germination (100%) after 24 hours of incubation at the highest concentration used (10mM) . The concentration (100 µM) was the breaking point for all sugars to induce significant increase in conidial germination.

The experiment was repeated twice; data for the germination rates of *B. cinerea* isolates under different sugars is presented in Figures 15, 24 and in appendix 2.

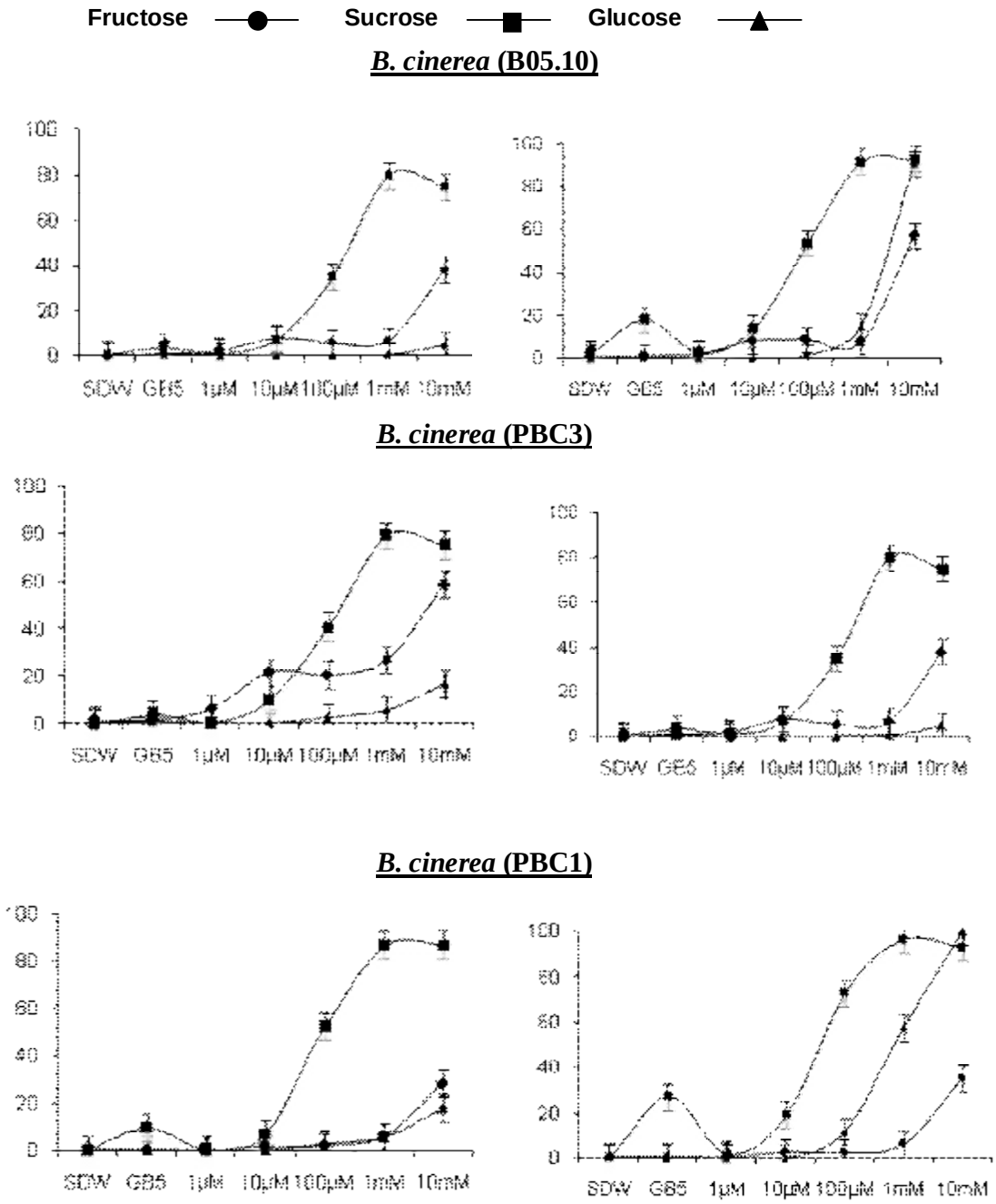


Figure 15. Influence of Fructose, Sucrose and Glucose solutions on germination of *B. cinerea* conidia. (LSD=10.168, n=4, p<0.001)

Experiment was done after 5 and 24 hours post inoculation in various concentrations at 20±1 ºC. SDW: Sterile distilled water, GB5: Gamborg's B5 basic salt mixture; hpi: hours post inoculation.

3.9 The effect of amino acids on germination of *B. cinerea* conidia

The amino acids (Tryptophan and Nicotinic Acid) had no influence on conidial germination of the different *B. cinerea* isolates (Table 5). Proline, however, weakly enhanced germination (39%) compared to the control with no obvious effect of amino acid concentrations or fungal isolates. Alanine moderately increased conidial germination (29-88%) but after 47 hours (Table 6).

Table 5. Conidial germination of *B. cinerea* isolates in response to tryptophan, proline and nicotinic acid after 5 and 24 hours post inoculation.

- 5hpi

	Tryptophan			**Proline**			**Nicotinic Acid**		
	B. cinerea isolates			*B. cinerea* isolates			*B. cinerea* isolates		
	PBC3	**PBC1**	**B05.10**	**PBC3**	**PBC1**	**B05.10**	**PBC3**	**PBC1**	**B05.10**
Con.	5h	5h	5h	5h	5h	5h	5h	5h	5h
SDW	1 jklmn	0 jklmn	0 jklmn	0 jklmn	0 jklmn	1 jklmn	0 jklmn	0 jklmn	0 jklmn
GB5	2 ijklm	1 jklmn	1 jklmn	4 ghijk	10 ef	4 ghijk	1 jklmn	0 jklmn	1 jklmn
1µM	1 jklmn	0 jklmn	2 ijklm	1 jklmn	29 a	17 bc	0 jklmn	1 jklmn	0 jklmn
10µM	1 jklmn	0 jklmn	1 jklmn	1 jklmn	1 jklmn	3 hijkl	0 jklmn	3 hijkl	0 jklmn
100µM	1 jklmn	0 jklmn	2 ijklm	7 efgh	11 de	11 de	0 jklmn	1 jklmn	0 jklmn
1mM	6 fghi	0 jklmn	3 hijkl	2 ijklm	8 efg	5 ghij	0 jklmn	0 jklmn	0 jklmn
10mM	5 ghij	1 jklmn	2 ijklm	7 efgh	20 b	15 cd	0 jklmn	0 jklmn	0 jklmn

- 24 hpi

	Tryptophan			**Proline**			**Nicotinic Acid**		
	B. cinerea isolates			*B. cinerea* isolates			*B. cinerea* isolates		
	PBC3	**PBC1**	**B05.10**	**PBC3**	**PBC1**	**B05.10**	**PBC3**	**PBC1**	**B05.10**
Con.	24h	24h	24h	24h	24h	24h	24h	24h	24h
SDW	2 ijklm	0 klm	1 klm	1 klm	0 klm	2 ijklm	0 klm	0 klm	0 klm
GB5	2 ijklm	1 klm	1 klm	5 ghij	27 b	18 c	1 klm	0 klm	1 klm
1µM	2 ijklm	0 klm	2 ijklm	5 ghij	39 a	23 b	1 klm	1 klm	0 klm
10µM	2 ijklm	0 klm	1 klm	2 ijklm	2 ijklm	4 hijkl	3 ijkl	3 ijkl	3 ijkl
100µM	2 ijklm	0 klm	2 ijklm	11 ef	25 b	17 cd	2 ijklm	1 klm	0 klm
1mM	6 ghi	0 klm	2 ijklm	5 ghij	13 de	8 fgh	2 ijklm	0 klm	0 klm
10mM	5 ghij	2 ijklm	3 ijkl	9 efg	26 b	17 cd	0 klm	0 klm	0 klm

97 Data are means of germination percentages; means followed by the same letter in the same column and row are not significantly different at LSD=12.646, P <0.001, n=4. SDW, Sterile distilled water; GB5; Gamborg's B5 – basic salt mixture; hpi: hours post inoculation

Table 6. Conidial germination of *B. cinerea* isolates in response to L- Alanine after 47 hours post inoculation.

