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Manzoor Ali Abro

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GENERAL INTRODUCTION

In recent years the importance of combining agricultural productivity and sustainability has risen to become one of the most important issues in agriculture. Meanwhile, plant diseases continue to represent a major limiting factor in agricultural production (Dordas, 2008).. The control of plant diseases using classical pesticides has raised serious concerns about food safety, environmental quality and pesticide resistance, which have dictated the need for alternative pest management techniques and their combined use in the framework of integrated protection (Atkinson and McKinlay, 1997). One such alternative could be a rational use of fertilization to reduce the susceptibility of a crop to its pathogens or to improve the efficacy of different control methods. Mineral elements are routinely applied to boost crop yields and improve over all plant health and quality. They also have marked effects on numerous plant diseases, and in certain situations, their rational use can reduce disease to an acceptable level, or at least to a level at which further control by other cultural practices or conventional organic biocides is more successful and less expensive (Huber and Watson, 1974)

Among all elements, nitrogen (N) is by far the most extensively reported mineral nutrient affecting plant disease, it is also the nutrient element applied in the largest quantity and the most frequently deficient in cultivated soils (Datnoff et al 2007). A commonly accepted generality is that high N fertilization tends to increase disease (Huber and Thompson, 2007); however, this generality fails to take into account the effects of the rate of application, time of application, form of N, soil conditions and interaction with other elements.

Botrytis cinerea, the causal agent of grey mould can infect more than 200 plant species including tomato and lettuce which are economically important crops (Jarvis, 1992;

Williamson et al., 2007). Due to its broad host range this pathogen can cause huge losses, although the specific costs of damage are difficult to estimate (Dean et al., 2012). The fungus is considered as a typical necrotroph, which co-opts programmed cell death pathways in the host to achieve infection (van Baarlen et al., 2007)

Greenhouse tomatoes are susceptible to a wide variety of diseases and pests. Among all diseases, grey mould is one of the most important (Bardin et al., 2008; Nicot and Baille, 1996). Infections of the pruning wounds by *B. cinerea* result in stem cankers that can rapidly kill the plants (Decognet *et al*., 2010). On lettuce, a crop that is often grown in rotation with tomato in unheated greenhouses, grey mould is also a common diseasewhich can reduce both yield and quality Affected leaves are inedible and need to be removed at harvest. The worst case is when the lettuce is attacked at the base and slimy, yellowish red-brown rot forms, which can the kill the entire plant (Jarvis, 1992; Williamson et al., 2007).

On both crops, fungicides are widely used to control grey mould but due to limited number of fungicides available and the possible emergence of resistant strains complicating their use (Elad et al., 2004; Leroux, 2004), much research is dedicated to developing alternative control methods. A possible alternative could be the use of biocontrol agents, . Another possible alternative to reduce disease severity might be through the manipulation of plant fertilization. Availability of nutrients is known to affect plant disease development (Dordas, 2008; Walters and Bingham, 2007). Among common nutrients, nitrogen appeared to have the greatest effect on plant susceptibility to plant pathogens (Huber and Thompson, 2007). In case of *B. cinerea* N fertilization of the host plant influences the susceptibility of several crops against this pathogen (Dik and Wubben, 2004b; Yermiyahu et al., 2006).

Moreover, sporulation is very important for the reproduction and spread of *B. cinerea*, as it plays a key role in the development of epidemics, as this pathogen can produce millions of spores on diseases tissues within one week after inoculation especially in favorable

conditions, that are easily disseminated to large areas and thus play a key role in epidemics of grey mould (Nicot et al., 1996). Suppression of *B. cinerea* sporulation is a valuable component in *Botrytis* blight management in commercial greenhouses, because reduction in sporulation may lead to retardation of an epidemic (Morandi et al., 2003). So there is need to find a control measures how to reduce the sporulation of this fungus and N fertilization may be used to overcome this problem. Several studies have shown that high N fertilization increased the sporulation of *B. cinerea* on sweet basil (Yermiyahu et al., 2006). But little is known of possible effect of N fertilization on sporulation of the *B. cinerea* on tomato diseases tissues and on the pathogenicity of resulting secondary inoculums.

Biological control has gained increasing interest, but a lower and less efficacy as compared to pesticides, is often cited as one of the disadvantage of biocontrol agents and their wide use by growers (Nicot 2011). In order to enhance the biocontrol efficacy against fungal pathogens certain strategies such as adding various compounds like calcium salts, carbohydrates and amino acids were used to enhance the efficacy of biocontrol agents (El-Ghaouth et al., 2002; Janisiewicz et al., 1992). In addition, In vitro conditions studies have shown that the antagonistic activity of *Trichoderma harzianum* was stimulated against *Sclerotium rolfsii* in the presence of three nitrogen sources (Khattabi et al., 2004). According to our knowledge there are no studies on the possible role of N fertilization on the efficacy of biocontrol agents against *B. cinerea*.

Objectives of the thesis work:

The different objectives of the present work are the following:

1: The first objective was to evaluate the effect of different levels of N fertilization on the susceptibility of tomato plants to six strains of *B. cinerea* widely different in their

aggressiveness and to test the influence of initial inoculum density and fungal strain on the growth response of the fungus, both *in vitro* and *in planta*.

2: The 2nd objective was to evaluate the effect of different regimes of N fertilization on the susceptibility of lettuce to the same six strains of *B. cinerea* which were tested before on the tomato plants and to test whether the N fertilization effect on severity of disease was linked to the plant N status or was related to the main plant sugars. What is the link between the concentration of primary plant components at the time of infection and the severity of disease?

3: The 3rd objective was focused on the sporulation of *B. cinerea* on diseased plants and the aggressiveness of the secondary inoculum. The specific objectives were (1) to assess the effects of N nutrition of tomato on sporulation of *B. cinerea* on diseased tissues and on the pathogenicity of the resulting secondary inoculum, and (2) to correlate these effects with modifications in plant tissue composition.

4: The 4th objective of the study was to investigate a possible effect of N fertilization on the efficacy of two biocontrol fungi, *Microdochium dimerum* and *Trichoderma atroviride*, shown in previous work to provide protection of tomato pruning wounds against *B. cinerea*. To understand the mechanisms at cellular and histological level the final objective of this study was (1) is there effect of N fertilization on the integrity of *B. cinera* mycelim? (2) N fertilization has any effect on the mycelial structure of two BCA and ultimately on the efficacy of biological control agents *T. atroviride* and *M. dimerum* through cyto-histological techniques by means of light microscopy?.

Chapter 1

Review of literature

1. Botrytis cinerea: causal agent of grey mould:

B. cinerea is one of the most important plant pathogenic fungi, being the responsible of major crop losses on more than 200 plant species in temperate and subtropical regions worldwide. Based on its scientific and economic importance *B. cinerea* the causal agent of grey mould has been recently ranked as number 2 among the top 10 fungal pathogens in molecular plant pathology (Dean et al., 2012). It is a ubiquitous fungus which causes grey mould on many economically important crops including vegetables, ornamentals and fruits (Jarvis, 1992; Williamson et al., 2007). It can attack many organs including leaves, stems and fruits as a nectrotroph, often with heavy losses after harvest (Elad, 1997b). *B. cinerea* infects stem wounds of greenhouse tomatoes and can cause serious economic losses (Eden *et al*., 1996),infections of the pruning wounds result in stem cankers that can rapidly kill the plants (Decognet *et al*., 2010). In a study of 15 greenhouses in the south of France, (Nicot and Baille, 1996) showed that the incidence of *B. cinerea* between May and June 1991 ranged from 32-100% and plant mortality reached 46% in some greenhouses. In a larger survey carried out in the same area in late April 1993 (Terrentroy, 1994) showed that *B. cinerea* attacks were found in 58 of 73 tomato greenhouses studied and partial mortality of plants by the fungus was recorded in 31 of these greenhouses.

This pathogen can cause the partial or total destruction of the host plant and in some cases the whole crop. Economically, this fungus is considered such a major pest problem in viticulture worldwide (Martinez *et al.*, 2005). Estimated global losses due to *B. cinerea* on alone grapevine to $ 2 billion per year (Elmer and Michailides, 2004a). In addition, the rapid and insidious *B. cinerea* produces the annual destruction of crops on hundreds of hectares of vines (Bolay and Pezet, 1987). Estimated losses for vineyards in France amount to 15-40% of crops depending on weather conditions. In Champagne, infection rates can reach 15 to 25%

depending on the year (Cilindre *et al*., 2007). In protected corps, for example, tomatoes, peppers, lettuce and strawberries the risk of attack by this fungus is always standing on (Jarvis, 1992).

1.1 Taxonomic position:

Botrytis has been recognized as a genra by Micheli in 1729 where he was listed in his book " *Nova Plantarum Genera*." At the beginning it was sometimes confused with *Sclerotinia spp*. but details were made by (Smith, 1900) and the confusion was resolved by (Whetzel, 1945) in 1945. The genra, redefined by (Hennebert, 1973) and includes 22 species, most of which have a restricted host range, such as *B. tulipae* attacking on tulips, *B. fabae* on beans or *B. squamosa* on onion (Hennebert, 1973). In contrast, *B. cinerea* is ubiquitous and there are many species of plants on which it can cause serious damage before and after harvest. *Botrytis cinerea* name was given in 1801 by Persoon a pathogen of the vine. This fungus like many others experiencing double classification:

- Perfect form (teleomorph) *Botryotinia fuckeliana* (Barry) Wetzel. This is an Ascomycete, class of Discomycetes, the order of Leotiales and family of Sclerotiniaceae.

- An imperfect (anamorph), *Botrytis cinerea* Pers. This is a Deuteromycete the class of Hyphomycetes, order of Moniliales and family of Moniliaceae.

It is de Bary (1866) which established a genetic relationship between *Botrytis cinerea* Pers., asexual organism and *Botryotinia fuckeliana* originally called *Peziza fuckeliana*, sexual organism. Drayton and Groves (1939) observed for the first time, *in vitro*, the formation of apothecia by *B. cinerea* confirming the systematic relationship between the two forms of the fungus. However, it is the name of *B. cinerea*, widely known by mycologists and plant

pathologists was retained while generally the scientific name of a fungus is given by its sexual form.

1.2. Life cycle of the pathogen:

During its life cycle, *B. cinerea* can produce mycelium, asexual spores (conidia), sexual spores and sclerotia. The mycelium of *B. cinerea* includes articulated filaments, grayish or olive-colored, cylindrical, sometimes vesicular at the central partition, whose diameter varies considerably depending on the conditions for the development of hyphae (Faretra and Antonacci, 1987; Faretra et al., 1988). When the mycelium is at the stage of fruit, it produces clusters of gray conidiophores. Sometimes this method of multiplication may disappear and give way to a white mycelial growth which corresponds to the elongation of hyphae slender, hyaline that spread in the form of web (Beever and Weeds, 2004).. The mycelium can be stored in plant debris from the previous crop. When conditions become favorable *B. cinerea* grows to give conidia. The development of conidia is characterized by the production of conidiophores erect in tufts that often extended, forming an intense gray chain. Their release is favored by a humid climate, and then they are transported by wind, rain and insects (Holz *et al*., 2004). When conditions become unfavorable to the development of mycelium and conidia sclerotia are formed. They consist of aggregated white mycelium (Coley-Smith *et al*., 1980).. As young get older, they become hard and black. They are composed of a thick cortex of cells forming thin barrier pseudo-parenchymal cells and a large central medulla composed of filamentous hyphae. In spring, the sclerotia germinate and produce mycelium or conidia (Beever and Weeds, 2004; Delcan and Melgarejo, 2002; Faretra et al., 1988)

The fungus overwinters as sclerotia or as mycelium in plant debris and may be seedborne as spores or mycelium in a few crops. Other crops may also serve as sources of the pathogen and are likely to cross-infect. Conidia are airborne and may also be carried on the surface of splashing rain drops (Williamson et al., 2007). In the field, spores landing on

tomato plants germinate and produce an infection when free water from rain, dew, fog, or irrigation occurs on the plant surface (Fig.1). Dying flowers are a favorable site for infection, but infections can also result from direct contact with moist infested soil or plant debris. In the greenhouse, stem lesions develop either by direct colonization of wounds or through infected leaves. The presence of external nutrients, such as pollen grains in the infection droplet, can markedly increase infection (Elad et al., 2004).

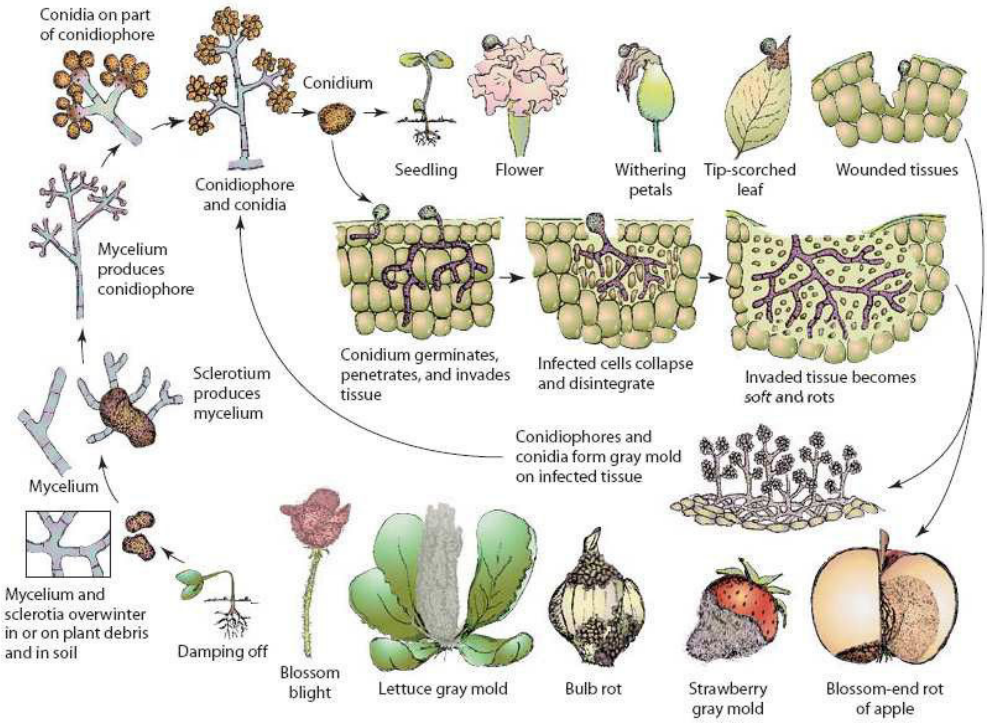

Figure1: Life cycle of *Botrytis cinerea* on different crops (Agrios, 2005).

1.3. Pathogenicity and host range:

B. cinerea produces a range of cell-wall-degrading enzymes, toxins and other low-molecular-weight compounds such as oxalic acid. This fungus secrete a variety of compounds among which oxalic acid, peptidases, and a pool of toxic metabolites(Williamson et al., 2007). These compounds allow the pathogen to modify the host redox status, perturb the host defence, alter

the cell integrity and macerate the plant tissues (Alghisi and Favaron, 1995; Godoy et al., 1990; Riou et al., 1991).

B. cinerea has the vide host range of host plants, over 200 mainly dicotyledonous plant species and can cause grey mould on different plant organs, including flowers, fruits, leaves, shoots and soil storage organs (i.e. carrot, sweet potato), Vegetables (i.e. cabbage, lettuce, broccoli, beans) and small fruit crops (grape, strawberry, raspberry, blackberry) are most severely affected (Jarvis, 1992; Williamson et al., 2007). Culture of plants out-of-season in heated or unheated greenhouses and under plastic tunnels used increasingly to supply fruits, vegetables, herbs and flowers in northern latitudes greatly increases the risk of infection, especially in tomato, lettuce, cucumber and sweet pepper (Jarvis, 1989).

1.4. Symptoms:

B. cinerea is responsible for a very wide range of symptoms. The most characteristic symptom is a grey-brown furry mould, which are masses of spores of the grey mould fungus, covering the infected area. When shaken, clouds of spores are released from these infected areas. On tomato, the infected areas can expand rapidly covering whole stems, leaves or petals. Stem infections can girdle the whole stem and cause wilting of the plants above the infected area (Fig. 2). On lettuce a quick-spreading grey mould appears on lettuce leaves, which renders them inedible. If the fungus strikes at the base of the plant, it turns yellowish-brown and becomes a slimy rot (Williamson et al., 2007).