	B. cinerea isolates		
	PBC3	PBC1	B05.10
SDW	2 o	0 o	3 o
10mM	66 defg	44 jk	40 jkl
15mM	74 bcd	70 bcdef	39 klm
20mM	73bcde	59 gh	29 n
25mM	79 ab	49 ij	55 hj
30mM	88 a	30 mn	77 bc

Means followed by the same letter in the same column and raw are not significantly different at (LSD= 10.935, n=4).

3.10 The effect of cations on germination of *B. cinerea* conidia

The cations Ca^{2+}, Mg^{2+}, K^{+}, and Fe^{2+} had no influence on conidial germination of *B. cinerea* isolates (B05.10 and PBC1) at the concentrations (0.001-1mM). At 10mM concentration, Fe reduced germination dramatically. At higher concentrations (>10mM), all cations showed toxicity and totally inhibited conidial germination (Fig. 16). Concerning germ tube elongation, only Fe was able to enhance germination at low concentrations, but as concentration increased germ tube elongation decreased until totally inhibited at high concentrations (>10mM). All the other cations (Ca^{2+}, Mg^{2+} and K^{+}) had no influence on germ tube elongation whatsoever at all concentrations tested (Fig. 16).

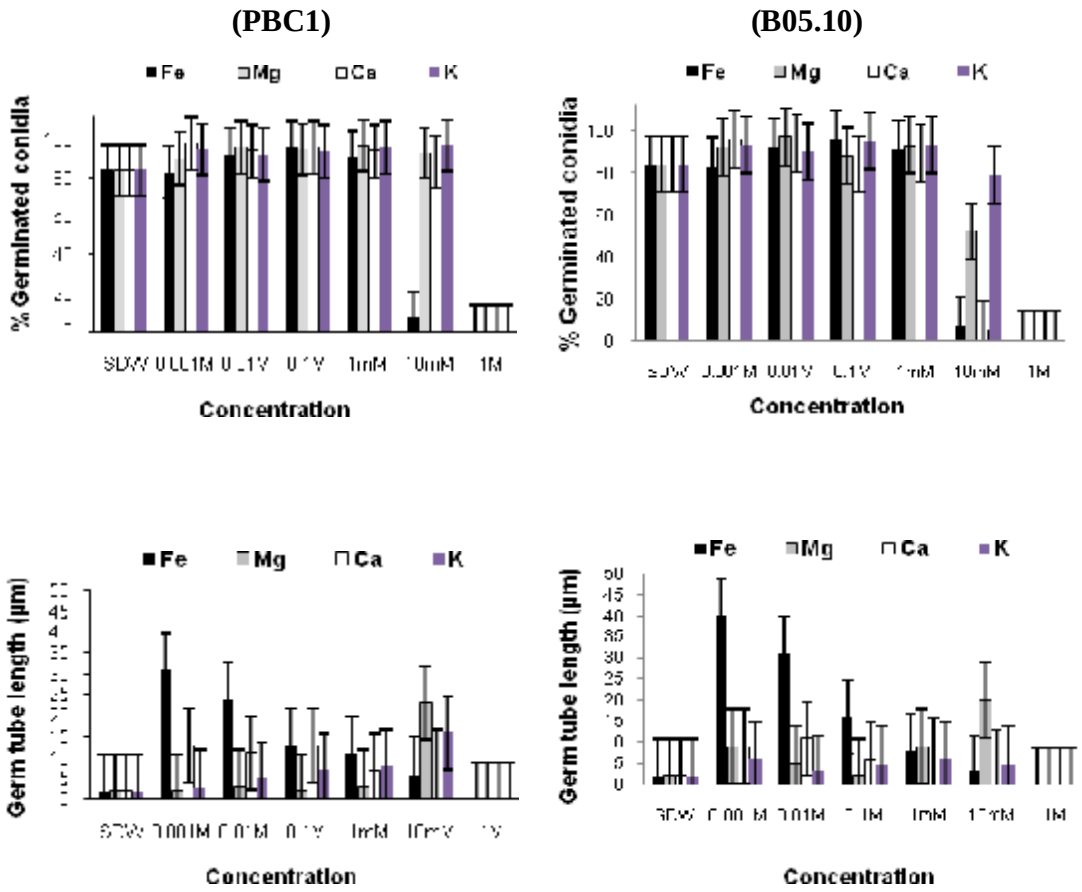


Figure 16. Influence of Ca^{2+}, Mg^{2+}, K^{+}, Fe^{2+} in various concentrations on conidial germination and germ tube elongation of *B. cinerea*. Conidial germination (LSD=13.527, n=4) ; Germ tube elongation (LSD=8.815, n=10).

3.11 The effect of inorganic nitrogen forms on germination of *B. cinerea* conidia

The effect of NH_4 and NO_3 on germination of *Botrytis cinerea* (B05.10 and PBC1) conidia and germ tube lengths was investigated (Fig 17). Inorganic nitrogen forms had no influence on germination percentages of *B. cinerea isolates* at all concentrations tested. However, germ tube length growth was dramatically influenced by both nitrogen forms positively; germ tube length increased by almost 99% at the highest concentration of NH_4 (1M) compared to the control (SDW). In addition, NH_4 enhanced germ tube growth to a larger extent than NO_3 form of N for both *B. cinerea* isolates. Both *B. cinerea* isolates responded almost similarly in respect to percentage germination and germ tube growth.

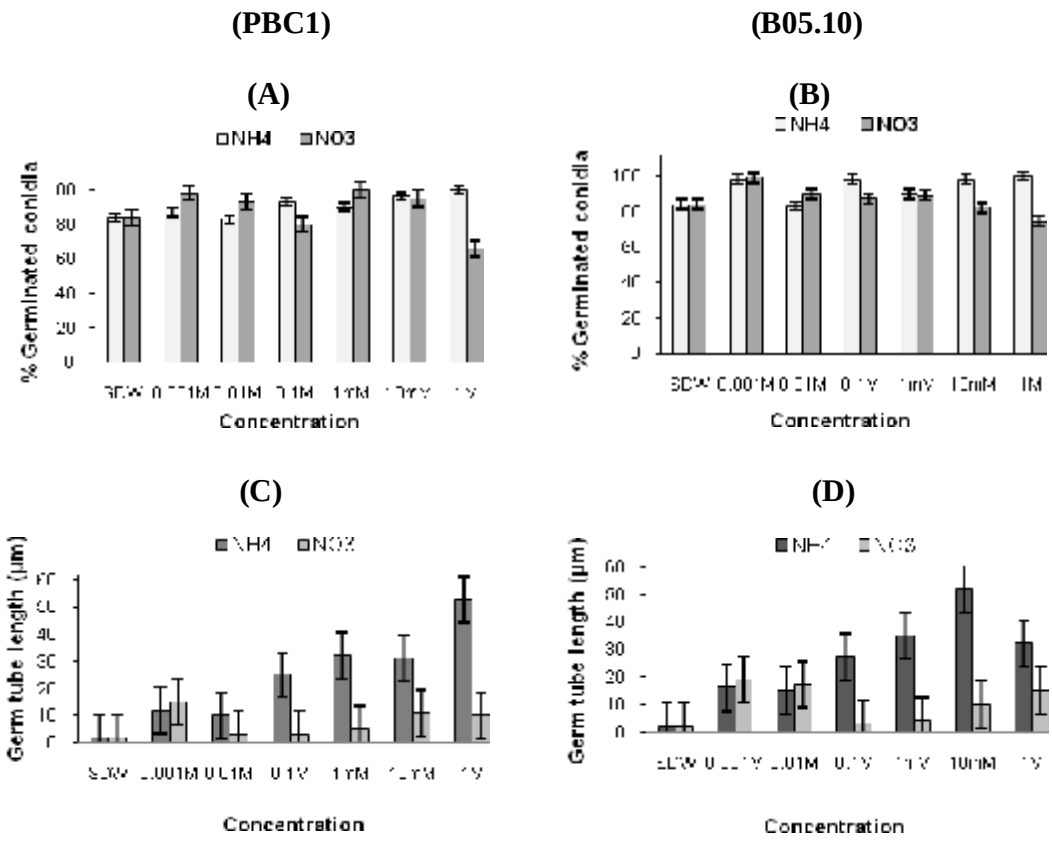


Figure 17. Influence of NH_4 and NO_3 in various concentrations on conidial germination and germ tube elongation of *B. cinerea* PBC1 (A,C), and B05.10 (B,D). Differences between means of germination percentages were not significant; bars represent the standard error of the mean. Germ tube elongation (LSD=8.489, n=10).

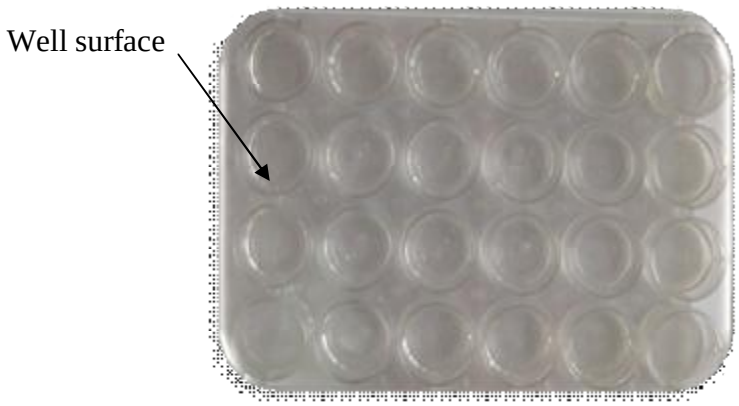


Figure 18. Sarstedt microtitre plate (Sarstedt, Newton. USA), used throughout the study for germination assays of *B. cinerea* conidia

Figure 19. Inverted microscope (Olympus® Hamburg, Germany) used throughout this study for monitoring germination of *B. cinerea* conidia.

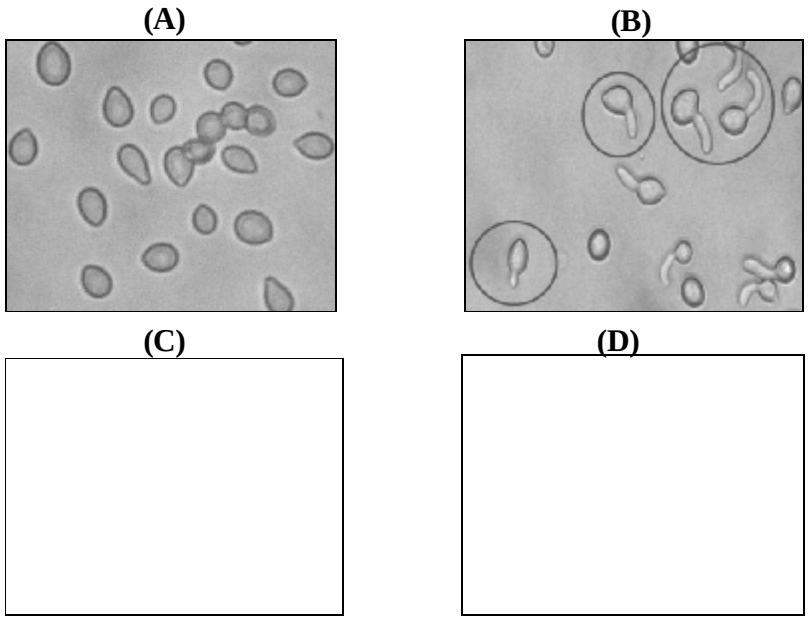


Figure 20. Standardization of conidial germination of *B. cinerea* conidia under the inverted microscope at 200X.

(A): Non-germinating conidia after 5 hpi in SDW; (B): Germinated conidia incubated in 10mM Fructose solution after 5 hpi, notice the variation in germ tubes lengths under the same field germ-tubes; (C): Non-germinating conidia after 24 hpi in SDW; (D): Germinated conidia incubated in 10mM Fructose solution after 24 hpi.

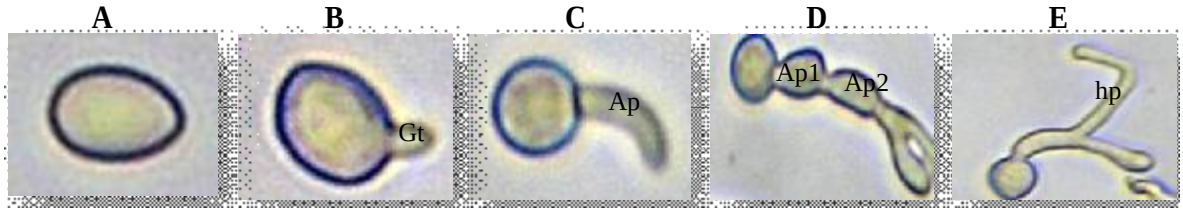


Figure 21. Germination of *B. cinerea* –isolate B05.10 conidia in 10mM fructose solution under the inverted microscope at 200X mp.

(A): Non-germinated conidium; (B): Germination starts after 2 hpi; (C): Germinated conidium after 5 hpi, notice the variation in germ tubes lengths (D), (E): Germinated conidia after 24 hpi. Notice the appressorium formation and hyphal branching. Gt: Germ tube; Ap. Appressorium, hp: hyphae

A B C D E

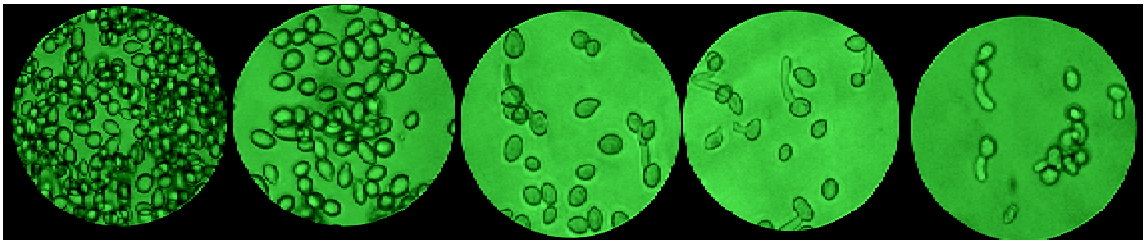


Figure 22. *B. cinerea* (B05.10) conidial germination using different concentrations of conidia at 200X.

(A): Conidial concentration= 5×10^6 Conidia/ml; (B): 1×10^6 Conidia/ml; (C): 4×10^5 Conidia/ml; (D): 2.5×10^4 Conidia/ml and (E): 5×10^3Conidia/ml.

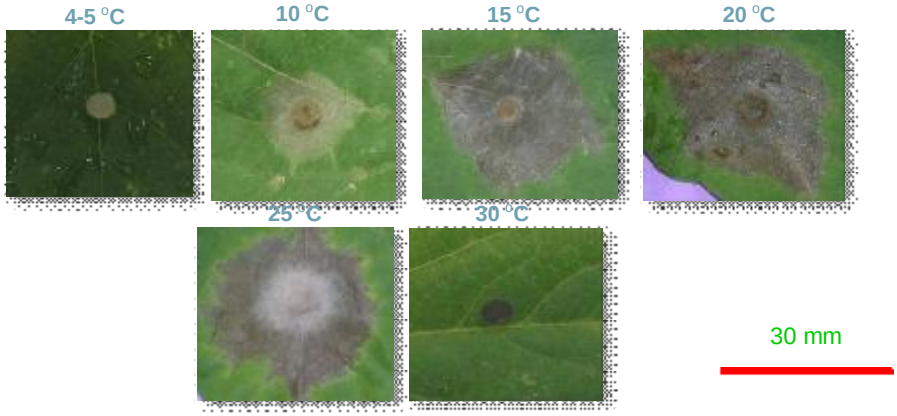


Figure 23. Spreading lesions following infection of Beans (Cultivar: Celina) with *B. cinerea*- isolate B05.10 under different incubation temperatures.