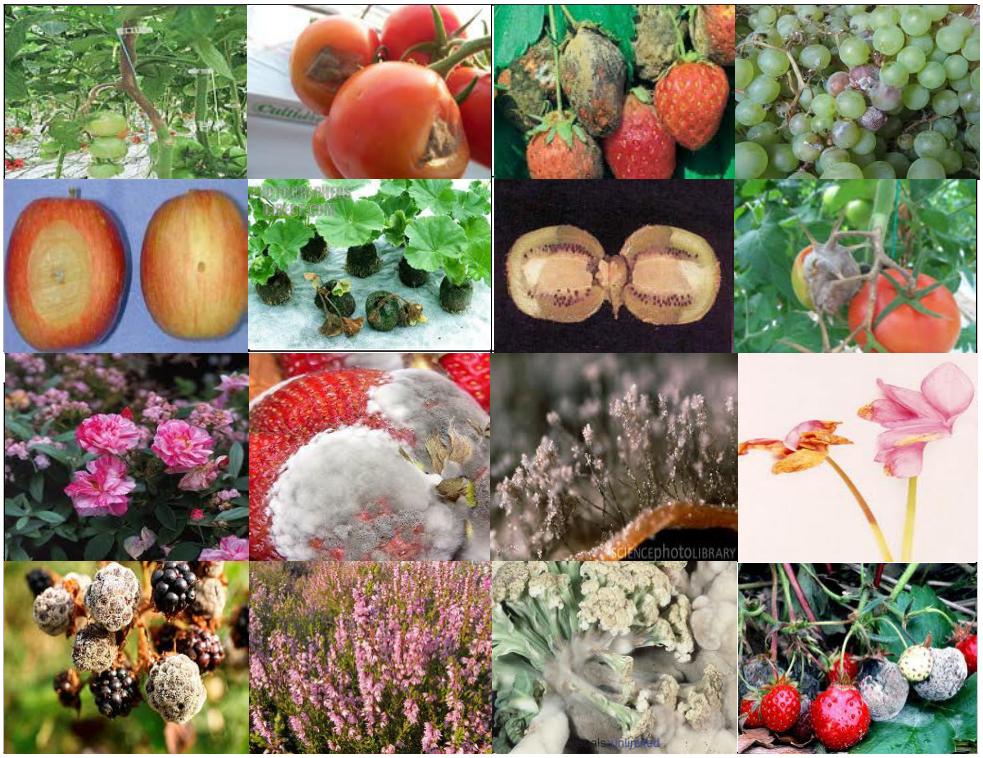

Figure 2: Symptoms development of *Botryts cinerea* on different host plants.

1.5. Factors affect the development of the pathogen:

There are so many factors that greatly influence the development of the *B. cinerea* during the early stages of infection, the disease development and sporulation of the fungus (Elad and Yunis, 1993; Yunis *et al*., 1990) For example, nutrient requirements, light, climatic factors etc.

1.5.1. Nutrient requirements:

Among other factors, nutrient requirement of the pathogen play a key role in the development of the pathogen. Because of the lack of endogenous energy reserves, the conidia of *B. cinerea*, require an exogenous source of nutrients to germinate (Yoder and Whalen, 1975). According to (Blakeman, 1975), the presence of both carbon and nitrogen is necessary. The presence of nutrients such as glucose and fructose promotes germination and elongation of the germ filament and allow the elderly to regain their conidia germination

(Clarck and Lorbeer, 1977). Thus the addition of sucrose, maltose, lactose, mannose, galactose or xylose stimulates the germination of conidia of *B. cinerea* (Shiraishi *et al*., 1970). Spraying onion leaves with conidia of *B. cinerea* suspended in the water does not result in injuries but the addition of a source of nutrients (sugars and minerals) leads to the germination of spores and leads to the formation of lesions (Clarck and Lorbeer, 1977). The molecular mechanisms of induction of germination by carbon sources were studied in detail by (Doehlemann *et al*., 2005; Doehlemann *et al.*, 2006)

Nutrients are also required for the infection process of the pathogen and can be provided in the form of glucose, leaf extract of cabbage, PDA medium) or injury to the point of inoculation. The concentration of nutrients influences the ability of *B. cinerea* to cause rotting of tissues (Yoder and Whalen, 1975). Nutrients also play a key role in the sporulation of the *Botrytis*. For example, (Maas and Powelson, 1972) demonstrated that *B. convoluta* sporulate abundantly on glucose, sucrose, and fructose media as compared to maltose, galactose and starch media, sporulation was also increased by the casein hydrolysate, asparagine, ammonium tartrate, and ammonium sulfate, less profuse with glutamine and potassium nitrate, and absent with glycine, urea and no nitrogen.

1.5.2. Effect of light:

Light is an important factor to consider for the control of *B. cinerea* in greenhouse crop protection. Germination of conidia of *B. cinerea* occurs better in light as compared to darkness, on the condition there is water and nutrients provided in sufficient quantity (Blakeman, 1980). (Nicot *et al*., 1996) showed that the germination of spores of *B. cinerea* on PDA medium has no difference when the spores were placed under a selective ultraviolet filter film (UV) or when placed in a film that does not absorb UV. Sporulation of *B. cinerea* is dependent on the quality of the light received and especially UV (Elad, 1997a; Nicot et al.,

1996; West et al., 2000). Spore production in a selective filtering ultraviolet film in a petri dish represents 0.05% of the spores under a non-control film for several weeks after inoculation. This demonstrates that the absence of UV inhibits sporulation rather than delay it (Nicot *et al*., 1996). Finally, it appears that some isolates are able to sporulate in the dark and are not affected by the quality of light (Dik and Wubben, 2004b). (Nicot *et al*., 1996) estimate that more than 15 million spores are produced in 7 days on a tomato stem segment of 2 cm and that reduces sporulation in a radical way to wait only 6 to 18,000 spores in a selective UV-filtering film. In greenhouse crops, the light filtering using a polyethylene film of green or pink and can inhibit sporulation of *B. cinerea* from 35 to 75%, respectively (Elad, 1997a). The spread of symptoms associated with this pathogen could be effectively limited by the effective management of light in protected crops, due to the reduction of spore production (Sutton, 1983; Sutton and Peng, 1993). The survival of conidia in the air is also influenced by the quality of light.

1.5.3 Climatic factors:

Environmental conditions, especially relative humidity and temperature play a key role for plant infection by *B. cinerea* and development of the disease. *B. cinerea* can thrive under a range of temperatures between 2 and 30oC (Elad and Yunis, 1993). The optimum temperatures for the different growth phases are range from 12–30 ^{o}C. *B. cinerea* will therefore always be a potential threat in greenhouse crops. Other studies have also evaluated the temperature requirements for the spores of *B. cinerea* germination. (Jarvis, 1977) reported that conidia of *B. cinerea* germinate to 100% at 20 °C, 15 ^{o}C and 5 ^{o}C with 100% humidity.

A 95% relative humidity, only 80% of conidia of *B. cinerea* germinate at 15 ^{o}C and 5 ^{o}C, however100% of the spores germinate at 20 ^{o}C. At 90% relative humidity, 85% of conidia germinated at 20 ^{o}C, and germination is stopped when the relative humidity and

temperature are lower (Jarvis, 1977). Under conditions of relative humidity of about 80 to 100%, the incidence of the disease is more severe on cucumber in dry condition (Yunis *et al*., 1990). The aerial mycelium and sporulation develop a more rapid at 21 ^{o}C, 94% relative humidity (Thomas and Marois, 1986). An area of open water or high relative humidity (95%) even seems necessary for conidial germination and penetration of germinal filaments, and the success of the infection (Williamson *et al*., 1995). According to (O'Neill *et al*., 1997), the sporulation phase is favored by high relative humidity and the interruption of these conditions causes a delay in sporulation.

2. Strategies for protecting plants against *B. cinerea*

2.1. Cultural control

Cultural methods that ensure ventilation and drying of plant canopy after rain, whilst maintaining adequate water supply to the roots, are the most effective means developed so far for prevention of *Botrytis* epidemics (Elad and Shtienberg, 1995). Increasing public awareness of some potential drawbacks of chemical fungicides was addressed by the development of alternative control measures making use of microbial antagonists that are capable of disease suppression (Dubos, 1992). Cultural measures can be a powerful means to suppress plant diseases in greenhouses where the value of crops is high and the farmers make considerable efforts during long cropping seasons. Such measures are usually aimed at altering the microclimate in the canopy and around susceptible plant organs, prevention of inoculums entrance into the greenhouse and its build up and rendering the host plants less susceptible to diseases. Calcium loading of plant tissues and alteration of nitrogen fertilization reduce susceptibility to *Botrytis* (Elad and Shtienberg, 1995a). Cultivars resistant to *B. cinerea* are not available. In straw berry only the combination of a one-row-system, leaf

sanitation and fruit sanitation decreased almost half gray mould damage in the first crop year compared to a two-row-system without leaf and fruit sanitation. *B. cinerea* damage correlated significantly and positively with the biomass of plants (Schmid *et al.*, 2005).

2.2. Chemical control

The most common means for disease management is by application of chemical fungicides. Both spraying of fungicides and application of fungicides directly to sporulating wounds is practiced. However, high activity of several fungicides is being lost, at least in part, due to the development of resistance. As fungicides still remain an important tool for control of epidemics caused by *B. cinerea*, it is important to monitor populations of the pathogen for their resistance towards potential fungicides (Elad and Shtienberg, 1995a) Chemical control remains the main way to reduce the incidence of grey mould and other *Botrytis* diseases on major crops. The use of botryticides is an efficient way to protect crops against *Botrytis* spp. The most common interventions consist of spraying aerial parts of plants with fungicides. The applied doses vary from 2000-3000 g/ha (e.g. maneb, thiram, dichlofluanid) to 400-500 g/ha (e.g. carbendazim, fludioxonil, pyrimethanil). The number of treatments during a season ranges from one or two, to more than twenty.

2.3. Biological control

Biocontrol of *Botrytis*-incited diseases has been extensively investigated over the last 50 years. Biocontrol of *Botrytis*-incited diseases with filamentous fungi, bacteria and yeasts has been intensively studied over the last two decades (Blakeman, 1993; Elad and Shtienberg, 1995b; Tronsmo, 1991). Biocontrol offers an attractive alternative or supplement to the use of conventional methods for disease control since microbial biocontrol agents (BCAs) are perceived to be less demanding to the environment and their generally complex mode of action reduces the risk of resistance development. For example *Ulocladium atrum* showed a

real potential to protect stem wounds from *B. cinerea*. The level of protection provided by the BCA was as good as that provided by the fungicide both in heated glasshouse and in unheated plastic tunnels (Fruit and Nicot, 1999). In 16 greenhouse trials conducted in southern France, an antagonistic strain of *Fusarium sp*. significantly reduced the incidence of stem lesions caused by *B. cinerea* on tomatoes (Decognet *et al.*, 1999).

The efficacy of combined biological control against *B. cinerea,* it is concluded that *M. dimerum, L. lecanii* and *R. sachalinensis* extract are compatible for application on tomatoes (Bardin *et al*., 2004). Yeast isolates consistently reduced incidence of disease and sporulation of *B. cinerea* in tomato. Several isolates reduced disease by more than 75% in all experiments (Dik *et al*., 1999) *U. atrum* was most effective in suppressing *Botrytis spp*. Colonization fruit rot of strawberries was significantly reduced by weekly applications of *U. atrum* in 3 out of 4 field experiments. The antagonist was as effective as fungicide treatments (Kohl and Fokkema, 1998). *U. atrum* is a strong competitor on necrotic above-ground plant tissues. In onion leaf spot the potential of the antagonist to reduce colonisation of necrotic leaf tissue by *Botrytis spp*. and subsequent sporulation was studied in two field experiments. *U. atrum* colonised necrotic tissues and consistently reduced the sporulation of fungal competitors.(Kohl *et al*., 2003)

The effectiveness of *Trichoderma harzianum* in suppression of tomato stem rot caused by *B. cinerea* was examined on tomato stem pieces and on whole plants, ten days after inoculation with *B. cinerea* and *T. harzianum*, the incidence of infected stem pieces was reduced by 62–84%, the severity of infection by 68–71% and the intensity of sporulation by 87%. Seventeen days after inoculation of wounds on whole plants, the incidence of stem rot was reduced by 50 and 33% at 15 and 26 °C, respectively, and the incidence of rot at leaf scar sites on the main stem was reduced by 60 and 50%, respectively. Simultaneous inoculation and pre-inoculation with *T. harzianum* gave good control of *B. cinerea* (50 and 90%) disease

reduction, 10 days after inoculation (O'Neill *et al*., 1996). *Trichosporon sp.* controlled grey mould and blue mould of apple fruits more effectively than *C. albidus*. Apple fruits treated with *Trichosporon sp*. and *C. albidus* had a lower incidence of grey mould rot than blue mould rot in the same storage conditions. Biocontrol efficacy of the yeasts for controlling gray mould and blue mould was better in apples than in pears (Tian *et al*., 2002a).

3. Effect of nutrition on the susceptibility of plants to Pathogens

Fungal pathogens are developing strategies to parasite their hosts to collect various nutritional resources needed for their growth. A traditionally distinction is biotrophic fungi, which thrive in the host plant without causing cell death through structures called haustoria, and the necrotrophic fungi, they secret the enzymes and toxins designed primarily to cause necrosis and collect resources from dead cells. The availability of minerals and mineral nutrition of the plant resulting act at three levels in the host-pathogen relationship:

1 The nutrients have an effect on growth and development of the plant, and thus how it interacts with its environment and that the pathogen;

2 The plant nutrition affects the distribution, quantitative and qualitative, molecules that can be used by the pathogen *in planta*;

3 The mineral nutrition of the plant modulates its ability to implement defence mechanisms, whether constitutive or induced

Pathogenic fungi are implementing a wide variety of mechanisms for infecting their hosts. It follows from the complexity of these interactions that influence the nutrition of a host on the development of the parasite that attacks is very variable. Thus, increasing the nitrogen content of tomato causes increased severity of infection by *Oidium lycopersicum*, but has no effect on *Fusarium* wilt caused by *Fusarium oxysporum*, while it decreases susceptibility to *B. cinerea* (Hoffland et al., 1999b; Hoffland et al., 2000a). However, general trends are suggested in the literature. For example, assume that the susceptibility of plants to rusts of cereals, mildews, and many other foliar pathogens tends to increase with the increase in the level of nitrogen nutrition (Huber and Thompson, 2007; Walters and Bingham, 2007). It is also considered that, generally a good availability of potassium; calcium and magnesium

limits the severity of epidemics. But understanding the exact role of nutrition in the host-pathogen interaction and therefore the use of nutrition as a way of limiting parasite pressure must be done by considering each specific pathosystem. We propose here a review of literature of major nutrition influences to plant known in host-pathogen relationships and focusing the study on nitrogen. In a second step, we examine more specifically the sensitivity of *B. cinerea* to the nutritional status of various plants that may infect.

3.1 Effect of nitrogen nutrition on the host pathogen relationship

Nitrogen is an essential element for plant growth and a key element of agricultural inputs. Rapid increases of crop yields became possible when synthetic N fertilizer became available after the discovery of the Haber–Bosch process in the early 20th century (Smil, 1999). However, increased use of N fertilizers has also led to increased N losses from agro ecosystems, especially since the 1950s. Distribution of fertilizer N across the globe is very uneven. In some areas N is used excessively and leads to N pollution, causing a lot of problems for human and ecological health. Other parts of the world suffer from reduced soil fertility, diminished crop production, and other consequences of inadequate N supply (Mosier *et al*., 2004). We can consider the effect of plant nutrition on host-pathogen relationships, the pathogen life cycle and interactions that promote infection, disease development in the host, and secondary spread of the pathogen and their interaction that facilitate infection.

3.2 Relationship between the form of nitrogen and host susceptibility

A wide range of plant hosts and their pathogens are influenced by N, but the form of N available to the host plant or the pathogen may greatly influence the disease reaction, independently of the rate of N available, for example disease susceptibility in strawberry was affected by both N-concentration and N-source. *B. cinerea* lesions were largest in ammonium nitrate > ammonium sulphate > calcium nitrate. This data suggest that calcium nitrate may be a suitable source of nitrogen, helping growers to reduce disease risk (Walter et al., 2008a). In

wheat crop when compared with the nil N treatment, ammonium nitrogen fertilizers, either as ammonium sulphate or ammonium chloride drilled with the seed, lowered the severity of take-all of wheat (Brennan, 1992). Availability or failure to act, on both growth and organogenesis nitrogen is the main growth factor element in the plants. An increased nitrogen supply has a strong impact on leaf area and foliage density. Ammonia may have fungicidal effects on *V. dahliae* and some *Fusarium* (Davis *et al*., 1976; Tenuta and Lazarovits, 2002). The use of organic nitrogen base may also improve the competition between certain fungi and micro-organisms antagonistic to pathogens in favor of the latter, and thus limit disease outbreaks (Van Bruggen, 1995). In cases where ammonium has an unfavorable effect on the development of a disease, the use of nitrification inhibitors may be a way to limit the risk of infection while continuing to provide the host plant with sufficient nitrogen (Huber and Thompson, 2007).