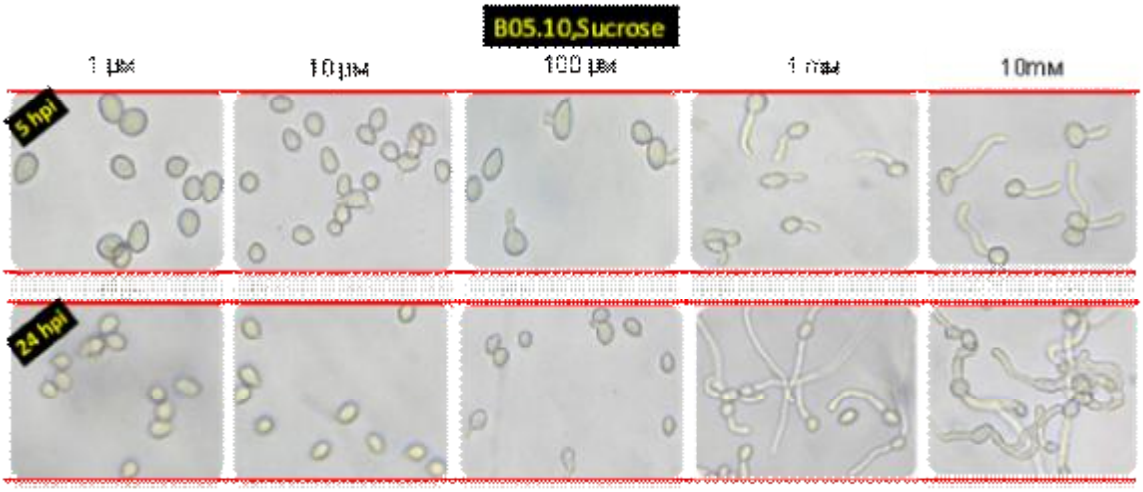


Figure 24. Conidial germination of *B. cinerea* (B05.10) in different concentrations of sucrose after 5 and 24 hours at 200X.

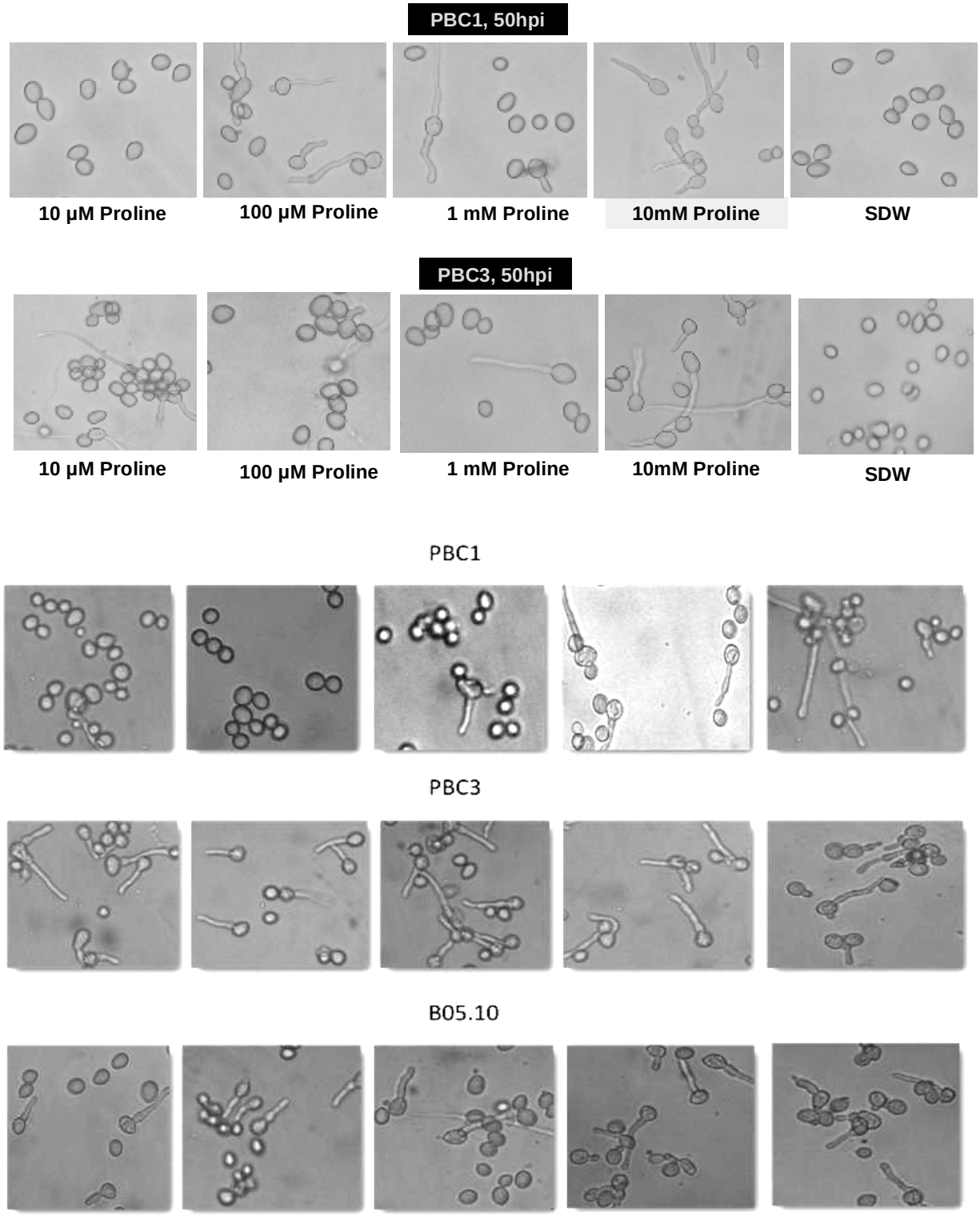


Figure 25. Conidial germination of *B. cinerea* in various concentrations of Proline, and Alanine at 200X.

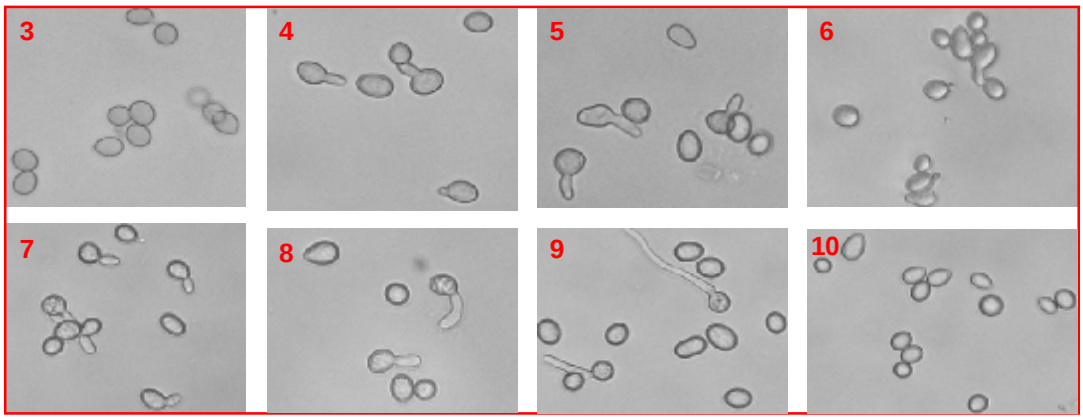


Figure 26. Conidial germination of *B. cinerea* –isolate B05.10 under different pH values in 1mM fructose solution at 200 X .

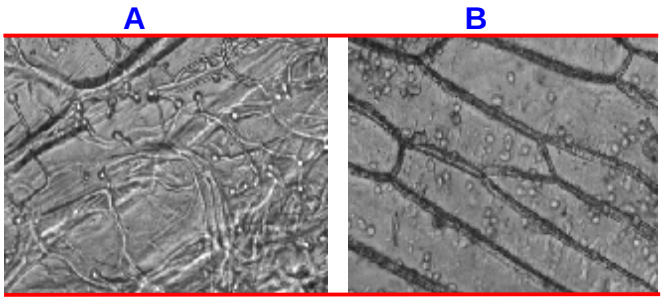


Figure 27. Conidial germination of *B. cinerea*-B05.10 on onion inner epidermis after 24 hours.

(A): at 15°C-Infection is visible and onion cells are macerated by elongated hyphae (B): at 5 °C - No conidial germination is suppressed by low temperature, At 200X.

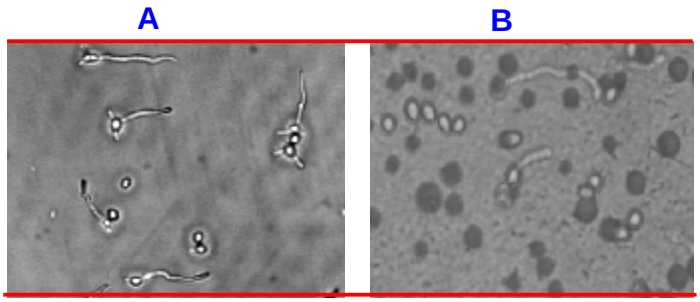


Figure 28. Conidial germination of *B. cinerea*-B05.10 on two hydrophobic surfaces.

(A): on Sarstedt surface; (B): on polypropylene surface. At 200X.

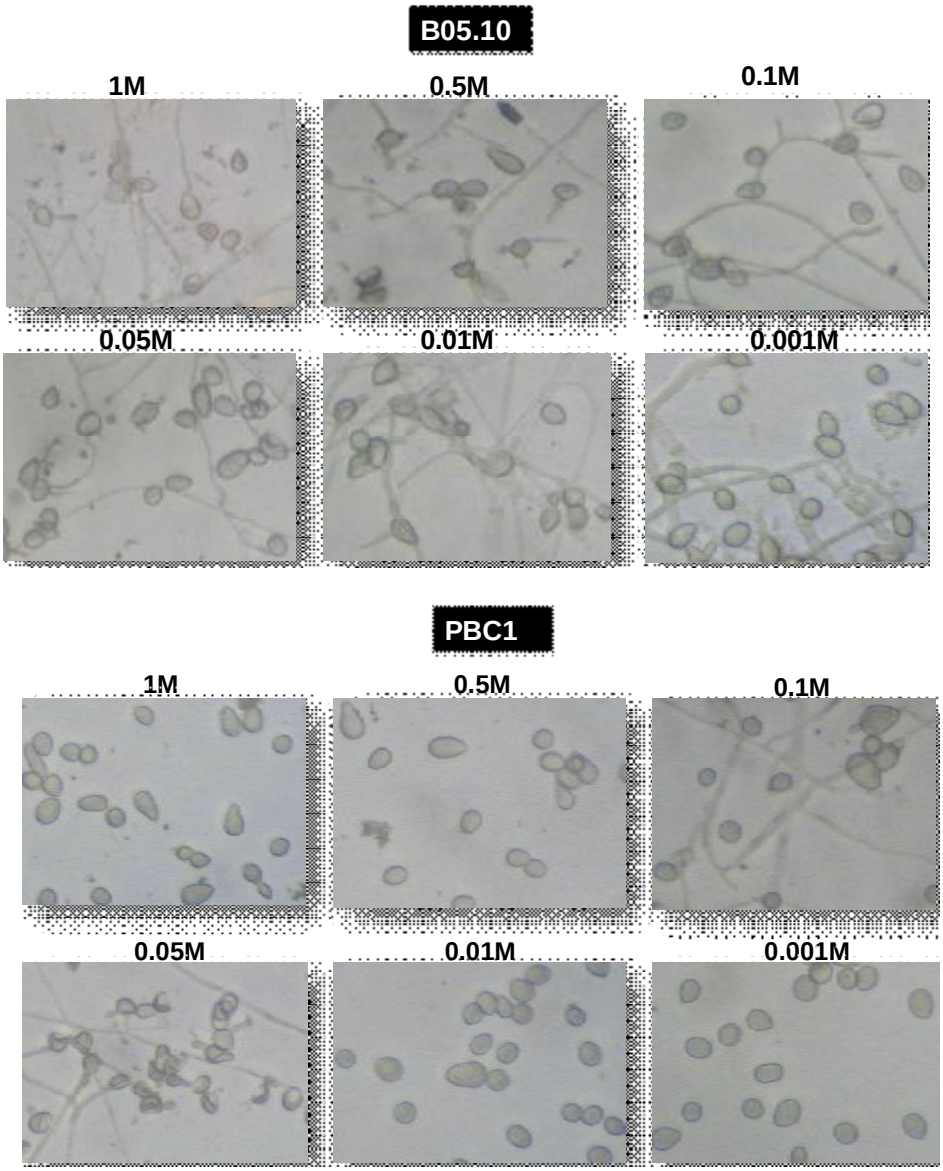


Figure 29. Conidial germination of *B. cinerea* in different concentrations of Calcium after 40 hours at 200X.

Isolate: PBC3

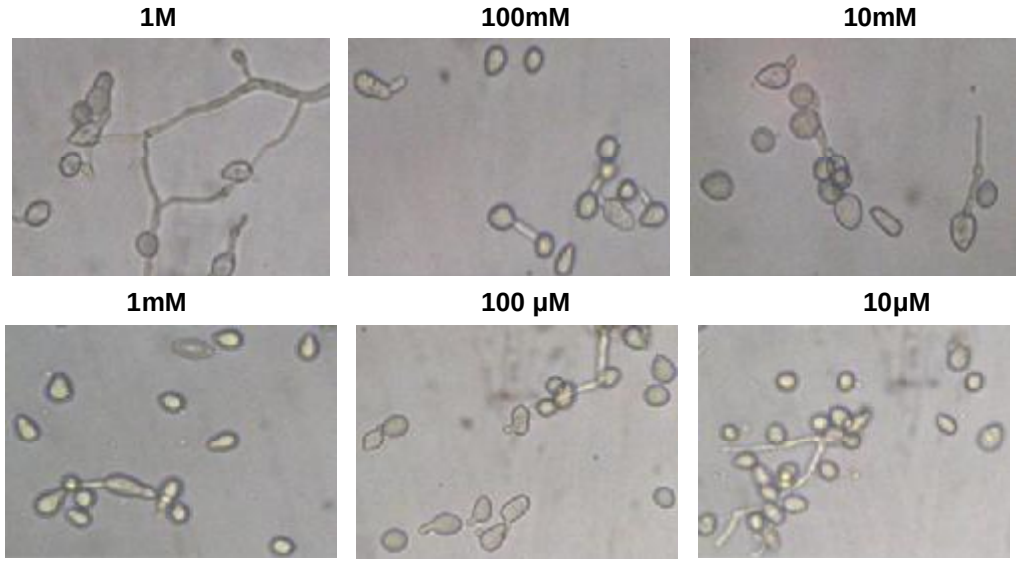


Figure 30. Conidial germination of *B. cinerea* in different concentrations of Magnesium after 40 hours of incubation at 200X.