Many authors have suggested that further development of the canopy could induce a favorable microclimate in infectious process (Champeil *et al*., 2004; Danial and Parlevliet, 1995; Leitch and Jenkins, 1995; Oerke and Schonbeck, 1990). This hypothesis is supported by the fact that a target size of leaves or fruit may limit the deleterious effect of nitrogen on an outbreak of *B. cinerea* (R'Houma et al., 1998b). Proposed contradictions about the effect of nitrogen on the development of certain diseases can be overcome by considering the nitrogen supply in the form of ammonia or nitrate (Huber and Watson, 1974; Huber and Thompson, 2007). The germination and penetration into the host can be promoted or inhibited by the presence of leaf or root exudates from the host. For example, root exudates promote the germination of *Fusarium solani f. chlamidospores sp. phaseoli*, and the quantity and chemical composition of these exudates may be influenced by the type of nitrogen nutrition (Huber and Watson, 1974).

Table I. Effect of form of N on disease severity of several diseases.

Host	Pathogen/disease	NH_4	NO_3	References
Rice	*Magnaporthe grisea*	Decrease	Increase	Huber et al 2000
Tomato	*Fusarium oxysporum*	Increase	Decrease	Duffy and Défago 1999
Beets	*Rhizoctonia solani*	Increase	Decrease	Elmer 1997
Strawberry	*Pratylenchus penetrans*	Decrease	Increase	Elmer and Lamondia 1999
Asparagus	*Fusarium oxysporum*	Increase	Decrease	Elmer 1989
Pea	*Fusarium* spp	Increase	Decrease	Sagar and Sugha 1998
Tobaco	*Thielaviopsis basicola*	Decrease	Increase	Harrison and shew 2001
Black walnut	*Gnomonia leptostyla*	Decrease	Decrease	Neely 1981
Many plants	*Sclerotium rolfsii*	Decrease	Decrease	Punja 1989
Egg plant	*Verticillium dahliae*	Decrease	Increase	Elmer 2000

3.3 Biological interactions between nutrition and soil microorganisms.

A nitrogen supply often leads to a significant limitation of the severity of epidemics. Diseases of the cortex and roots, caused by *Fusarium, Rhizoctonia, Aphanomyces, Cercospora,* and *Armillaria* are limited by the nitrate (Huber and Watson, 1974). As in most agricultural soils, because of active nitrification by soil bacteria, ammonia is almost completely converted to nitrate; nitrogen fertilization reduces the risk of developing infection. On the other hand, when a pathogen attacks early on root systems, better supply of nitrogen in the plant allows it to stimulate his organogenesis to create alternative structures and continue to keep its hydro power mineral while activating jointly metabolic defence mechanisms (Walters and Ayres, 1980; Walters and Ayres, 1981). Generally, it has often been reported that the transition from one level of sub-optimal nitrogen sufficient has limited outbreaks in cereals, for example *Pyrenophora tritici* ,*Gaeumannomyces graminis*, or *Septoria, nodorum* caused by *Stagonosphora.* Similarly in tomato, reduced availability of nitrogen promotes blight caused by *Alternaria solani* and damping-off caused by *Pythium spp* infections (Snoeijers et al., 2000b). Often, the kinds of nutritional effects of above reported are only partial, and can not seems to cause the severe epidemics, For example, the previously reported

effect of ammonium on resistance to *V. Dehliae* disappears with very high levels of inoculum (Elmer and Ferrandino, 1994).

3.4 Trophic host-pathogen interactions

Nitrogen is found in many forms in the plant, in elementary oxidized forms (NO_3^-) or reduced (NO_2^-, NH_4^+) or incorporated after reduction with carbon skeletons to form amino acids or amides or protein. The distribution of different forms of nitrogen is not homogeneous in the plant and pathogens find different sources of nitrogen, according to their characteristics (surface or underground, biotrophic or necrotrophic).(Walters and Bingham, 2007) Many correlations between susceptibility or resistance to diseases and various indicators of nitrogen status of the plant (amino acids, nucleic acids, phenols, total N, protein content, C / N, etc) have been reported (Huber and Thompson, 2007).

The successful colonization of a pathogen is related to its ability to draw resources from the plant. Several authors support the hypothesis that biotrophic fungi, which have limited access to nitrogen resources, are more sensitive to nitrogen nutrition than necrotrophic, who have access to the entire cellular content of the plant. But some necrotrophic, fungus-like non obligatory fungus *Magnaporthe grisea*, the pathogen of rice blast, can be stimulated by excess nitrogen (Kurschner *et al.*, 1992; Long *et al.*, 2000). The example of *M. grisea* in the same way refuses the hypothesis supported by (Dordas, 2008), that the obligatory parasites are favoured by excess nitrogen, while facultative parasites have increased severity at low level N. In fact, it seems that one can generalize the rule that biotrophic are favoured by nitrate and inhibited by ammonium, while in contrast the necrotrophic are favoured by nitrate and inhibited by ammonium(Solomon *et al.*, 2003). The means employed by the pathogen for appropriate resources of the host is subject to questions and controversies (Solomon *et al*., 2003).

The fact that the expression of genes essential for pathogenicity is induced in vitro by reduced concentrations of N and this expression is controlled by regulatory genes of nitrogen metabolism inducing ability to acquire nitrogen from secondary sources, has led some authors to the hypothesis that fungi *in planta*, find limited nitrogen concentrations(Talbot et al., 1997b; Thomma et al., 2006). In addition, the expression of many genes involved in pathogenicity is modified by the nitrogen status of the host (Snoeijers et al., 2000b). However, the cellular concentrations of nitrate and amino acids in the host are well above those required for proper growth of pathogenic fungi in vitro (Solomon *et al.,* 2003). Studies of the tomato apoplast during infection by *Cladosporium fulvum* showed the presence of many amino acids, and proteins at high nitrate concentration (Solomon and Oliver, 2001)).

The contradiction between the studies could be explained by the fact that some biotrophic in the early phase of infection find poor-nitrogen environments and are forced to activate the secondary nitrogen metabolic pathways (Divon and Fluhr, 2007). A significant portion of nitrogen resources of *C. fulvum* could derive amino acids from the host apoplast (Solomon *et al*., 2003). This is the fact that fungal genes encoding transporters of amino acids are activated during infection (Mendgen *et al*., 2000; Solomon *et al*., 2003) Some non-protein amino acids like GABA or ornithine are involved in defence mechanisms of the plant and accumulate in infected tissues. Pathogenic fungi would therefore use these host responses to these molecules affect their own defensive metabolism (Divon and Fluhr, 2007). Mutants of non-users nitrate have a full pathogenicity (Lau and Hamer, 1996) which reinforces the hypothesis that amino acids are the main source of nitrogen supply of pathogenic fungi. However, some mutant auxotrophic for methionine or arginine show a reduced or no pathogenicity (Divon and Fluhr, 2007) (Solomon *et al*., 2003), which proves that the synthesis of certain amino acids by the pathogen is necessary for infection The type of plant nitrogen nutrition, nitrate or ammonia, has an influence on its amino acid content. Increase of the

nitrate content of the plant increase the proline content and promote *Fusarium* in tomato and ammonium increased concentration of asparagine, which promotes the development of *Rhizoctonia solani* in beet (Huber and Watson, 1974) The increased severity of epidemics of rust and mildew on cereals where abundant nitrogen supply is explained by the higher content of amino acids into leaf tissue (Huber and Thompson, 2007).

3.5 Effect of nitrogen nutrition on the capacity of host defence

The plant is implementing four major types of defence following infection by a pathogen:

(I) a hypersensitive reaction leading to an oxidative burst, characterized by the synthesis of compounds such as H_2O_2 near sites of infection that cause cell death;

(II) The establishment of structural barriers in the cell walls, particularly through the production of callose and lignin, preventing the fungus to grow in the plant;

(III) The synthesis of secondary metabolites with antifungal properties. These compounds may be constitutive (Phytoanticipins), or synthesized following new infection (Phytoalexins)

IV) The synthesis of proteins to detoxify fungal toxins or alter the metabolism of the pathogen PR proteins (Pathogenesis Related Proteins). The availability of nitrogen strongly affects the expression of defence mechanisms, both constitutive and induced.

The availability of nitrogen strongly affects the expression of defence mechanisms, both constitutive and induced. Theories are meant to predict the relative importance of types of secondary compounds for defence according to the environment of the plant (Bryant *et al.*, 1983; Ruohomaki *et al*., 1996). An environment that promotes photosynthesis and limiting nutrients promote the acquisition of defensive compounds based on carbon, such as terpenes or phenols. An environment poor in light and rich in nutrients favour the contrary, the

accumulation of nitrogen compounds such as alkaloids. This assumption contributes to a more general theory, called the growth-differentiation balance (Herms and Mattson, 1992)), suggesting a negative correlation between tissue growth and synthesis of secondary compounds.

In environments rich in resources, they are a priority to growth and secondary metabolism is limited, however, when stress limits the growth more than photosynthesis, the theory predicts a larger allocation of resources for secondary metabolism. It was suggested; however (Hoffland et al., 1999b; Walters and Bingham, 2007) that the low concentration of defensive compounds and their accumulation located in the body makes them dependent on the overall balance of carbon and nitrogen resources of the plant. A compound as-α tomatine, and saponin fungi-toxic is produced in relatively large quantities in tomato, seems its concentration positively correlated with C / N of the plant, then it is considered a nitrogen-based molecule (Hoffland et al., 1999b).

The literature reports conflicting results regarding the effect of nitrogen on the expression of defence molecules. a negative correlation has been found, for example, in grapevine, between the amount of resveratrol a phytoalexin phenolic of stilbene family and nitrogen fertilization (van Baarlen *et al.*, 2004). Stout *et al.* (1998) showed that the total concentration of phenolic compounds was lower with high nitrogen nutrition. These findings seem to illustrate a general situation. But for the terpenes and alkaloids, relationship with N nutrition are more variable (Dietrich *et al*., 2004) While a deficient nitrogen supply limits the overall content of the plant protein, a variation of the nitrogen in tomato plants did not alter the induction of proteinase inhibitor activity following a leaf stress (Stout *et al*., 1998) This contradicts the hypothesis that favoured the induction of nitrogen based defence molecules at high nitrogen nutrition. However, another study (Dietrich *et al*., 2004) showed that the availability of nitrogen in the plant modulates both the constitutive and induced levels of

certain defensive proteins, and their speed of induction in case of stimulation of a chemical elicitor. But all the PR proteins do not have their increased synthesis by a higher nitrogen content of the plant, which undermines the general theories to predict the importance of defensive reaction to the level of nutrition.

3.6 Effect of nitrogen on the development of plant disease

Nitrogen plays an important role in development of plant diseases. The effect of different nitrogen levels on disease severity of genotypes of wheat varying in quantitative resistance to yellow rust caused by *Puccinia striiformis,* in both years the disease severity increased strongly with increased N levels. The infection types also increased with increased N levels (Danial and Parlevliet, 1995). In the field excessive amounts of N fertilizer had a marked tendency to increase the size of the late blight lesions on the potato leaves. In the greenhouse excessive use of N fertilizer did not increase the late blight lesions on the potato leaves.(Awan and Struchtemeyer, 1957). By increasing the amended N, there was a linear increase of 34.8% for AUIPC (Area under incidence progress curve) and 34.3% for AUSPC (Area under severity progress curve) of Phoma spot of coffee. The dry matter of coffee seedlings increased linearly with the increase in the amended N. By increasing the amended N, a corresponding increase in shoot N content was observed (De Lima *et al*., 2010).

Table II. Effect of N level on disease severity of several diseases.

Pathogen/disease	Low N	High N	References
Puccinia graminis	decrease	increase	Howard et al 1994
Erysiphe graminis	decrease	increase	Büschbell and Hoffmann 1992
Oidium lycopersicum	decrease	increase	Hoffland et al. 2000
Plasmodiophora brassicae	decrease	increase	Kiraly 1976
Tobacco mosaic virus	decrease	increase	Singh 1970
Pseudomonas syringae	decrease	increase	Hoffland et al 2000
Xanthomonas vesicatoria	increase	decrease	Chase 1989
Alternaria solani	increase	decrease	Blachinski et al. 1996
Fusarium oxysporum	increase	decrease	Woltz and Engelhar 1973
Botrytis cinerea	increase	decrease	Lecompte et al 2010

4. Relationship between nutritional status of the host plant and susceptibility to *Botrytis cinerea*.

4.1 Nitrogen nutrition of the host and Botrytis

The effect of nitrogen nutrition of plants on their susceptibility to the fungus is controversial (Dik and Wubben, 2004a; Yermiyahu et al., 2006). Some of the inconsistencies in literature is certainly what the different phases of the cycle of the pathogen were examined, and the response to nitrogen nutrition for the host is multifactor. There remains, however some inconsistencies. (Huber and Watson, 1974), refer to the bean infection 2.5 times greater with ammonium than with the nitrate nutrition. The authors explain that the addition of ammonium increased quantity and quality of leaf exudates (the richest in sugars and amino acids) and cell permeability of the host is increased with NH_4. These two factors contribute to a better germination of spores and increased infection of the host. Nitrogen fertilization promotes both leaf growth of vines and the development of clusters, making them more susceptible to infection by *B. cinerea* (Elmer and Michailides, 2004a; R'Houma et al., 1998b), but some authors also report no effect of nitrogen on the development of the pathogen (Elmer and Michailides, 2004a; R'Houma et al., 1998b), showed that the frequency of infection and the severity of the disease increased with intake of ammonium nitrate from 200 kg / ha to 700 kg / ha, but the size of some of the leaves and clusters allowed removing the increased susceptibility with nitrogen. It seems that at least part of the susceptibility of the vine is the result of the abundant fertilization with establishment a favourable microclimate for infection. However, a negative correlation was found between nitrogen fertilization and resveratrol content of the plant (van Baarlen *et al*., 2004). This compound is active against *Botrytis in planta;* it is also possible that the increased susceptibility is related to a lower production of defence compounds in plants well supplied with N. Increased susceptibility of several

legumes to *Botrytis* (*B. cinerea* or *B. fabae*) with increasing doses of nitrogen has also been reported (Davidson *et al*., 2004)..

The potential role of the microclimate in the infection is not known. The effect of nitrogen nutrition of the host on the severity of infections after artificial inoculation of the pathogen is mixed. A logarithmic relationship between the nitrogen content in the nutrient solution (between 1.8 and 28 mM NH_4NO_3) and the incidence of sporulation on stem segments of basil has been reported by (Yermiyahu *et al*., 2006). But in this study neither the incidence nor the growth of cankers were significantly affected by nitrogen. (Pitchay et al., 2007) have reported a much higher incidence of lesions in the begonia for fertilization plethoric 42 mM N. For lower doses (between 1 and 28 .7 mM), a hump-shaped relationship between nitrogen content and frequency of cankers was shown, with a maximum severity at 7 mM. A quadratic susceptibility response of the fungus to the nitrogen nutrition has been reported in chrysanthemum (Hobbs and Waters, 1964). But in the tomato, it has been consistently reported a negative correlation between nitrogen nutrition and susceptibility to *B. cinerea* (Hoffland et al., 1999b; Lecompte et al., 2010; Verhoeff, 1968b). (Hoffland et al., 1999a) showed a strong positive correlation between the C / N of the plant and its susceptibility, the latter being estimated by the number of lesions on inoculated leaves after sampling. A proposed correlation was more questionable between soluble carbohydrates content and susceptibility. These authors also showed that tomatine content was correlated to the susceptibility of the plant, and assumed that the nitrogen nutrition of the host was not likely to affect other defence compounds (phytoalexins and PR proteins) active on *Botrytis.* In contradiction with these results, it has been shown, in source-sink manipulations on tomato, as cankers on shaded plants (with a content of soluble carbon likely lower than the un shaded control) grew faster than control, while the rate of pathogen development was slowed when

the fruits were removed from the plant (probably leaving a carbon content in the stems higher than controls (Shtienberg *et al.*, 1998).

4.2 Effect of form of nitrogen on severity of *B. cinerea*

A wide range of pathogens and their hosts are influenced by N, but the form of of N available to the pathogen or the host may influence the disease reaction, independently of the rate of N available (Huber and Watson, 1974). The influence of the different forms of N on the severity of *B. cinerea* is well documented. For example in *in vitro* conditions when 8 different nitrogen sources were tested, the best development of *B. cinerea* was observed on L-leucine aminoacide and DL-asparagine, monohydrate amide substrates. The least favorable nitrogen sources were DL-norvaline and L-asparagine, substrates on which the test fungus grew and sporulated very weakly. Among 13 carbon sources tested on Czapek culture medium supplemented with yeast extract 0.2%, the most metabolized were the polysaccharides cellulose and inulin, and the least was sorbose (Stefan, 2001). The disease susceptibility in strawberry was affected by both N-concentration and N-source. *B. cinerea* lesions were largest in ammonium nitrate > ammonium sulphate > calcium nitrate. These data suggest that calcium nitrate may be a suitable source of nitrogen, helping growers to reduce disease risk caused by *B. cinerea* (Walter et al., 2008b).