Isolate: PBC3

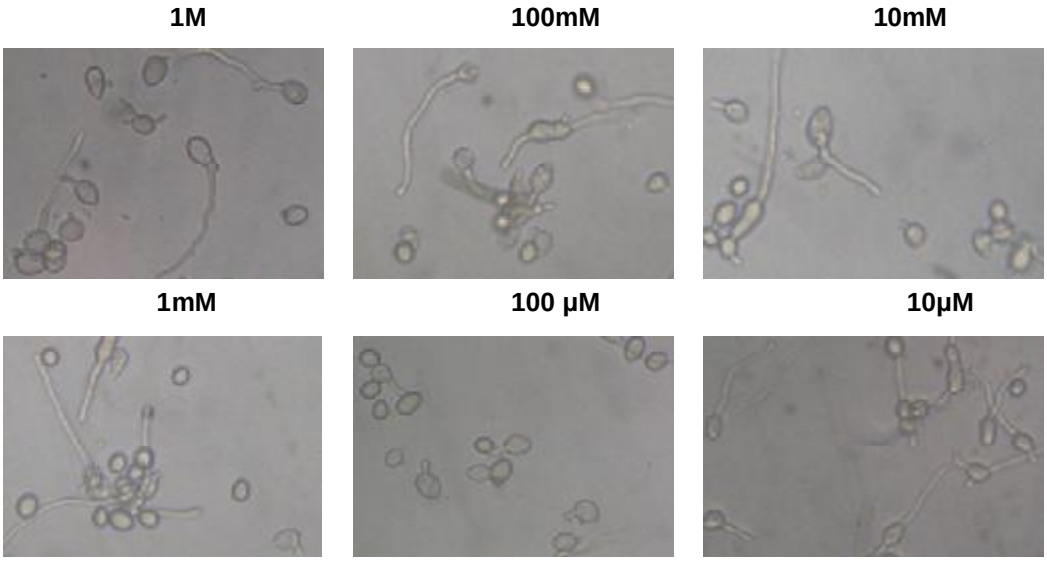


Figure 31. Conidial germination of *B. cinerea* in different concentrations of Potassium after 40 hours of incubation at 200X.

Isolate: PBC3

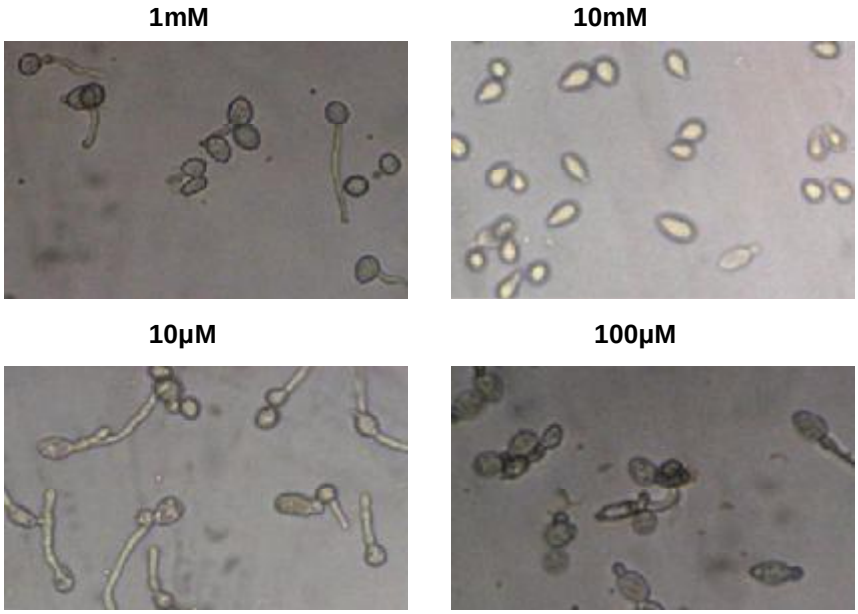


Figure 32. Conidial germination of *B. cinerea* in different concentrations of Ferrous after 40 hours of incubation at 200X mp.

Chapter Four. Discussion

The work in this thesis describes different physical, chemical, nutritional factors and surface properties that influence conidial germination of the fungal plant pathogen *B. cinerea*. The ability of fungi to adhere to and germinate on leaves and other substrata is well documented and is thought to represent an important early event in plant-microbe interactions (Braun and Howard, 1994; Jones, 1994). Spore germination in *Botrytis cinerea* follows a developmental sequence of spore swelling, localized outgrowth of the germ tube and subsequent polarized growth of the new hyphae.

Botrytis cinerea growth parameters in culture media

Potato dextrose agar medium fortified with bean leaves filtrate improved significantly growth of *B. cinerea in vitro* in terms of conidial germination and rate of sporulation when compared to PDA alone with no significant variation within isolates. Bean leaves filtrate has been widely used in *Botrytis* growth media as enhancer component for growth and sporulation. (Kokkelink, 2008; Doehlemann *et al.*, 2006; Schumacher *et al.*, 2008). Kepczytiska, (1993) has reported that colony diameter could reach 50 mm after 72 hours of incubation on PDA medium. In the same direction, Barakat and Al-Masri, (2005) reported that the average mycelium growth rate on PDA medium could reach (7.2 mm/day) under 20 ˚C.

Conidial concentration

Conidial germination rates of *B. cinerea*-isolates decreased with increasing spore concentrations without significant differences between isolates. At concentrations above 4×10^5 conidia/ml, conidia were unable to germinate and

appeared in clots. Sharrock, *et al.*, 2001 found that conidia of *B. cinerea* exhibit a self inhibition strategy during germination at high concentrations (10^6 conidia/ml) or more. It is assumed that at high concentrations, conidia tend to produce specific germination and/or growth inhibitors regardless of the richness of the substrate.

Kritzman, 1999 reported that spore germination of *Botrytis* was influenced by the spore concentration and the viscosity of the germination medium and mentioned an evidence for the existence of a germination-inhibiting factor which is produced in the germinating cultures.

Furthermore, several germination-self-inhibitors in other fungal species such as *Puccinia*, *Uromyces*, *Colletrotrichum*, *Dictyostelium*, *Fusarium*, *Aspergillus* were investigated and reports shown that they can be volatile or non-volatile (Allen, 1955; Bacon *et al.*, 1973; and Barrios-Gonzales, *et al.*, 1989). It was also concluded that self-inhibitors can affect other fungal processes, such as prevention of appressorium induction which make conidial germination unlikely to occur.

Age of conidia

Spore age could be another factor involved in early conidial germination in fungi. It was found that conidial germination was significantly reduced in older conidia (67% at 14days) compared to younger conidia (91% at 5 days) when germination was tested on a hydrophobic surface (Polypropylene), while no differences were noticed when germinating the spores in Fructose and GB5. This suggests that nutritional factors may mask the effect of age and older conidia can germinate as well as younger conidia if the growth substrate was supplied with a nutritional source. Using different germination conditions, Shirashi *et al.*, 1988 found that young *Botrytis* conidia, in general,

germinated well at 20°C compared to old conidia. Furthermore, using the same germination conditions, Kokkelink, 2008 (unpublished data) reported that conidia from various ages revealed almost similar germination rates in the presence of a nutritional supplement (i.e 10mM fructose).

Effect of temperature

The highest germination rate (93%) was obtained at 20 ºC; germination was almost zero at 5 and 30 ºC. Pathogenicity on detached bean leaves followed the same trend over the same temperatures tested (5-30 ºC); the highest disease severity was obtained at 20-25 ºC. Similar results were found by Tomioka *et al.*, 1999 using 3 different isolates of *Botrytis cinerea*. They observed very low germination rates (5%) at 5ºC. The temperature requirements for germination are usually in the same range as for growth, but the differences in the optimum for germination and growth maybe existing between or within fungal species (Griffin, 1994). Elad and Younis (1993) , however, found that germination and the infection process in *Botrytis cinerea* occur at a wide range of temperatures up to 25 °C. Furthermore, and in the same direction, the temperature range in which *B. cinerea* could germinate and grow *in vitro* under climate controlled chambers was 5-30 °C (Kerrssis A., 1994). In addition, flowers had more lesions of *B. cinerea* at temperatures of 20 and 25°C than at 10 and 15 °C (Kerrssis A., 1994).

Surface hydrophobicity

It was found that even in the absence of nutrients, conidial germination was efficiently induced by contact with a rigid hydrophobic surface; polypropylene surface coated with grease induced almost complete germination of B05.10 conidia (94%) after 18 hours.

Doehlemann *et al.*, (2006) using the same germination procedure reported high germination rates (91-99 %) on onion epidermis and polypropylene hydrophobic surfaces. Coertze *et al.*, (2001) has observed such high rates of *B. cinerea* conidia germination (90 to 98%) using different dry inoculation procedures after 6 hours of incubation. In the same direction, Kim *et al.*, 1998 reported that in the fungus *Colletotrichum gloeosporioides*, hard surface contact was required to prime conidia for the perception of plant signals that induce germination and appressorium formation. Furthermore, several researchers have reported similar findings for other plant pathogenic fungi such as *Colletotrichum graminicola* (Chaky *et al.*, 2001) and *Phyllosticta ampelicida* (Kuo and Hoch, 1996).

Microclimate pH

Conidial germination was significantly impaired by extreme pH ranges (below 6 and above 8). Conidia germinated well at pH ranging from 6-8 with the highest germination rate at pH=7. In this direction, fungi very often can dynamically alter the local pH to fit its enzymatic arsenal, with the level of pathogenicity being related to the efficiency of the pH change. (Prusky *et al.*, 2001).

Generally, *Botrytis cinerea* is classified among acidic fungi (Prusky and Yakoby, 2003) and similar to other pathogenic fungi, such as *Penicillium expansum*, *P. digitatum*, *P. italicum*, and *Sclerotinia sclerotiorum* that use tissue acidification in their attack (Vautard and Fevre, 2003). This investigation, however, was restricted to the conidial germination *in vitro*.

The ability of *B. cinerea* to germinate at various pH values emphasizes the previous findings stating that *Botrytis* spp. change the medium or site pH to facilitate the enzymatic activities.

Sugars

Germination of *Botrytis cinerea* conidia was stimulated in the three different sugars (fructose, sucrose and glucose) at various concentrations compared to SDW. Almost all sugars have induced full germination (100%) after 24 hours of incubation at the highest concentration used (10mM) knowing that the concentration (100 µM) was the (breaking point) for all sugars to induce significant increase in conidial germination. Addition of sugars at relatively low concentrations (i.e 10mM) also induced early swelling of conidia and enhanced early germ tube branching. It has been shown that Fructose induced germination of *B. cinerea* conidia more efficiently than any other monosaccharide (Blakeman, 1975). However, in this *in vitro* germination assay on glass surfaces, it was found that sucrose was the best to enhance germination of conidia. Germination induction by sugars was concentration dependent, and fructose was more effective than glucose. Of the sugars, fructose has been pointed out as the best inducer of germination in *B. cinerea*, being more effective than glucose and other hexoses or disaccharides (Blakeman, 1975).

One explanation for the particular important activity of fructose in conidial germination could be that this sugar is preferentially taken up by a fructose-specific transport system. This is surprising since glucose is usually the most efficient hexose not only as a nutrient, but also as a signalling compound (Doehlemann *et al.*,2005).

Using almost the same protocol for germination, Doehlemann, *et al.*, 2006 found similar results after incubation for 24 hours. Induction of conidial germination by nutrients, in particular sugars, is well known in saprotrophic fungi (Osherov and May, 2000). In rich media, most fungi germinate

quickly, including phytopathogens such as *F. solani*, *C. graminicola* and *C. gloeosporioides* (Ruan *et al.*, 1995; Chaky *et al.*, 2001; Barhoom and Sharon, 2004).

The mechanism of sugar sensing by *B. cinerea* conidia is unknown. As diverse sugars and acetate induce germination with similar efficiency, it appears unlikely that nutrient sensing occurs by plasma membrane proteins (Forsberg and Ljungdahl, 2001).

Amino acids

The effect of amino acids on conidial germination was not fully clear in this study. While Tryptophan and Nicotinic Acid showed no influence, Proline weakly enhanced germination (39%) compared to the control. Alanine on the other hand, moderately increased germination (29-88%) but ofter longer period of incubation (47 hours). Barakat and Almasri, 2009 (unpublished data) reported that Tryptophan have no influence on germination even at high concentrations (i.e 1M). They found that Alanine inhibited germination at concentrations above 600µg/ml whilst Proline had no significant influence on germination at concentrations below 1000 µg/ml with variations in germ tube lengths.

Furthermore, Joseph *et al.*, 1975 reported that high levels of readily metabolizable compounds such as glutamic acid, proline, trehalose and polyols have been found in dormant spores of *Neurospora crassa*. He suggested that spores contain endogenous storage compounds that are used for the initiation of germination. and that the primary effect of many of the organic and inorganic initiators may be to activate the metabolism of these storage compounds.

Cations and nitrogen forms

From looking at the results, it was obvious that the tested cations (Ca^{2+}, Mg^{2+}, K^{+}, and Fe^{2+}) had no influence on conidial germination at a wide range of concentrations (0.001-1mM). However, at high concentrations (>10mM), germination declined sharply, especially with Fe^{2+} which suggests a level of toxicity induced at high concentrations. It is very likely that conidia before germination is not affected at low concentrations of cation availability in the growth substrate. However, after germination, germ tube growth becomes more sensitive to a wide range of cation concentrations in the growth media. However, Fe^{2+} seems to provide an important nutritional source for germ tube growth at low concentrations (0.001 M). Barakat and Almasri, 2009 (unpublished data) found that at high concentrations (i.e. 1M) all these cations inhibited germination of *Botrytis* conidia and the level of toxicity varied between isolates. Shirani and Hatta (1987), found that at the concentration (5×10^4 conidia/ml) conidial germination of *Botrytis cinerea* was optimum (100%) in the presence of Ca^{2+} ($CaCl_2$) and was relatively high (66%) in Mg^{2+} ($MgSO_4$) at the concentrations (0.1-0.7 g/liter).

Conidial germination responded almost similarly to nitrogen forms. While N-forms had no influence on germination, germ tube growth and elongation responded positively with increasing concentrations of both forms. This suggests that conidia may depend more on available energy inside the spore to germinate but after germination, germ tube growth greatly depend on nutritional elements available in the growth substrate.

Conclusions

It is very difficult at this stage to draw up strong conclusions about *B. cinerea* germination and infection; however, several points can be highlighted:

- ⇒ Potato dextrose agar amended with bean leaves extract improved significantly growth and spore production of *B. cinerea in vitro* with no significant variation within isolates.
- ⇒ Germination-self-inhibition is associated with increased spore concentrations above (4.5×10^5 conidia/ml) tested *in vitro* without significant differences between isolates.
- ⇒ Spore age was not significant germination in rich medium.
- ⇒ The optimum temperature requirements for germination (20-25 ˚C) was in the same optimum range for growth and pathogenicity.
- ⇒ Conidial germination is efficiently induced by contact with a rigid hydrophobic surfaces.
- ⇒ Conidial germination was significantly impaired by extreme pH values (below 6 and above 8).
- ⇒ Conidial germination is strongly induced (>90% after 24hours) in the presence of fructose and sucrose at 10mM concentration.
- ⇒ The cations (Ca^{2+}, Mg^{2+}, K^{+}, and Fe^{2+}) had no influence on conidial germination at a wide range of concentrations (0.001-1mM), while ferrous was found to be supporting germ tube growth and elongation.
- ⇒ Conidial germination responded similarly to nitrogen forms with no visible effect influence on germination, but germ tube growth and elongation responded positively with increasing concentrations of N-forms.

⇒ The results and data of this study have definitely shed some light on early events in germination and infection of *B. cinerea*, but further investigations are still needed at the molecular level to delineate the cascades of the "early events" in pathogenesis of this important plant pathogen.

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Appendices

Appendix 1. Conidial germination of *B. cinerea* (B05.10) under different incubation temperatures in 10mM Fructose solution

Temperature (°C)	5	10	15	20	25	30
Fructose concentration	**(%) Germinated conidia**					
1µM	0h	1h	3 h	50de	3h	0h
10µM	0h	2h	9g	72b	3h	0h
100µM	0h	4h	15g	85a	33f	0h
1mM	0h	8gh	40 ef	90a	33f	0h
10mM	0h	17g	58cd	93a	62bc	0h

- Means followed by the same letter in the same column and raw are not significantly different at LSD ($\alpha = 0.050$). n=4.