4.3 Nitrogen increases the susceptibility of crops to *B. cinerea*

Nitrogen is by far the most extensively reported element affecting plant disease caused by *B. cinerea* by increasing the susceptibility of plants. Several studies demonstrated that high N increased the susceptibility of plants to *B. cinerea*. For example, the susceptibility of winter rape infection by *B. cinerea* increased with increasing N rate, while infection by *S. sclerotiorum* and shedding of flower buds and flowers due to water and heat stress decreased as N rate increased (Jankowski and Budzynski, 1997). In Rape crop differences in N fertilizer application did not affect the presence of *S. sclerotiorum*, but infection with *B. cinerea*

increased with increasing amounts of N (Lemanczyk *et al.*, 1997). Elevated levels of N supply from 7.15 to 57.1 mM also increased the susceptibility of begonia to *B. cinerea* disease by 10-80% in stems and 3-14% in leaves, the increase in susceptibility also corresponded with increased tissue energy content and altered leaf orientation (Pitchay *et al.*, 2007). In grapevines high N predisposed grapevines to infection by *B. cinerea* bunch rot had increased disease severity, latent infection of cups and berries, as well as visible infection of clusters increased as the rate of ammonium nitrate amendment increased (R'Houma et al., 1998a). The high N nutrition also increased the susceptibility of sweet basil to *B. cinerea* , disease incidence, lesion size and rate of disease progression were erratic and rarely significant (Yermiyahu *et al*., 2006).

(Lacroux *et al*., 2008) reported that high N fertilization also enhances aroma expression, but it also increases vine vigour and susceptibility to grey mould, grapes may be apparently healthy when packed, but bunches from vigorous vines grown with excess N could develop high levels of *B. cinerea* rot during and after storage (Chambers *et al.*, 1993). In other study high N increased vegetative growth but not improve the quality & yield of grapes there was no visible effect of *B. cinerea* (Chambers *et al*., 1995). High N causing increased disease severity and canopy density, which in turn causes a microclimate more conducive to the development of botrytis bunch rot in grapes (Mundy and Beresford, 2007). Further more in strawberries high N increased severity of *B. cinerea*, optimising cultural methods may, however be of greater importance in susceptible strawberry cultivars with their greater potential losses due to *Botrytis* (Daugaard *et al*., 2003). In straw berry elevated nitrogen concentrations in the fertilizer solution increased disease severity of *Botrytis* rot (Nam et al., 2006). High N increased *Botrytis* store rot in Kiwi fruit and fruits were more susceptible in cold storage (Pertot and Perin, 1999). High N also increased the susceptibility of maize to grey leaf spot, AUDPC was higher with higher nitrogen (Caldwell *et al.*, 2002).

4.4 Reduction of plant susceptibility to *Botrytis cinerea* with nitrogen fertilization

The N nutritional status of the plant is known to decrease the susceptibility to *B. cinerea* in different host plants. In a very careful study conducted by Lecompte et al (2010) on the effect of different levels of N on the susceptibility of tomato to six isolates of *B. cinerea* the results showed that the overall disease severity was lower for all isolates on plants with higher N fertilization regimes, regardless of inoculum concentration. Disease onset was delayed on all plants with higher nitrogen inputs, but the response was greater for isolates with lower aggressiveness on tomato. The daily rate of stem lesion expansion decreased with increasing nitrogen fertilization levels for the more aggressive isolates, while it increased for the less aggressive isolate (Lecompte et al., 2010). Furthermore earlier studies also confirmed this phenomenon, for example, when leaves of tomato plants grown at low N availability were about 2.5 times more susceptible to primary lesion formation by *B. cinerea* compared to plant grown at high nitrogen availability (Hoffland et al., 1999a; Verhoeff, 1968a).

In other study on pepper plants the results showed that increasing nitrate supply was positive to suppress *B. cinerea* on stems. With regard to nitrogen analysis, the leaves had more nitrogen content than stems and roots. The total nitrogen concentrations in plants increased with increasing calcium and nitrate supply, results of this study indicate that the increasing calcium and nitrate in nutrient solution positively affected vegetative growth and also effective against *B. cinerea* of sweet pepper plants (Yoon *et al*., 2010). High N fertilization rates or late timing application increased the resistance of pepper plants (Carballo et al., 1994a). In raspberry plants high N decreased *B.cinerea,* N + mixtures of fungicides combine give better results (Cruz A, 1993). In some cases the application of 80 and 240 kg N/ha and a mixture of benomyl + captan on *B. cinerea* development on raspberry reduced conidial number in ripe and unripe berries and percentage of infected berries (Cruz A, 1993).

4.5 High N nutrition increase the susceptibility of crops during storage

The high N nutrition also have the effect on *Botrytis* rots during storage for example in grapes during the storage excess N could develop high level of *Botrytis* rot because due to high N grapes becomes healthy and vigorous and they were more susceptible to rot (Chambers *et al*., 1993). Preliminary investigations have shown that excessive N-fertilization significantly increases the incidence of rot in kiwifruit in cold storage, since the incidence of rot varies from year to year in fruit from plants not receiving high rates of N (Pertot and Perin, 1999). In other study in vine yards showed that during infection the quantity of nitrogen used did not have a visible effect on the infection rates of *B. cinerea* in the vineyard, but after harvest there was a greater presence of the fungus in grapes from vines treated with high N (Chambers *et al*., 1995).

5. Effect of nitrogen nutrition on fungal sporulation

The most important aspect in the epidemiology caused by fungi is the time of onset of sporulation and also it is a major factor for determining rates of disease progress because spore is the major vehicle for the development and the maintenance of epidemic within a crop (Dahlberg and Van Etten, 1982a; De Vallavieille-Pope et al., 2000; Meredith, 1973). Fungal sporulation is of great interest to workers in many scientific disciplines. Sporulation is important to plant pathologists and mycologists since spores are the major vehicle for dissemination of fungal diseases. Nitrogen nutrition of the plant has the effect on sporulation of many fungi. The severity of an epidemic in cultural conditions also depends on the ability of the fungus to form and spread a secondary inoculum. Studies linking the level of sporulation of the fungus to the nitrogen nutrition of the host are few, but they usually lead to the conclusion that high nitrogen content in the host promotes sporulation of the pathogen (Hoffland et al., 2000a; Jensen and Munk, 1997; Robert et al., 2002).

Robert et al. (2002) showed that despite the nitrogen and carbon contents of wheat seedlings were very different, spores of *P. triticina* was relatively constant. In contrast, a comparable density of spores, the number of spores per unit area was significantly weakened in the nitrogen deficient host. If sporulation can be induced in vitro by depletion of the medium carbon and nitrogen analysis of transcription during sporulation in *in planta* showed that transcripts are similar to those found during sporulation in vitro in rich media. It does not seem to be any mention in the literature of variability in aggressiveness of spores from a primary infection on plants with access to different food resources. The canopy structure, which depends on nitrogen nutrition, may affect the dispersal of secondary inoculum. Lovell *et al*. (1997) showed that the severity of infection by *S. tritici* on wheat crop was dependent on both the ability of spores to grow vertically into the canopy and the latency time between cycles of infection. Despite a canopy structure more conducive to limiting the spread of

inoculum to the upper leaf canopy in the case of good nitrogen availability, a reduced duration of the infectious cycle in plants well supplied with N have led to a more severe infection of wheat by *S. tritici*.

The composition of medium *in vitro* and also type and quantity of nutrients available for the fungi play an important role in the spore production of different fungi but nutritional requirements for fungal growth and sporulation vary among different fungi (Dong and Yao, 2005; Schisler et al., 1991a; Steyaert et al., 2010). For any fungal biocontrol agent to be commercially viable, artificial substrates with different composition are very important for their mass production. In this regard several studies have been conducted to investigate the effect of medium nutrients on mass production of spores of several biocontrol fungi for example *Trichoderma viride*; *Paecilomyces lilacinus* (Gao et al., 2007); weed biocontrol agent *Colletotrichum truncate* (Schisler et al., 1991a); the insect pathogenic fungus *Metarhizium anisoplia* (Shah et al., 2005); for red yeast fungus *Monascus. purpureus* a combination of glucose and sucrose greatly enhanced sporulation and cell mass production (Ajdari et al., 2011).

For plant pathogens like *Botrytis sp* according to (Maas and Powelson, 1972) *B. convoluta* sporulate abundantly on media containing glucose, sucrose, and fructose media as compared to maltose, galactose, and starch media and absent with glycine, urea and no nitrogen. As for as *B. cinerea* is concerned generally this pathogen infects healthy plant tissues but mycelial growth and production of spores occurs in necrotic tissues after infection . *B. cinerea* is a polycyclic pathogen and produced epidemics in the field usually are polycyclic, and involve cycles of infection and sporulation. The timing and abundance of sporulation by *B. cinerea* on dead plant tissues contributes to the development and the maintenance of epidemic within a crop (Hausbeck and Moorman, 1996). For understanding the development of epidemics and predicting yield losses the description of spore production

and the factors affecting the rate and pattern of pathogen sporulation is necessary (Robert et al., 2004);(Bastiaans, 1991). Therefore the reduction in sporulation of *B. cinerea* may result in a retardation of the polycyclic epidemics and rate of disease development (Yermiyahu *et al*., 2006). In addition the severity of epidemic condition in crops also depends on the ability of the fungus to spread and form a secondary inoculum. In absence of efficient control, *B. cinerea* have the ability to produce massive amount of spores on diseased plants. In favorable conditions it can produce millions of spores per gram of fresh tissues of tomatoes within a week after inoculation (Nicot et al., 1996).

Regarding the influence of N fertilization of the host plant on the sporulation of *B. cinerea*, the results showed that the nitrogen concentrations in the standard irrigation solution (7.2 and 1.3 mM, respectively) were varied in a series of experiments conducted in pots and large containers. Effect of the N nutrition on grey mould incidence of sweet basil, lesion size and rate of disease progression were erratic and rarely significant, but decreasing the concentration of N and increasing the concentration of Ca in the irrigation solution decreased sporulation of *B. cinerea* (Yermiyahu *et al*., 2006).

5.1 Effect of type of substrates on the sporulation of different fungi

For plant pathogens, plant substrates can also play an important role in the production of spores but surprisingly little or no information on effect of plant fertilization, although it must modify the content of nutrients available to plant pathogens. The effect of substrate composition on spore production by fungi, including *B. cinerea*, has been fairly well documented (Dahlberg and Van Etten, 1982b; Dallemole-Giaretta et al., 2011; Gao and Liu, 2010; Maas and Powelson, 1972; Nicot et al., 1996; Winder, 1999). It has been shown that sporulation is generally favored in the substrates with nutritional conditions that restrict growth. Some fungi have specific carbon and nitrogen requirements for sporulation, others sporulate only upon starvation or nutritional depletion (Dahlberg and Van Etten, 1982a).

5.2 Effect of nutrient substrate on aggressiveness of fungi

Composition and type of substrate has been shown to affect the aggressiveness of different fungi. A few *in vitro* studies have shown that aggressiveness of fungi can be affected by the composition of the spore production medium. For plant pathogens for example, aggressiveness of *B. cinerea* spores to rose flowers was shown to increase with increasing concentrations of glucose in the inoculum production medium (Phillips *et al*., 1987). Other effects of substrate have also been reported on the aggressiveness of other plant pathogens such as *Fusarium avenaceum* and *Phytophthora infestans* (Winder, 1999; Zarzycka, 1995) and the entomophagous fungus *Beauveria bassiana* (Bena-Molaei *et al.*, 2011).

Some results indicate that incubation of conidia under conditions of nutrient stress can attenuate the aggressiveness and germinability of *B. sorokiniana* conidia (Arora *et al.*, 1985). The cultural substrates types also affect the production of spores and virulence of the fungi (Schisler et al., 1991b). Earlier studies shows that when inoculum of *Fusarium avenaceum* was produced on puffed wheat was twice as virulent as that produced on marsh reed grass straw amended with malt extract, while straw cultures generally produced a higher proportion of macro conidia (Winder, 1999). When fungus has enter into the plant tissues the available nitrogen source also depend on the infected tissues of the plant being colonized like leaves, stems and roots of the same plant has different amount of nitrogen sources (Snoeijers et al., 2000a).

5.3 Effect of nitrogen supply on sugars, acids carotinoids and phenolic compounds.

Nitrogen supply has a wide range effects on the plants primary and secondary metabolism. The levels of amino acids, tricarboxylic acid cycle intermediates, sugars, sugar alcohols, and representative compounds of secondary metabolism revealed substantial changes under the various growth regimes applied in tomato. The nitrate nutrition has wide-

ranging effects on plant leaf metabolism with nitrate deficiency resulting in decreases in many amino and organic acids and increases in the level of several carbohydrates and phosphoresces as well as a handful of secondary metabolites (Urbanczyk-Wochniak and Fernie, 2005). Lowering nitrogen supply had a low impact on tomato fruit commercial yield but it reduced plant vegetative growth and increased fruit dry matter content, improving consequently fruit quality. Fruit quality was improved due to lower acid (10-16%) and increased soluble sugar content (5-17%). The content of some phenolic compounds (rutin, a caffeic acid glycoside, and a caffeic acid derivate) and total ascorbic acid tended to be higher in fruit with the lowest nitrogen supply, but differences were significant in only a few cases.

With regard to carotenoids, data did not show significant and univocal differences related to different levels of nitrogen supply (Benard *et al.*, 2009). In apple lower phenolic compounds were found at higher N rates and also high N increased the susceptibility of apple to scab (Leser and Treutter, 2005). Potato plants supplied with high N showed significantly increased biomass as compared to plants without additional N fertilisation, C/N ratio was lower and protein content was higher in leaves of the high N variant. The concentration of chlorogenic acids and flavonols was significantly lower in leaves from the high N variant. Whereas resistance to *Alternaria solani* increased when plants were supplied with additional nitrogen, these plants were more susceptible to *Phytophthora infestans* (Mittelstrass *et al*., 2006).

But in case of Barley higher phenol content was found at higher N levels and high N and K levels were positively correlated with a high level of resistance against barley leaf spot (Jalal and Sarhan, 1988). In basil higher phenolic contents observed when nutrient availability was limited at the lowest (0.1 mM) applied nitrogen treatment. Similarly, basil treated at the lowest nitrogen fertilization level generally contained significantly higher rosmarinic and caffeic acid concentrations than basil treated at other nitrogen levels. Nitrogen

fertilization also affected antioxidant activity with basil treated at the highest applied nitrogen level, 5.0 mM, exhibiting lower antioxidant activity than all other nitrogen treatments (Nguyen and Niemeyer, 2008). In oil seed rape the oil content was negatively correlated with N fertilizer rate and the increase in the N fertilizer rate resulted in the increase in infection by *Peronospora parasitica*. Protein content also increased with the increase in N fertilizer rate (Sochting and Verreet, 2004).

5.4 Effect of nitrogen nutrition on growth, transpiration and nutrient uptake of the plants.

The optimum N concentrations for pepper for maximum stem and leaf dry matter production were in the range of 8.0 to 9.2 mM. The optimum $N\text{-}NO_3$: $N\text{-}NH_4$ ratio for maximal stem DM production was 3.5.(Bar-Tal *et al.*, 2001) The optimum value of N concentration for total fruit DM production was 9.4 mM. The N concentration, but not N source, affected leaf chlorophyll content. Shorter plants with more compacted canopies were obtained as the $N\text{-}NO_3$: $N\text{-}NH_4$ ratio decreased. The effect of N concentration on transpiration was related to its effect on leaf weight and area. Nitrogen uptake increased as the N concentration in the solution increased (Bar-Tal *et al*., 2001).

Levels of NO_3^- and Ca_2^+ exceeding normal concentration resulted in decreased seedling growth, shorter internodes, and lower shoot/root relations. These effects are presumably due to the increase in solution salinity and the greater osmotic potential in the growing medium (Guzman and Sanchez, 2003). An adequate balance between growth rates and transplant quality parameters was obtained with 8 meq. L-1. NO_3^- and 5 meq. L-1 Ca_2^+ in nursery fertigation solution. When different cultivars of transplanted pepper (Capsicum annuum L) were subjected to very high concentrations of NO_3^- (32 meq.L-1) and low concentrations of Ca_2^+ (5 meq.L-1) in nutrient solution, the vegetative growth rate, leaf area expansion

parameters and leaf biomass production were positively affected (Guzman and Sanchez, 2003).

6 Effect of nutrition on the enhancement of biological control

Biological control has advanced greatly during the last few years and microbial antagonists have been reported to control several diseases (Janisiewicz and Marchi, 1992). Reliability and cost are two major factors that will determine the feasibility of any biocontrol system (Janisiewicz *et al*., 1992). In order to be a successful competitor at the wound site, an antagonist has to be better adapted than the pathogen to various environmental and nutritional conditions, to grow rapidly at the wound site, to use nutrients effectively at low concentrations, and survive and grow at the infection site at range of temperatures, pH and osmotic conditions. Enhancers are chemicals that serve as a food base for antagonistic microbes or are selectively toxic or fungicidal to pathogens (Spurr, 1994). Therefore, enhancers can be used to manipulate the antagonist populations on fruit and can greatly improve biocontrol levels.