Appendix 2. Conidial germination of *Botrytis cinerea* in different concentrations of Fructose, Sucrose and Glucose on after 5 and 24 hours.

- After 5 hpi

	Fructose			Sucrose			Glucose		
	B. cinerea isolates			*B. cinerea* isolates			*B. cinerea* isolates		
	PBC3	PBC1	B05.10	PBC3	PBC1	B05.10	PBC3	PBC1	B05.10
SDW	1mn	0 n	0 n	0 n	0 n	1mn	0 n	0 n	0 n
GB5	4klmn	1mn	0n	4klmn	1mn	0n	4klmn	1mn	0n
1µM	6 jklmn	0 n	2 lmn	1mn	1mn	2 lmn	0 n	0 n	0 n
10µM	22 hi	2 lmn	8 jk	10j	7 jkl	7 jkl	0 n	0 n	0 n
100µM	21 i	2 lmn	6 jklmn	41e	53 d	35 f	3 klmn	3 klmn	0 n
1mM	27 gh	6jklmn	7 jkl	79b	87 a	80 b	6 jklmn	6 jklmn	1mn
10mM	59 c	29 g	38 ef	75 b	87 a	75 b	17 i	18 i	5 jklmn

- Means followed by the same letter in the same column and raw are not significantly different at (LSD =5.828, n=4).

- After 24 hpi

	Fructose			Sucrose			Glucose		
	B. cinerea isolates			*B. cinerea* isolates			*B. cinerea* isolates		
	PBC3	PBC1	B05.10	PBC3	PBC1	B05.10	PBC3	PBC1	B05.10
SDW	2 jk	0 k	1 k	1 k	0 k	2 jk	0 k	0 k	0 k
GB5	5jklmn	5jklmn	1k	5jklmn	5jklmn	1k	5jklmn	0jk	1k
1µM	10 hijk	0 k	2 jk	4 jk	2 jk	3 jk	0 k	0 k	0 k
10µM	33 ef	3 jk	8 ijk	19 gh	19 gh	15 hi	0 k	0 k	0 k
100µM	27 fg	2 jk	9 hijk	61 d	73 c	54 e	4 jk	12 hij	1 k
1mM	38 ef	6 ijk	8 ijk	95 a	96 a	92 ab	43 e	58 d	15 hi
10mM	83 bc	35 ef	57 d	100 a	93 ab	93 ab	98 a	100 a	91 ab

- Means followed by the same letter in the same column and raw are not significantly different at (LSD =10.168, n=4).

Appendix 3. ANOVA tables

Experiment	**DF treat.**	**DF resid.**	**DF total**	**SS treat.**	**SS resid.**	**SS total**	**MS treat.**	**MS resid.**	**F**
Mycelium growth rates of *B. cinerea* grown on two types of media	5	18	23	364.5	38.3	402.8	72.9	2.1	34.3
Sporulation of *B. cinerea* isolates under fixed conditions	5	48	53	9.9	1.4	11.3	1.9	0.03	67.4
Influence of spore concentration on germination of *B. cinerea* conidia	11	24	35	19177.6	254.7	19432.3	1743.4	10.6	164.3
Influence of spore age on germination of *B. cinerea* conidia	4	13	17	25.6	76	101.6	6.4	5.8	1.095
Time vs. germination of *B. cinerea* conidia	17	36	53	75882.8	604	76486.8	4463.7	16.8	266.1
Influence of temperature on germination of *B. cinerea* conidia	11	36	47	54129.2	3131.8	57260.9	4920.8	86.9	56.6
Influence of temperature on pathogenicity of *B. cinerea* isolates	17	153	170	22708.3	1120.5	23828.8	1335.8	7.3	182.4
Influence of sugars on germination of *B. cinerea*-isolates B05.10, PBC3 and PBC1	63	192	255	267311.3	8643.8	275955.1	4243	45	94.3
Influence of amino acids on germination of *B. cinerea*-isolates B05.10, PBC3 and PBC1- Proline, Tryptophan and Nicotinic acid	63	192	225	16587.2	2483.3	19070.5	263.3	12.9	20.4
Influence of the amino acid L-Alanine on germination of *B. cinerea*-isolates B05.10, PBC3 and PBC1	14	45	59	4809.7	3548.3	51640.9	3435.2	78.9	43.6
Influence of microclimate pH on germination of B05.10 conidia.	15	32	47	8352.667	941.333	9294.000	556.8	29.4	18.9
Influence of surface hydrophobicity on germination of *B. cinerea*	4	15	19	332.3	108.3	440.6	83.1	7.2	11.5
Influence of cations (Ca, Mg, Fe, K) on germination of *B. cinerea*	25	78	103	103705	7202.7	110907.5	4148	92	44.9
Influence of cations (Ca, Mg, Fe, K) on germ tube elongation of *B. cinerea*	38	117	155	16143	4636.2	20779.6	424.8	39.6	10.72
Influence of Nitrogen forms (NO3, NH4) on germ tube elongation of *B. cinerea*	13	112	125	9250.4	38945.9	29695.5	2284.3	82.6	27.7

الملخص

العوامل المؤثرة على إنبات الأبواغ والعدوى بفطر البوترايتس ساينيريا

إعداد

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إشراف

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يعتبر فطر البوتريتس أحد الفطريات الرمية والممرضة للنبات. وبسبب أهمية هذا الفطر اقتصاديا دعت الحاجة إلى دراسة وبائية المرض واثر العوامل العديدة التي تتحكم في بيئة الإصابة المبكرة على أسطح العوائل المختلفة. أشارت النتائج إلى أن بيئة الـــ PDA المدعمة بمستخلص أوراق الفاصولياء أثرت بشكل دال إحصائيا على نمو الفطر وقدرته على إنتاج الأبواغ. كما لم تشر النتائج لوجود اثر للأعمار المختلفة للأبواغ (5-14 يوما) على الإنبات في محلول الفركتوز بتركيز 10 مليمول. بالإضافة لذلك، لوحظ أن التثبيط الذاتي للإنبات يرتبط بقوة بتركيز الأبواغ المـــــستخدمة (أعلى من 2.5×10^4 سبور/مل) دون ان يكون هناك فروق معنوية بين العزلات المستخدمة. أما بالنسبة لأثر الحرارة (5 و10 و 15 و 20 و25 و 30 م°)، فقد دلت النتائج أن الإنبات يبدأ على 10م° وكان أعلى ما يمكن على درجة حرارة 20 م° في حين توقف بالكامل على درجة حرارة 30 م°. إن أعلى انتشار للعدوى كان على درجات حرارة (20_25 م°). لقد أشارت النتائج أيضا الى انه حتى بدون إضافة المغذيات، تم تحفيز الإنبات بشكل واضح (88-96%) على الأسطح الصلبة الطاردة للماء. وعن اثر درجة الحموضة فقد دلت الدراسة أن إنبات الأبواغ يقل بشكل واضح بفعل مستويات الحموضة المتطرفة الأقل من 6 والأكبر من 8. كما أشارت النتائج إلى أن إضافة

السكريات بتركيز اكبر من 100 مليمول تحفز الإنبات بشكل كبير(أكثر من 90% بعد 24 ساعة) في حين ان ايونات الكالسيوم، والمغنيسيوم، والبوتاسيوم لا اثر لها على إنبات الأبواغ بالتراكيز المستخدمة (0.001-1 مليمول). وبنفس التراكيز المستخدمة لوحظ أن الإنبات يكون متماثلا وغير دال إحصائيا في أشكال النيتروجين غير العضوي المستخدمة لكن هذه المواد أثرت و بشكل ايجابي على نمو واستطالة أنبوبة الإنبات بزيادة التراكيز المستخدمة.

بسم الله الرحمن الرحيم

جامعة الخليل

كلية الدراسات العليا والبحث العلمي

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