In order to replace recommended synthetic fungicides with biocontrol agents it will be necessary to develop other methods to enhance the efficacy of biocontrol (Droby, 2001; Droby *et al*., 1997; Wisniewski *et al.*, 1995). For this to enhance biocontrol activity of antagonists against fungal pathogens, certain strategies, such as adding calcium salts, carbohydrates, amino acids and other nitrogen compounds to biocontrl treatments are proposed (Janisiewicz *et al*., 1992) Some exogenous substances, such as chitosan, amino acids, antibiotic, calcium salts and carbohydrates have been studied to enhance biocontrol capabilityof antagonists against fungal pathogens (El-Ghaouth *et al.*, 2002). Nutritional manipulation has been shown to enhance biocontrol activity of several antagonists against several pathogens for example biocontrol with yeast against *B. cinerea* rot in apple was enhanced when *Trichosporon sp*. even at low concentration of 10(5) was applied in the presence of 1-2% CaCl2 in an aqueous suspension (Tian *et al*., 2001). Biocontrol activity of yeasts against *Rhizopus* in peach is also improved (Tian *et al*., 2002b). Another study reported that *Candida saitoana* plus glycol chitosan

enhanced the control of postharvest decay on apples and citrus (El-Ghaouth *et al*., 2000; El-Ghaouth *et al.*, 2002) Sodium bicarbonate also increased the effectiveness of post harvest decay of apple control by each antagonist alone or in combination (Conway *et al*., 2004). (Janisiewicz *et al*., 1992) demonstrated that, when using the antagonist *Pseudomonas syringae*, the addition of specific amino acids (L- asparagine and L-proline) enhanced biocontrol activity against the blue mould of apple caused by *Penicillium expansum.* It was suggested that the enhancement of *P. syringae* activity was due to the ability of the bacterium to outcompete the fungal pathogen for a specific nitrogen source, leading to enhanced growth of the bacterium in the wound site.

Previous *in-vitro* screening of the nutritional requirements of the antagonist and the pathogen identified several amino acids that were selectively utilized by the antagonist and not by the pathogen. (McLaughlin *et al.,* 1990) reported that calcium salts improved the efficacy of yeast biocontrol agents against *Botrytis* and *Penicillium* rots of apples. Suspensions of yeast cells at concentrations as low as 10^6 to 10^7 CFU/ml effectively inhibited disease development in the presence of different calcium salt solutions. In the absence of the salts, a yeast cell concentration of at least 10 and usually 100 times greater was required to achieve the same level of protection. They further demonstrated that the effect was specifically due to the calcium cations rather than other cations or anions. Salt solutions applied to apple wounds without the antagonist cells failed to reduce the incidence of the rot.

The authors suggested that the effect of calcium on the biocontrol activity of the yeast antagonists was due to some interaction with the yeast or its metabolic products at the wound site rather than a direct effect of calcium on the pathogen or the fruit tissue. In contrast, (Wisniewski et al., 1995) have indicated that calcium chloride reduced germination and germ tube elongation of *B. cinerea* and *Penicillium expansum in vitro*. The inhibition was again demonstrated to be due to the presence of the calcium cation rather than the chloride anion

(e.g., $MgCl_2$ had no effect). The application of $CaCl_2$ with the yeast antagonist *Candida oleophila* enhanced the biocontrol activity of the yeast against both pathogens on apple. In other study the effect of foliar fertilizers containing various macro and microelements on mycelial growth, spore germination and antagonism of *Trichoderma* isolates. It was found that foliar fertilizers cause changes in the development and antagonism of *Trichoderma spp*. Foliar fertilizer cause change in mycelial growth, spore germination and antagonism rate of *T. harzianum* isolate. It was found that potassium Alkalin and Wapnovit fertilizers significantly increased the growth rate of *T. harzianum* mycelium. Potassium alkalin is a fertilizer which favourably affects the antagonism of *Trichoderma* isolates towards *B. cinerea* and *R. solani* (Duzniewska, 2008).

7 Possible mechanisms involved in disease suppression by nitrogen

7.1 Physiology of the plant

Nitrogen is one of the main factors which are involved in the plant's physiological functions for the growth and also for the disease resistance. Nitrogen influences many plant constituents like amino acids, proteins, total N, phenolics, nucleic acids and ratio of carbohydrates to N and these plant components are directly correlated to susceptibility or resistance to disease (Huber, 1980). The plant diseases are influenced by the nitrogen nutrition in several ways. Nitrogen influence physiology of the plants by causing changes in their structures, it also affect the growth and virulence of the pathogen and also cause changes in the biotic and abiotic environment of the plants. In addition to this N nutrition affects the vegetative growth of the plants and also on the chemical composition of the plants for example, in tomato low nitrogen increased soluble content and also the content of some phenolic compounds (rutin, a caffeic acid glycoside, and a caffeic acid derivate) and total ascorbic acid tended to be higher in fruit with the lowest nitrogen supply. It was reported that primary and secondary metabolites could be affected as a result of a specific response to low nitrogen, combined with a lower degree of vegetative development, increasing fruit irradiance, and therefore modifying fruit composition (Benard et al., 2009). In sweet basil at high N phenolic compounds are decreased and also higher N fertilization lowers the antioxidants (Nguyen and Niemeyer, 2008).

Passive and active mechanisms of disease control are activated through nutrient management. Mineral nutrients are the components of plants and regulate metabolic activity associated with resistance of a plant and virulence of a pathogen. Adequate nutrition is generally required to maintain a high level of disease resistance. Plants contain preformed anti-microbial compounds and have active response mechanisms where inhibitory

phytoalexins, phenols, flavonoids, and other defense compounds accumulate around infection sites of resistant plants if the nutrients required for the synthesis or induction of those compounds are adequate.

The interaction of fungal disease and fertilizers is of great practical interest and there have been numerous studies into the interaction of (in particular) nitrogenous fertilizer and disease. Application of nitrogen above the recommended rate has often been shown to significantly increase disease incidence and lesion area. This applies to both necrotrophic *Magnaporthe grisea* (Long et al., 2000) and biotrophic pathogens *Blumeria graminis f. sp.tritici*; and tomato mildew *Oidium lycopersicum* (Hoffland et al., 2000b; Jensen and Munk, 1997). The nature of the nitrogen also has an effect, Huber and Watson's (Huber and Watson, 1974) review noted that nitrate consistently increased and ammonium decreased the incidence of hemi-biotrophs (mildew, *Colletotrichum* and rust) whereas nitrate decreased and ammonium increased the incidence of the necrotrophs (*Cochliobolus*, *Magnaporthe* and *Botrytis*). Sub-optimal nitrogen and also lead to more disease. In the grey mould pathogen *B. cinerea*, the formation of primary lesion on tomatoes was more frequent with low nitrogen levels (Hoffland et al., 1999a). These plants have a high physiological carbon/nitrogen ratio and a large amount of soluble carbohydrates in tissue (Hoffland et al., 1999a), indicating that the growth of the pathogen was dependent on high soluble carbohydrate levels. The hypotheses being tested in the above studies can be redefined as follows;

(A) An insufficiency of N fertilization leads to susceptibility by weakening a plant and promoting invasion and disease.

(B) Superabundant N fertilization leads to hyper-susceptibility by either

1 providing excess nutrient that is directly available to support the growth of pathogens,

2 promoting the growth of ill-defended plant tissue, or

3 Diverting metabolic resources into the assimilation of the nutrient.

Studies that might distinguish these possibilities have given conflicting results. Matsuyama and Dimond (1973) showed that the application of nitrogenous fertilizers to rice led to a reduction in the activities of enzymes in the phenyl proponed pathway and the production of lignin, suggesting that fertilizers compromised the intensity of secondary plant defenses. This is consistent with hypothesis B3. The positive effect of ammonium on necrotrophs is consistent with hypothesis B1, whereas the positive effect of low nitrogen on *Botrytis* is consistent with hypothesis A. Clearly more work is needed in this area and it is, of course, likely those different fungi will respond in different ways to the application of N fertilization. It is important that future studies combine measurements of fungal growth and symptom production with plant biomass and in plant nutrient levels.

7.2 Pathogen growth and virulence

Plant pathogens germination, growth, and virulence may be influenced by the amount and also the form of N. Obligate fungal pathogens mostly rely on the nutrients supplied by the host plant, so that changes in the plant constituents can greatly affect the growth, reproduction and virulence of the pathogens. For example, few studies have addressed the effect of N fertilization on the infection of fungal pathogens. Work on powdery mildew of barley showed that infection of *Blumeria* (syn. *Erysiphe*) *graminis* was enhanced with increasing N fertilization (Bainbridge, 1974; Jensen and Munk, 1997). Similarly, disease severity by *Oidium neolycopersici* on tomato leaves increased with increasing nitrogen supply to the plants (Hoffland et al., 2000b) and leaf rust uredospore production per lesion was higher in leaves with a high-nitrogen content (Robert et al., 2004). In case of the facultative saprophytes their survival, growth, and survival may also be greatly influenced by N nutrition. For example, the presence of both carbon and nitrogen is necessary for the germination of spores of the *B. cinerea* (Blakeman, 1975), because of the lack of endogenous energy reserves

present in the conidia of *B. cinerea*, the fungus requires an exogenous source of nutrients to grow (Yoder and Whalen, 1975). Nitrogen may affect the virulence of the pathogen by stimulation or inhibiting enzyme synthesis or activity required for pathogenesis. This type of mechanism could be especially important in macerating diseases.

7.3 Modification of the biotic or a biotic environment

Nitrogen fertilization can affect the growth of plants growth, development, chlorophyll content, leaf angle, nutrient uptake, and thus influence the microclimate conditions within the canopy or vegetation that might then affect disease progress (Huber and Watson, 1974). For example, high N predisposed grapevines to infection by *B. cinerea* and increased disease severity. Latent infection of cups and berries, as well as visible infection of clusters increased as the rate of ammonium nitrate amendment increased. Conversely, removal of leaves around clusters, when practiced 2 or 3 times during the season, and thinning of berries, significantly reduced disease development and resulted in less latent and visibly infected clusters and berries. These green pruning practices also attenuated the beneficial effects of N fertilizers on disease development (R'Houma et al., 1998c).

7.4 Effect of nitrogen on gene expression of fungal pathogens

For successful infection of plants by fungal pathogens requires efficient utilization of nutrient resources available in host tissues. Several studies have been reported that fungal genes are specifically induced during pathogenesis and under nitrogen-limiting conditions *in vitro*. For example, avirulence gene *Avr9* of the biotrophic fungal pathogen *Cladosporium fulvum* is both induced *in planta* and during nitrogen starvation *in vitro* (Perez-Garcia et al., 2001). In the entomopathogenic fungus *Metarhizium anisopliae* the products encoded by the genes *pr1A* and *pr*2 show protease activity and their expression is subjected to both carbon and nitrogen repression (Smithson et al., 1995). In addition *mpg1* of *Magnaporthe grisea*

(Talbot et al., 1997a), *pCgGS* of *Colletotrichum gloeosporioides* (Stephenson et al., 1997) are also induced during infection of the host and during nitrogen starvation *in vitro*.

Further studies are thus required to understand the mechanisms involved at the level of gene expression. Expression of the sporulation-regulating *brl*A gene of *Aspergillus nidulans* was reported to be induced by either glucose or N starvation, but with different kinetics and with differing phenotypical outcomes (Skromne et al., 1995). Similar studies with *B. cinerea* would be possible, as orthologs of genes known to function upstream of *blr*A (including *fad*A, *flu*G, *flb*C and *flb*D) have been reported in the genome of this fungus (Amselem et al., 2011).

Trophic interactions related to primary metabolism are unlikely to be the only mechanisms implicated. Other compounds related to plant defense mechanisms would merit further attention as they could influence fungi indirectly through their effect on plant tissue colonization by the pathogen. N nutrition has a large effect on the amino acid content (Le Bot et al., 2001) and constitutive phenolics (Stout et al., 1998) involved in various stages of the infection process in tomatoes. In other plant models, peroxidase and chitinase (two enzymes implicated in plant defense) were lower when N was limiting (Dietrich et al., 2004). Further work could also include the effect of N (and resulting differences in plant tissue constituents) on the expression of pathogenicity-related genes of *B. cinerea*. Nitrogen-limiting conditions can influence the expression of such genes for several plant pathogenic fungi (Snoeijers et al., 2000a; Solomon et al., 2000) and possibly for human pathogens such as *Aspergillus fumigatus* (Panepinto et al., 2002).

Chapter 2

Publication 1

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Contrasted responses of *Botrytis cinerea* isolates developing on tomato plants grown under different nitrogen nutrition regimes

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The nutritional status of a plant is known to influence its susceptibility to pathogens. In the case of *Botrytis cinerea*, the role of nitrogen fertilization of various host plants on disease development appears to be variable. This study was carried out to characterize possible variability associated with isolates and inoculum density of *B. cinerea* in its ability to infect leaf-pruning wounds and to develop stem lesions on tomato plants, as affected by the nitrogen input. Six isolates differing in their aggressiveness to tomato were compared. They all had similar reaction patterns *in vitro* in response to differential nitrogen levels. In tests on plants grown with contrasted regimes of nitrate fertilization, overall disease severity was lower for all isolates on plants with higher nitrogen inputs, regardless of inoculum concentration. However, differences among isolates were observed in the effect of plant nitrogen nutrition on infection and on lesion expansion. Disease onset was delayed on all plants with higher nitrogen inputs, but the response was greater for isolates with lower aggressiveness on tomato. The high est contrast among isolates was observed with the colonization of stems. The daily rate of stem lesion expansion decreased with increasing nitrogen fertilization levels for the more aggressive isolates, while it increased for the less aggressive isolate. Hypotheses to explain these results are discussed in light of the possible physiological effects of nitrogen fertilization on nutrient availability for the pathogen in the host tissue and of possible production of defence metabolites by the plant.

Keywords: Grey mould, host resistance, *Lycopersicon esculentum*, *Solanum lycopersicum*

Introduction

It has long been recognized that the nutritional status of a plant can play a role in its susceptibility to pathogenic fungi. Nitrogen, in particular, is deemed to strongly influence the host-pathogen interactions (Huber & Watson, 1974; Huber & Thompson, 2007). However, the nitrogen (referred to as 'N' in the rest of this paper) status of a plant can be either favourable or unfavourable to the infection process, depending on the pathosystem (Huber &Thompson,2007). This variable effect can be explained by the fact that plant N nutrition may have an influence on many factors involved in the epidemiological cycle. Such factors include the molecules involved either in host defence or in the virulence and aggressiveness of the pathogen, the quantity and nature of host N-based substrates acquired by the pathogen and the microclimate around the plant (through an effect on plant vigour and architecture). The N level of plant tissues has often been correlated to host susceptibility, one explanation being that at high plant N content ore substrate is available for the development of the pathogen (Jensen & Munk, 1997;Neumann et al., 2004; Walters & Bingham, 2007). In contrast, it has been shown in *Arabidopsis thaliana* that the constitutive and induced levels of some proteins involved in plant resistance to infection are higher at high N nutrition (Dietrich et al., 2004). But other defence compounds and molecules acting as structural barriers against pathogens can be lowered at a low C/N ratio, which are the consequence of a high N nutrition (Taluk- der et al., 2005). It has also been suggested that plant solu- ble carbohydrate content, which is negatively correlated with N nutrition, has a positive influence on plant susceptibility (Hoffland et al., 1999).

Although these contradictory effects highlight the need for pathosystem-specific studies, a general rule has been suggested (Solomon et al., 2003): the development of biotrophic fungi would be enhanced by nitrate and inhibited by ammonium, while the contrary would be true for necrotrophic fungi. Regarding the influence of N nutrition on the necrotroph *Botrytis cinerea*, the situation is not as clear (Dik & Wubben, 2004). Higher plant susceptibility to *B. cinerea* was reported at high N fertilization rates in legumes (Davidson et al., 2004), grape (R'Houma et al., 1998), sweet basil (Yermiyahu et al., 2006) and begonia (Pitchay, 2007). In three of these studies (R'Houma et al.,

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1998;Yermiyahu et al., 2006; Pitchay, 2007), the N source was nitrate or a mixture of nitrate and ammonium. In contrast, a high N nutrition seems to lower the level of disease in tomato (Verhoeff, 1968; Hoffland et al., 1999). It has been reported that the susceptibility of bean to *B. cinerea* was 2–5 fold higher with an ammonium nutri- tion compared to a nitrate-based source of N (Huber & Watson, 1974). The explanation for this was that ammonium enhanced cell permeability and increased leaf exudates, both factors being favourable to infection (Huber & Watson, 1974). For grape, pruning of leaves and fruits lead to a reduction in infection, even at high N, suggesting that the effect of N was mainly to increase leaf surface, thus rendering the microclimate around infection sites more conducive to infection (R'Houma et al., 1998). For sweet basil, the percentage of stems carrying sporulating lesions was higher at higher nitrate concentration in the fertigation solution, but the percentage of infected plants, the lesion size and the rate of disease progression were not affected (Yermiyahu et al., 2006). For begonia, disease incidence was higher at 42 mmol L^{-1} N (brought as ammonium nitrate), a concentration hardly found in agricultural situations. However, between 1–7 and 28 mmol L^{-1} N, the relationship between N nutrition and susceptibility was quadratic, with a maximum at 7 mmol L^{-1} N (Pitchay, 2007). Thus the effect of plant internal N content on *B. cinerea* infection and lesion growth appears to be highly dependent on the host spe cies. This suggests that there could be a trade-off between a 'trophic component' and a 'defence component' of the host-pathogen interaction. For example, a high plant N status could raise the level of nutrients accessible to the pathogen, while at the same time enhance host defences. An unexplored hypothesis to explain the reported variability could be that the host-pathogen interaction could be differentially affected by N fertilization depending on the isolate of *B. cinerea*. The response to N availability, *in vitro* and *in planta*, of *B. cinerea* isolates of contrasting aggressiveness, as well as the effect of inoculum density, should help to better understand the nature of this interaction.

In the present study, the objective was to confirm that the N content of nitrate-fed tomato plants has an influence on their susceptibility to *B. cinerea*, and to test the influence of initial inoculum density and fungal isolate on the growth response of the fungusboth in vitro and in planta.

Materials and methods

Three experiments were conducted with tomato (*Solanum lycopersicum*) plants produced under differ ing N nutrition regimes between May and July 2008 (experiment E1), between June and August 2008 (experiment E2) and between March and May 2009 (experiment E3). Although comparable in its design, experiment E3 differed from E1 and E2 by the number of N treatments and isolates of *B. cinerea* tested. Additionally, two in vitro tests with *B. cinerea* on contrasting nutrient medium were conducted in summer 2009.

Production of plant material and fertigation treatments

Tomato seeds (cv. Swanson, De Ruiter Seeds) were sown in 1 cm^3 rock wool cubes in a greenhouse. Ten days after sowing, the cubes, each containing one plantlet, were transferred onto rock wool blocks 7–5 7–5 6 cm (Grodan). During the first month, the plants were ferti- gated twice a day with a standard commercial nutrient solution (Duclos International). After that period, the plants (bearing 3–4 leaves) were placed on the top of 2 L pots filled with a mixture (1:1 V / V) of vermiculite and pozzolana (inert crushed volcanic rock) to start the nutrition treatments. Thirty plants were used in each treatment. In experiments E1 and E2, three levels of NO_3^- concentrations were tested: 0–5, 5 and 15 mmol L^{-1} NO_3^-; in E3, five levels were tested: 0–5, 2, 5, 10 and 20 mmol L^{-1} NO_3^-. The equilibrium in electric charges was maintained by replacing nitrates with sulphates in the solutions with less nitrate. The concentration of other major nutrient elements was kept constant, at the following levels: 11 mmol L^{-1} K, 3–5 mmol L^{-1} Mg, 3–5 mmol L^{-1} Ca and 1 mmol L^{-1} P. Oligo-elements were also added at the following concentrations (in μmol L^{-1}): 20–6 B, 0–5 Cu, 10–7 Fe, 11–6 Mn, 0–28 Mo and 3–2 Zn. The plants were fertigated with a drip irrigation system (one dripper per pot) up to six times a day depending on the climatic demand, with 1 min pulses. Three pots chosen at random were weighed continuously to evaluate their loss of water, and thus the climatic demand in the greenhouse. The pH was adjusted to 6 in each treatment by addition of H_2SO_4. Plants were grown with those solutions for four (in E1) or three (in E2 and E3) weeks and were then inocu lated.

Evaluation of plant susceptibility to *Botrytis cinerea*

Two monoconidial isolates of *B. cinerea* (BC1 and BC21), previously collected in commercial greenhouses and used routinely in the laboratory, were used in E1 and E2. Four additional isolates (BC43, BC44, BC84 and NHPm4) were added in experiment E3. From previous work in the laboratory, isolates BC1, BC43 and BC44 were known to have a high level of aggressiveness on tomato, while isolates BC21, BC84 and NHPm4 had a medium to low level of aggressiveness (Ajouz, 2009; Ajouz et al., 2010). For each isolate, inoculum was produced on potato dextrose agar medium (39 g L^{-1}, Difco) in a growth chamber (18°C night, 22°C day, and 14 h daylight). Conidia were collected in sterile distilled water from the surface of 14-day-old cultures. Each suspension was filtered through a 30 μm mesh sterile filter to remove mycelium fragments and adjusted to the desired concentration with the help of a haemocytometer. Three infec tion concentrations were tested in E1 and E2: 10^5 (e5), 10^6 (e6) and 10^7 spores mL^{-1} (e7). Only concentrations

e6 and e7 were retained in experiment E3. The third, fourth, fifth and sixth leaves of five randomly selected plants were excised, leaving 5–10 mm petiole stubs on the stems. The wounds were inoculated with 10 μL aliquots of a spore suspension. In E1 and E2, isolate BC1 was inoculated on petioles 3 and 5 and isolate BC21 on petioles 4 and 6, with different groups of plants for the different inoculum concentrations. A total of 15 plants were inoculated per N treatment. In E3, each plant was inoculated with one isolate only, at concentration e6 on petioles 4 and 6 and at concentration e7 on petioles 3 and 5. A total of 30 plants were inoculated per N treatment. After inoculation, the plants were placed in a growth chamber for 7 days. The chamber was set at 21°C, 90% RH and 14 h daylight. During this period, the plants were irrigated manually, twice a day, using the same fertilization solutions as those used before inoculation. Symptoms were assessed between the third and seventh day after inoculation. The incidence of stem lesions and the length of developing lesions (in mm) were recorded daily. Area under the disease progress curves (AUDPC) were computed as described by Aissat et al. (2008) and Decognet et al. (2009), as:

$$\text{AUDPC} = [(Y_1/2) + \sum_{2}^{n-1} Y_j + (Y_n/2)]\times l$$

where Y_j was the observed lesion length (in mm) at the j^{th} observation time, n was the total number of observations, and l the interval between each observation (in days). These values were computed for individual pruning lesions for n = 5 observation dates at daily intervals during the period from the third to the seventh day after inoculation.

Plant nutrition assessment

At the end of the growing period in the greenhouse, just prior to the assay of susceptibility to *B. cinerea*, five additional plants were randomly sampled in each treatment for nutrient analysis. The wet and dry weights (after 72 h at 70°C) of stem and leaves were measured. Subsamples were ground, calcined at 400°C for 12 h and then mineralized in boiling H_2SO_4. In experiments E1 and E2, the K, Ca and Mg concentrations in the filtrates were measured with an atomic absorption spectrometer (Varian AA100). In all experiments, plant P, N and C content was assessed, with a spectrophotometer (Perkin-Elmer Lambda) for P and a as analyzer (Thermo Finnigan 1112) for C and N.

Fungal growth *in vitro*

The six isolates used in the inoculation treatments were grown on an agar medium amended with varying concentrations of NH_4NO_3. A minimal medium adapted from Weeds et al.(1998) was used, containing 2·5 g Na_3 Citrate, 5 g KH_2PO_4, 0·2 g $MgSO_4$, 15 g sucrose and 15 g Bacto™ Agar (Difco) per litre. Fourdoses of NH_4NO_3 were compared: 0, 0·1[as in the minimal medium of Weeds et al. (1998)], 2 and 10 g L^{-1}. For each medium, five 90 mm diameter Petri plates (containing 15 mL of medium) were inoculated with a 2 μL drop of spore suspension deposited in their centre. For all isolates, the suspension was adjusted to 10^7 spores mL^{-1}. The plates were incubated at 21°C for 5 days and the colony diameter was recorded daily. Two independent repetitions of the test were conducted. There was no difference between these two experiments, and the pooled data were used for analysis. The colony radial growth between day 3 and 4 was used for statistical analysis.

Data analysis

Significant differences between the experiments were assessed by analysis of variance. When significantly different, results from different experiments were treated separately. In the experiments with tomato plants, the latency period was calculated as the number of days between inoculation and lesion appearance on the stem. If a lesion was visible on the first day of symptoms assessment (third day after inoculation), the latency period was given a value of 2 days; if no symptoms were apparent on the last day of measurement, it was given a value of 7 days. For each inoculation point, the rate of lesion expansion was calculated as the average daily increase in lesion length between the day of symptom appearance on the stem and the last day of measurement. As a result, the estimated rate of expansion of an individual lesion was based on 1 to 4 measurements depending on the date of appearance of the lesion. There was no systematic effect of the day of measurement on the rate of lesion expansion, thus the mean rate of lesion expansion was used in the statistical analyses as a variable following a normal law. For each isolate, the effect of the inoculum concentration (e5, e6 and e7) and the nitrate nutrition were assessed by analysis of variance. Lesions on the same stem did not overlap and were assumed to appear and to grow independently. In each experiment, the number of inoculated wounds, for a given inoculum concentration and plant nutrition treatment was 10. The SAS software package was used for data analysis.

Results

Fungal growth *in vitro*

All the isolates were significantly affected ($P < 0·0001$) by the concentration of NH_4NO_3 in the agar medium (Fig. 1). On the medium containing no added N, their radial growth was strongly reduced and the mycelial mats were composed of sparse and very thin filaments which contrasted with the dense growth observed on the media supplied with N. On media amended with NH_4NO_3, all isolates were similarly affected by N concentration. For a given isolate, radial growth was similar or slightly faster at 2 g L^{-1} NH_4NO_3 than at 0·1 g L^{-1} NH_4NO_3, and reduced at 10 g L^{-1} NH_4NO_3 (Fig. 1).

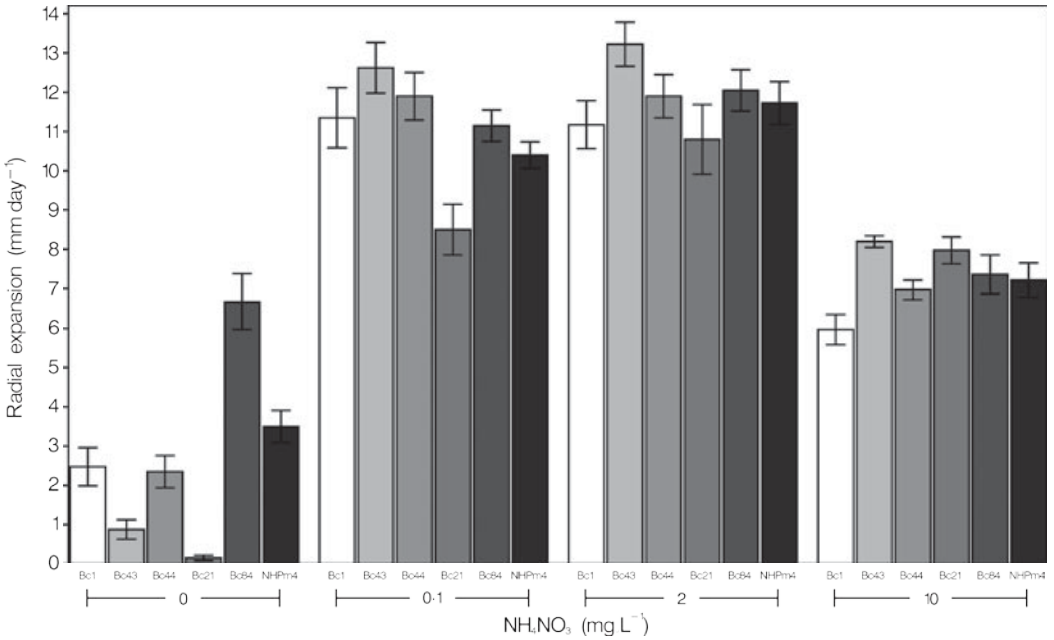

Figure 1 Radial growth of Botrytis cinerea isolates on agar medium at different concentrations of NH_4NO_3. Bars represent the standard errors.

Plant growth and mineral content

As expected, the N fertigation treatments in the three experiments with tomato plants consistently resulted in contrasted dry matter contents of the plants at the time when they were used for the assays of susceptibility to *B. cinerea* (Table 1). It appeared from experiment E3 that the optimal concentration for growth was close to 10 mmol L^{-1}. At higher N nutrition, plant N content still increased, but not plant weight. In experiments E1 and E2, the K, Mg and Ca contents were measured. They were positively correlated with the nitrate concentration in the fertigation solution (data not shown). Conversely, the phosphorous content was negatively correlated with nitrate nutrition. Plant C content did not vary signifi cantly, except in E1 where a small decrease was noticed at high N (Table 1).

Overall disease severity

The AUDPC for the two groups of isolates appeared rather different (Fig. 2). For fast growing isolates there was a steep decrease of AUDPC between 0·5 and 10 mmol L^{-1} and no significant difference between 10and 20 mmol L^{-1}. A quadratic regression gave a better fit to

Table 1 Dry matter (g), N and C content of 7-week-old (experiment E1) or 8-week-old (experiments E2 and E3) tomato plants, fertigated with solutions containing different NO_3^- concentrations. Data are means and standard errors of five replicates per treatment

	Nitrate concentration (mM L^{-1})					
Experiment	0·5	2	5	10	15	20
Dry matter (g)						
E1	10·3 ± 0·6a		35·8 ± 1·0b		45·9 ± 2·0c	
E2	5·1 ± 0·5a		20·4 ± 0·7b		24·3 ± 1·3c	
E3	6·4 ± 0·4a	11·4 ± 0·2b	20·0 ± 0·7c	29·1 ± 1·7d		26·9 ± 2·4d
N (% dm)						
E1	1·0 ± 0·1a		2·0 ± 0·1b		3·7 ± 0·2c	
E2	1·1 ± 0·1a		1·9 ± 0·1b		3·8 ± 0·2c	
E3	1·4 ± 0·1a	1·8 ± 0·1ab	2·2 ± 0·1b	3·4 ± 0·2c		4·1 ± 0·2d
C (% dm)						
E1	39·7 ± 0·1a		40·5 ± 0·6a		38·4 ± 0·34b	
E2	39·7 ± 0·1a		40·1 ± 0·3a		39·1 ± 0·2a	
E3	37·8 ± 0·2a	38·0 ± 0·1a	37·8 ± 0·2a	37·7 ± 0·2a		36·8 ± 0·6a

Different letters in a line indicate significant difference between treatments (SNK test, a = 0·05).

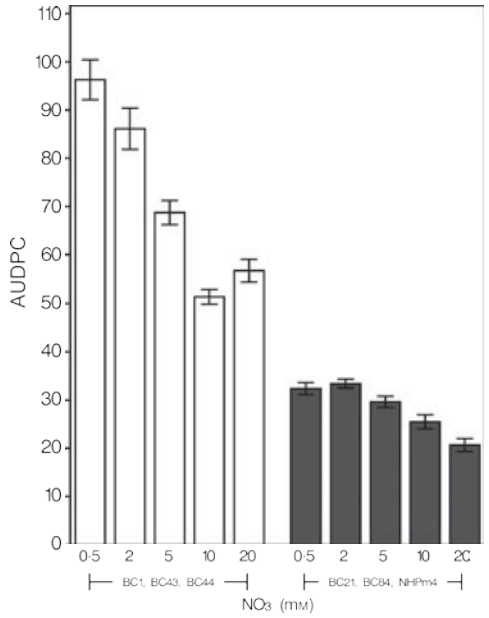

Figure 2 Area under disease progress curve (AUDPC) for stem lesions of *Botrytis cinerea* measured on tomato plants grown at various nitrate nutrition in the growing medium. Data were pooled for fast-growing isolates (BC1, BC43 and BC44) and slow-growing isolates (BC21, BC84 and NHPm4). Data are from experiment 3. Bars indicate standard errors.

the data compared to a linear regression (r^2 of 0·33 and 0·21,respectively). For slow growing isolates AUDPC was not significantly altered at low N nutrition rates, but decreased steeply between 5 and 20 mmol L^{-1} at higher nitrate nutrition. A quadratic model did not improve the regression compared to a linear model (r^2 of 0·19 in both cases).

Kinetics of stem infection

There was an overall highly significant effect ($P < 0·0001$) of inoculum concentration and plant nutrition on the latency period before symptom appearance. Details for the different isolates are given in Table 2. For BC1 and BC21, results were not different in experiments E1 and E2 ($P = 0·22$), and were pooled. For each isolate, at each inoculum concentration, there was an effect of N nutri tion on the delay before symptom appearance: high nitrate nutrition delayed symptoms by 0·5–2·5 days. This increase in latency period was more pronounced for less aggressive isolates (Table 2). An increase in inoculum concentration resulted in a shorter latency period, but the effect was more pronounced at low N levels. The time necessary to obtain symptoms on 100% of the inoculated wounds also increased with N concentration (data not shown). After 7 days, all wounds showed stem lesions, with the exception of plants inoculated with isolate

Table 2 Latency period (days) between inoculation of *Botrytis cinerea* and symptom appearance on the stem of tomato plants grown at various nitrate concentrations, for six different isolates, and different inoculum concentrations (e5: 10^5 spores mL^{-1}, e6: 10^6 spores mL^{-1} and e7: 10^7 spores mL^{-1}). Data are mean and standard errors of 20 replicates (experiments 1 and 2) or 10 replicates (experiment 3)

Isolate	Inoculum concentration	Nitrate concentration (mM L^{-1}) 0·5	2	5	10	15	20
Experiment 1 and 2							
BC1	e5	3·1 ± 0·1a,A		3·3 ± 0·1a,A		4·3 ± 0·3b,A	
	e6	2·2 ± 0·1a,B		2·3 ± 0·1a,B		2·8 ± 0·1b,B	
	e7	2·0 ± 0·0a,B		2·0 ± 0·0a,C		2·4 ± 0·2b,B	
BC21	e5	3·9 ± 0·3a,A		4·0 ± 0·1a,A		5·7 ± 0·2b,A	
	e6	3·3 ± 0·2a,AB		3·0 ± 0·1a,B		4·7 ± 0·3b,B	
	e7	2·9 ± 0·3a,B		2·4 ± 0·2a,C		4·7 ± 0·3b,B	
Experiment 3							
BC1	e6	3·0 ± 0·0a,A	3·0 ± 0·0a,A	3·0 ± 0·0a,A	3·0 ± 0·0a,A		3·2 ± 0·1b,A
	e7	2·4 ± 0·2a,B	2·6 ± 0·2ab,B	2·9 ± 0·1ab,A	2·8 ± 0·1ab,A		3·1 ± 0·1b,A
BC43	e6	2·8 ± 0·1a,A	3·0 ± 0·0ab,A	3·0 ± 0·0ab,A	3·5 ± 0·3b,A		3·3 ± 0·2ab,A
	e7	2·3 ± 0·2a,B	2·9 ± 0·1ab,A	3·0 ± 0·0ab,A	3·0 ± 0·0ab,A		3·6 ± 0·4b,A
BC44	e6	2·7 ± 0·1a,A	2·9 ± 0·1ab,A	3·0 ± 0·0b,A	3·0 ± 0·0b,A		3·0 ± 0·0b,A
	e7	2·2 ± 0·1a,B	2·4 ± 0·2a,B	2·9 ± 0·1b,A	2·9 ± 0·1b,A		3·0 ± 0·0b,A
BC21	e6	3·2 ± 0·1a,A	3·6 ± 0·4a,A	4·3 ± 0·4ab,A	4·9 ± 0·3bc,A		5·8 ± 0·4c,A
	e7	2·6 ± 0·2a,B	3·0 ± 0·0a,A	3·6 ± 0·3a,A	4·7 ± 0·4b,A		5·1 ± 0·5b,A
BC84	e6	3·3 ± 0·2a,A	3·2 ± 0·2a,A	4·3 ± 0·3b,A	5·1 ± 0·2c,A		5·1 ± 0·1c,A
	e7	2·9 ± 0·2a,A	3·1 ± 0·1a,A	3·9 ± 0·3b,A	4·8 ± 0·2c,A		5·1 ± 0·1c,A
NHPm4	e6	3·0 ± 0·0a,A	3·3 ± 0·3ab,A	3·9 ± 0·2bc,A	3·6 ± 0·3abc,A		4·2 ± 0·3c,A
	e7	2·7 ± 0·2a,A	3·0 ± 0·0a,A	3·1 ± 0·1a,B	3·1 ± 0·1a,A		3·8 ± 0·3b,A

Lower case letters indicate significant differences between N treatments in a line, uppercase letters indicate significant differences between inoculum concentrations for a given isolate, in a column (SNK test, $\alpha = 0·05$).

BC21 for which lesions were observed on only 60–100% of the wounds, depending on the experiment and the N treatment.

Lesion expansion

The comparison of the rates of lesion expansion on tomato stems revealed contrasted patterns among isolates of *B. cinerea* in response to plant nutrient status. For the more aggressive isolates (BC1, BC43 and BC44), the rate of lesion expansion decreased with increasing nitrate concentrations in the fertigation solution up to 10–15 mmol L^{-1} (Fig. 3a).The response was similar for these three isolates (P = 0·12). In contrast, the isolates with lower aggressiveness (BC21, BC84 and NHPm4) were stimulated by increasing N concentrations, up to 10–15 mmol L^{-1} (Fig. 3b). The response was not significantly different among these three isolates (P = 0·13). The effect of inoculum concentration on lesion expansion was only significant for BC21, BC43, BC44 and NHPm4. However, in each experiment, when data of all isolates and N nutrition were put together, there was a highly significant effect of inoculum concentration on lesion growth rates (Fig. 4).

Discussion

The results show that the overall severity of the disease, as assessed by the AUDPC, was consistently lower at high nitrate nutrition (between 10 and 20 mmol L^{-1}) for six

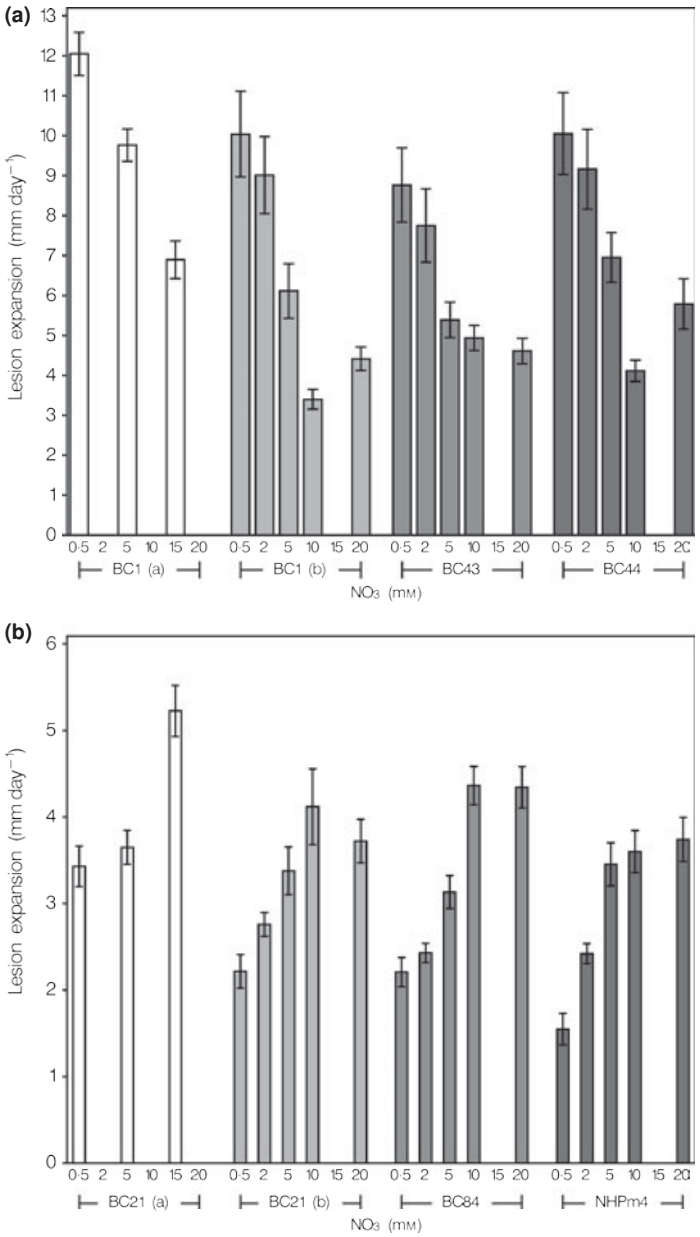

Figure 3 Daily rate of lesion expansion of six isolates of *Botrytis cinerea* on stems of tomato plants grown at various nitrate concentration in the growing medium. A separation was made between fast-growing isolates (a: BC1 in experiment 1 and 2 (BC1(a)) and experiment 3 (BC1(b)) and BC43 and BC44 in experiment 3) and slow-growing isolates (b: BC21 in experiment 1 and 2 (BC21(a)) and experiment 3 (BC21(b)) and BC84 and NHPm4 in experiment 3). Bars represent the standard errors.

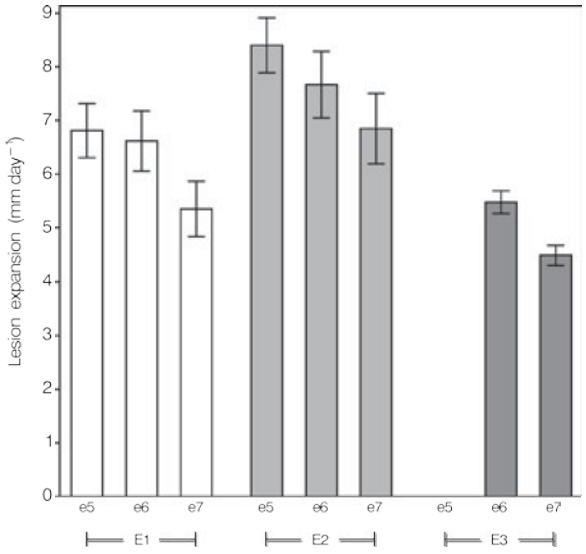

Figure 4 Daily rate of stem lesion expansion on tomato following the inoculation of leaf pruning wounds with spore suspensions of Botrytis cinerea containing 10^5 (e5), 10^6 (e6) and 10^7 spores mL^{-1} (e7). Data from all the isolates were pooled in each experiment, E1 to E3. Bars indicate standard errors.

isolates of *B. cinerea* differing widely in their aggressive ness to tomato. This confirms earlier results showing that a high N nutrition lowers the susceptibility of tomato to *B. cinerea* (Verhoeff, 1968; Hoffland et al., 1999). How ever, the correlation between N content and AUDPC was not linear, at least for aggressive isolates. The C/N ratio of the plants decreased steeply from low to high N treat- ments, as a result of roughly constant C concentration and increasing N concentration in the plant (Table 1). Hence, a linear relationship was not bserved between C/ N and susceptibility in this experiment, as proposed by Hoffland et al. (1999). Moreover, examining separately two key steps of pathogenesis (infection and lesion expansion) revealed a higher than anticipated level of complexity. The lesions of the three isolates with lower aggressiveness expanded faster with increasing plant N content, while those of the three more aggressive isolates were inhibited by high N content. In contrast, the increase with plant N concentration in the latency period before symptom appearance was more pronounced for less aggressive isolates. This may explain why, in fine, the relationship between N nutrition and AUDPC was overall similar for both types of isolates.

Contrasted intra-specific pathogen responses to plant nutritional status has already been reported in other pathosystems. However, the correlation between nutri- ent concentration and plant susceptibility is, for the vari- ous isolates tested, always either positive or negative, but not opposite as observed here. For example, the infection efficiency of *Blumeria* (formerly *Erysiphe) graminis* on barley seedlings was, depending on the isolate, 2·5 to 7·8 times higher at 240 mg N per plant compared to 30 mg N per plant (Jensen & Munk, 1997). The inhibitory effect of calcium on *B. cinerea* has also been shown to depend on the isolate, but a decrease in fungal growth at high cal cium concentration was always noted (Chardonnet et al.,2000). Here, an opposite effect of plant N on the two types of isolates used in this study was consistently observed in three independent experiments.

It has been suggested that high nitrate nutrition increases host susceptibility to biotrophic fungi and decreases that of necrotrophic fungi. One explanation could be that biotrophic pathogens rely on the nutrient content of the apoplast, or on nutrient transfer from the host cells by a haustorium, while necrotrophic fungi that kill cells have better access to the host nutrients (Solomon et al., 2003; Divon & Fluhr, 2007). This paper shows that in vitro the isolates responded positively, and similarly, to the N content of the substrate, at least up to a concentration of 2g L^{-1} NH_4NO_3 (Fig. 1). As the plant N content increased with increasing N in the nutrient solution, it could be hypothesized that any repression of fungal growth at the higher host N content was the result of a better ability of the plant to defend itself against the path- ogen. This is consistent with reports that a high N nutri- tion can lead to production by the host of higher levels of some constitutive and induced defence molecules (Stout et al., 1998; Dietrich et al., 2004). The different responses of the isolates to the host N status could be explained by the fact that infection strategies of *B. cinerea* (types and levels of secreted fungal toxins, cell wall degrading enzymes or oxalic acid) are isolate-dependant (Siewers et al., 2005; Choquer et al., 2007). Thus the ability of the host to counteract, at high N content, the effect of patho gen metabolites could depend on the fungal phenotype. However, this study has shown that, regarding primary infection and latency, the effect of plant N was compara- ble for all isolates (Table 2): the delay before symptom appearance was higher at high plant N and lower at high inoculum concentration. Regarding early infection then, there appears to be no isolate-specific effect of host N status. A nitrogen deficit comparable to that imposed n the treatment with 0·5 mmol L^{-1} in this study leads to a steep decrease of the nitrate and amino acid content of the plant (Scheible et al., 1997; Le Bot et al., 2001), notably gluta- mine and glutamate which are the principal source of fun gal N nutrition. Although secondary N sources can be used by the pathogen, N scarcity induces specific responses in fungi, ultimately sporulation (Divon & Flu hr, 2007). Thus, even for necrotrophic fungi such as *B. cinerea*, a decrease in host N resources could impair growth. The hypothesis is that the increasing rate of lesion development of less aggressive isolates with increasing N may be related to a reduction in competition for N substrates. As the total plant volume colonized by the mycelium of less aggressive isolates is smaller than that colonized by more aggressive ones, N scarcity is likely to be more detrimental to the first group, as local resources are exhausted faster. The fact that low N reduced the latency period for both groups of isolates can be explained if one assumes that, at that time, fungal growth relied mostly on the nutrient content of the spore

(Divon & Fluhr, 2007). Thus the effect of high N availability could predominantly affect host defences, and not the pathogen nutrition, during early infection. Moreover, an unexpected decrease in lesion growth has been shown at higher inoculum concentration (Fig. 4). Usually, disease severity is higher when the conidial concentration in the infected wound is higher (see O' Neill et al. (1997)). This could indicate that the competition for N was higher when N resources were low, leading to less disease development and that conversely, it was attenuated when N resources were higher.

This study has shown that the effect of host N on *Botrytis* development depends on the isolate and the concentra- tion of inoculum leading to primary infection. One explanation for this could be that the fungal development depends both on trophic and defence processes, and that the availability of nutrients for the pathogen ultimately depends on its ability to explore new sources of unex- plored plant tissues. Upon N scarcity, low substrate avail- ability would impair growth of moderately aggressive isolates, those against which plant defences are more effi- cient, while resources available to aggressive isolates would not be limiting, whatever the nutrition status of the host.

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Chapter 3

Publication 2

F. Lecompte, **M. A. Abro**, and P. C Nicot. 2013. Can plant sugars mediate the effect of nitrogen fertilization on lettuce susceptibility to two necrotrophic pathogens: *Botrytis cinerea* and *Sclerotinia sclerotiorum*? Plant Soil.

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REGULAR ARTICLE

Can plant sugars mediate the effect of nitrogen fertilization on lettuce susceptibility to two necrotrophic pathogens: *Botrytis cinerea* and *Sclerotinia sclerotiorum*?

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Abstract
Aims Nitrogen (N) fertilization is known to modify a plant's susceptibility to necrotrophic diseases. However, the effect of N nutrition on defence is not well known. It was hypothesized that not only molecules related to the N metabolism but also main sugars could mediate the effect of plant fertilization on its susceptibility to pathogens.
Methods Two necrotrophic fungi, *Botrytis cinerea* and *Sclerotinia sclerotiorum* were inoculated on leaves of lettuce plants grown at five nitrate (NO_3^-) fertilization levels, in three independent experiments. Variations in plant composition at the time of inoculation were linked to the size of lesions observed after 5–6 days.
Results Both diseases were favoured by high NO_3^- fertilization. However, the highest disease levels were not found in the same experiment for *B. cinerea* and *S. sclerotiorum*. Among the components measured, NO_3^- and sucrose (SUC) were positively and negatively correlated to the two diseases in the three experiments, but the relationship between SUC and lesion size was more significant for *S. sclerotiorum*. Water content, N and total carbon (C) were also significantly correlated to the diseases, but the relation- ships were less straightforward. The ratios of SUC over total sugars and fructose (FRU) over total sugars fitted, very closely for *S. sclerotiorum*, a negative and positive exponential relationship respectively with le- sion size. Absolute or relative glucose levels were not linked to the diseases.
Conclusions Plant metabolic modifications induced by variations of N availability conferred the plant variable defence ability, which seemed, at least for *S. sclerotiorum*, mainly mediated by variations in host SUC and FRU levels. The generalization of these findings to other species would be of interest.

Keywords *Lactuca sativa* L · Plant disease · Pathogenic fungi · Sucrose · Fructose

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Introduction

Botrytis cinerea Pers. Fr. and *Sclerotinia sclerotiorum* (Lib.) de Bary are two phylogenetically close necrotrophic ascomycetes (Amselem et al. 2011), sharing many common characteristics in their development and pathogenicity. Both feed and grow on previously killed plant host cells (Williamson 2007; Bolton et al. 2006). Both fungi can infect a very wide range of plant species and,

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to kill their hosts, secrete a variety of compounds including oxalic acid, cell wall degrading enzymes, peptidases and a pool of toxic metabolites. These compounds allow the pathogen to modify the host redox status, perturb its defence, alter the cell integrity and macerate the plant tissues (Alghisi and Favaron 1995; Riou et al.1991; Godoy et al. 1990). Common patterns of host defence responses upon infection by the two necrotr- ophs have also been demonstrated (El Oirdi et al. 2011; Glazebrook 2005; Oliver and Solomon 2010; Robert- Seilaniantz et al. 2011). They lead, on the one hand, to the local production of reactive oxygen species which trigger the hypersensitive response and ultimately programmed cell death. On the other hand, the plant activates the secretion of fungal enzyme antagonists, including inhibitors of cell wall degrading enzymes (Juge 2006), anti-fungal secondary metabolites and cell wall strengthening molecules (Stotz et al. 2011; Kliebenstein 2004; van Baarlen et al. 2004). Whatever the similarities in infection strategies and stimulation of plant defences, the secretome diversity of both fungi allows them to adapt to their broad host range (Amselem et al. 2011; Choquer et al. 2007). Strain-specific aggressiveness, possibly linked to toxin synthesis and/or detoxification of plant defence metabolites, has been frequently reported (Choquer et al. 2007; Siewers et al. 2005). Although they are frequently asso ciated in the field, especially in lettuce cropping systems, only a few comparative studies of symptom development of these two fungi have been conducted.

Upon infection, a massive change in the host's genome expression is triggered (Katagiri 2004), which coincides with an enhanced use of plant primary metabolites towards defence-oriented pathways and the use of energy and C and N skeletons for defence (Bolton 2009; Berger et al. 2007). Negative or positive correlations between plant sugar status and suscepti bility to fungi have long been noticed (Levy and Cohen 1984; Horsfall and Dimond 1957), leading to the concept of low- or high-sugar pathogens (Horsfall and Dimond 1957). Several studies have reported a decrease in photosynthetic activity and an increase in leaf cell wall invertase activity after infections by either biotrophic or necrotrophic fungi (Berger et al. 2004; Scharte et al. 200 5; Kocal et al. 2008; Fotopoulos et al. 2003). A rise in the concentration of soluble sugars can induce the synthesis of defence related molecules (Johnson and Ryan 1990; Ehness et al. 1997; Morkunas et al. 2011) and the onset of the hypersensitive reaction (Essmann et al. 2008). However, hexoses are also thought to be the principal source of C for the pathogen, and both *B. cinerea* and *S. sclerotiorum* secrete their own invertases to exploit the host sugar content (Jobic et al. 2007; Dulermo et al. 2009). Along with the host carbohydrate metabolism, the plant N status largely determines the outcome of an infection. However, the relationship between N availability and plant constitutive or induced immunity is not straightforward (Walters and Bingham 2007). Generally, higher concentrations of secondary metabolites active in plant defence, notably polyphenols, are found in plants grown at low N availability (Stout et al. 1998; Le Bot et al. 2009; Lou and Baldwin 2004). Nevertheless, the accumulation of secondary metabolites in N-deficient plants is not readily linked with decreased susceptibility to necrotrophs such as *B. cinerea* (van Baarlen et al. 2004) or *Alternaria solani* (Mittelstrass et al. 2006). Indeed, low N availability is known to limit the plant contents of amino acids and proteins, which could include constitutive or inducible pathogenesis-related proteins (Dietrich et al. 2004). Also, the influence of the host N status on its ability to regulate nitric oxide synthesis, which has recently been reported as an important component of plant defence against *B. cinerea* and *S. sclerotiorum* (Asai and Yoshioka 2009; Perchepied et al. 2010), is not known. Furthermore, as for sugars, fungal pathogens retrieve N from their hosts, presumably with amino acids as preferential source (Solomon et al. 2003). Whether the N content of the host can be limiting for pathogen growth is still a matter of debate (Bolton and Thomma 2008; Solomon et al. 2003). The availability of plant-based N compounds might however depend on the plant N status and the speed of fungal expansion, as suggested from disease progress observations (Lecompte et al. 2010; Newton and Guy 1998). As a result of these multiple processes, the effects of host N content on its susceptibility to necrotrophic pathogens are variable, either positive, negative or neutral (Hoffland et al. 2000; Lecompte et al. 2010; Long et al. 2000; Huber and Thompson 2007). Based on the above evidence that the mobilization of plant primary metabolism can markedly affect the outcome of the host–pathogen relationship, and given the interdepen dence of carbon (C) and N metabolism (Nunes-Nesi et al. 2010), it is surprising that very few studies have examined the combined effect of C and N availability on fungal disease development. An experiment with

two levels of N at ambient and elevated CO_2 concluded that high N and CO_2 reduced epidemics caused by *Cercospora* sp. on *Solidago rigida* (Strengbom and Reich 2006). However, in this work, the various C- and N-based metabolites were not analyzed with regard to disease severity. We hypothesized that the effect of N nutrition on epidemic severity could be partially due to modifications of the plant C status, especially its sugar content, and to its impact on triggering or fueling host immunity. The questions we addressed were: (1) which mathematical function(s) gave a consistent relationship between lettuce NO_3^- fertilization and the symptoms caused by *B. cinerea* and *S. sclerotiorum*; (2) was the fertilization effect on each disease related to the plant N status or to the main plant sugars? We report an experimental study on lettuce grown at five N-supply levels, during three different seasons in a greenhouse, leading to marked variations in C and N contents. We assessed and compared the severity of disease caused by *B. cinerea* and *S. sclerotiorum* and related the development of lesions with total C, total N, NO_3^-, sugars and other nutrients in plant tissues. The relative proportion of sucrose (SUC) and fructose (FRU) were closely correlated, negatively, and positively, respectively, with lettuce susceptibility to *S. sclerotiorum*, while infec- tions with *B. cinerea* were correlated most closely with plant NO_3^- and total N.

Material and methods

Experimental design

Three batches of 200 lettuce plants were grown from March 31st to May 30th 2009 (experiment E1), September 7th 2009 to November 11th 2009 (experiment E2) and November 16th 2009 to January 14th 2010 (experiment E3). For all experiments, seeds of cultivar Faustina (Rijk Zwaan) were sown in 1-cm^3 rockwool cubes in a nursery greenhouse. Ten days after sowing, the cubes, each containing one seedling were transferred to 10 × 10 × 6 cm rockwool blocks (Grodan, Roermonds, The Netherlands) and placed in a second greenhouse dedicated to the experiments. Plants were then grown for approximately 1 month and irrigated twice a day with a standard commercial nutrient solution (Plantain, Duclos international, Lunel, France). After that period, the rockwool blocks (bearing plants with three to four developed leaves) were transferred on to the top of 2-L pots filled with a mixture (1:1 V/V) of vermiculite and pozzalana (inert crushed volcanic rock) to start the nutrition treatments. The experimental design was monofac- torial, with four randomized blocks of 10 plants per treatment. Five NO_3^- concentrations were tested in the fertilization solution: 0.5, 2, 5, 10 and 20 mmol L^{-1} NO_3 (abbreviated as mM in the rest of this paper). The composition of the five solutions, made up from simple salts, is given in Table 1. At NO_3^- concentrations below 10 mM, NO_3^- ions were replaced by sulphates, by the use of potassium sulphate instead of potassium nitrate. At the highest nitrate level (20 mM), the concentration of potassium nitrate was doubled. We considered that potassium nitrate was better than other NO_3^- salts to achieve the doubling of NO_3^- concentration in solution. The concentrations in solution of other macronutrients (calcium, magnesium and phosphorus) and trace elements (B, Fe, Cu, Mn) were kept constant. The ionic charge was neutral in all solutions when accounting for Cl^- and HCO_3^- ions already present in the irrigation water, but the electrical conductivity was 40 % higher at the highest NO_3^- level (20 mM). The pH of each solution was adjusted to 6.5 by adding H_2SO_4. Nutrient solutions were supplied via a fertigation network with an individual dripper into each pot. Three pots in an additional block with a

millimole per liter) of main nutrients in the fertilization solutions (Solutions S1 to S5, with increasing NO_3^- concentration from S1 to S5) used for lettuce

mmol L^{-1}	[NO_3^-]	[K^+]	[Ca^{2+}]	[Mg^{2+}]	[SO_4^{2-}]	[$H_2PO_4^-$]
S1	0.5	11	3.25	3.5	7.25	1
S2	2	11	3.25	3.5	6.5	1
S3	5	11	3.25	3.5	5	1
S4	10	11	3.25	3.5	2.5	1
S5	20	21	3.25	3.5	2.5	1

20 mM fertigation were continuously weighted to estimate daily water losses, which were replaced by fertigation pulses, up to six times a day, depending on the external radiation and the crop growth stage. All plants, whatever the nutrient solution, received the same amount of water. Excess water was lost in drainage. Plants were grown for three additional weeks with these different NO_3^- concentrations in the fertigation solution. At the end of the period, the 70-day-old plants were either used for inoculation with *B. cinerea* or *S. sclerotiorum* or for nutrient content assessment. Depending on the season, the greenhouse was either cooled or heated. The incoming radiation gradually increased from 1,000 to 2,300 $J\,cm^{-2}$ in E1, decreased from 1,800 to 750 $J\,cm^{-2}$ in E2 and remained around 500 $J\,cm^{-2}$ in E3. Daily average air temperatures increased from 17 to 27 °C in E1, fluctuated between 15 and 25 °C in E2 and decreased from 17 to 10 °C in E3.

Analysis of plant components

Five plants were randomly selected from each N nutrition regime in the greenhouse for determination of plant fresh and dry weight and for analysis of different minerals and primary metabolites just before inoculation. The plants were harvested at similar times of day in the three experiments, all around 8 AM. The fresh and dry mass of the aerial parts were measured, while roots were discarded. A subsample of three or four leaves from the intermediate crown was kept for plant analysis. Immediately after sampling, the leaves were placed in liquid N. Ten plant components (primary metabolites or elementary elements) were measured, namely: total N (N), total C (C), nitrate (NO_3^-), glucose (GLU), FRU, SUC, phosphorous (P), potassium (K), magnesium (Mg) and calcium (Ca). Sub samples of dry material were ground, calcined at 400 °C for 12 h and then mineralized in boiling HNO_3. The K, Ca and Mg contents were measured with an atomic spectrometer (Varian A220), and the P content with a spectrophotometer (Perkin-Elmer Lambda 2). Total N and C were measured with a gas analyzer (Thermo Finnigan EA1112), and NO_3^- with a NO_3/NO_2 analyzer (5000 FIAstar). Soluble sugars were determined with an enzymatic method in a microplate reader, as proposed by Gomez et al. (2007). Hexoses (FRU + GLU) and total sugars (FRU + GLU + SUC) were computed from these analyses, as well as the C/N ratio.

Inoculation and disease assessment

Six strains of *B. cinerea* and one strain of *S. sclerotiorum* were used in this study. The strains of *B. cinerea* (BC1, BC43, BC44, BC21, BC84, NHPm4) where chosen for their contrasted aggressiveness on tomato (Lecompte et al. 2010), but without preliminary information on their aggressiveness on lettuce. For each strain, the inoculum was produced on potato dextrose agar medium (39 $g\,L^{-1}$ Difco, Detroit, USA) in a growth chamber (Heliofroid, Le Beausset, France) at 21 °C with a 14 h photoperiod. For each strain of *B. cinerea* and *S. sclerotiorum*, one set of five plants per NO_3^- treatment (35 plants in total) was inoculated. On each of these 70-day-old plants, three leaves of the middle crown were inoculated. A 5-mm-diameter mycelial disk, excised from a 3-day-old colony, was placed in the centre of each leaf. The leaf inoculations were made on intact plants. Following inoculation, the plants were placed in a growth chamber and incubated for 7 days in conditions conducive to disease development (21 °C, RH above 85%, with a 14-h photoperiod). During this period, the plants were irrigated manually twice a day, using the same fertilization solutions as those used before inoculation. Very few of the 1,575 observed leaves failed to develop lesions, and 0 values were discarded. The size of the lesions was assessed 5 and 6 days after inoculation for *S. sclerotiorum* and *B. cinerea*, respectively. At these dates, the lesions had not yet covered the whole leaf surface. Each inoculated leaf was detached and photographed over a blue background. The image analysis software Assess 2.0 (APS Press, St Paul, MN, USA) was used to quantify the leaf area (mm^2) and the lesion size (mm^2).

Data analysis

For the five NO_3^- nutrition levels and 3 experimental replicates, 1,350 and 225 observations were analysed for *B. cinerea* and *S. sclerotiorum* respectively. The SAS statistical package was used for the data analysis. The first step was to analyse by general linear models (GLM) the effect of the three (or two) experimental factors related to *B. cinerea* (or *S. sclerotiorum*) lesion development: "NO_3^- concentration in solution", "experiment", "*B. cinerea* strain" (for *B. cinerea* only) and their interactions. Significant differences among treatments were determined by the Newman–Keuls test. Secondly, the type of mathematical function relating NO_3^- concentration with disease severity was assessed.

Six types of functions were tested, whose equations are as follows, where L is the lesion size (in millimeter), R the regressor, and a, b and c are parameters:

Linear: $L = a + b \times R$

Exponential: $L = a \times \exp(b \times R)$

Power: $L = a + b \times R^c$

Logistics: $L = \frac{a}{(1 + (\frac{a}{b} - 1) \times \exp(-c \times R))}$

Michaelis–Menten: $L = a \times \frac{R}{b + R}$

Hill: $L = a \times \frac{R^b}{c^b + R^b}$

The Gauss–Newton method was used to estimate parameter values, using the NLIN procedure of the SAS package (with 449 degrees of freedom (df) for each regression model for *B. cinerea* and 74 for *S. sclerotiorum*). For each disease, the model minimizing the mean square error of prediction (MSEP), was chosen.

Thirdly, the plant components linked to disease variability were investigated, using the fresh weight data. Variables whose distributions were highly skewed and significantly different from a normal distribution were log-transformed. As plant analyses and leaf susceptibility assays were not done on the same plants, the regressions had to be performed on mean values, providing 14 df for each model with the pooled data (for global multiple regression), and 4 df for regressions on individual plant components in each experiment. This means that for each N level in each experiment, the mean lesion area observed on leaves was related to the mean nutrient content of 5 other plants that received the same nutrient solution. However as the trend of the relationship between plant nutrient content and susceptibility was similar for all strains, the *B. cinerea* inter-strain variability was not considered in this part of the analysis and the overall mean from 90 measurements was used: three leaves × six strains × five plants. Fifteen measurements (three leaves × one strain × five plants) were used for *S. sclerotiorum*. In the linear regressions between lesion areas and individual plant component for each experiment, only those components which were, in the three experiments, highly correlated ($r^2 > 0.7$) to disease severity were retained. Among these components, several showed high auto-correlation levels. Repeated GLM analysis—with the pooled data from the three experiments—between disease severity and plant components were thus performed, with all possible combinations of variable orders, and the type I sums of squares (SS) were analyzed. When a significant F value for a given variable was obtained whatever the order of its entry in the model, it was assumed to be independent of other variables.

On a fourth step, the same six functions described above were tested to relate the influence of the selected plant components on both diseases.

Results

Lettuce growth, primary metabolites and ions contents

Leaf area increased asymptotically with NO_3^- concentration in the fertigation solution ($p < 0.0001$, Fig. 1). Significant differences were found between experiments ($p < 0.0001$), but in all cases no further increase was observed for concentrations beyond 10 mM. NO_3^- concentration also had a significant effect on fresh weight (in gram), dry weight (in gram) and plant water content (in gram water per gram dry matter), following the same pattern, with no increase beyond 10 mM NO_3^-. Water content almost doubled with the treatments, from 8.5 g g^{-1} at 0.5 mM NO_3^- to around 17 g g^{-1} at 10 and 20 mM NO_3^-, and was closely correlated with total N content (data not shown). NO_3^- nutrition had a very significant effect on the ten plant components measured, either on a fresh or on a dry weight basis (Table 2, data shown only on a fresh weight basis). All elements except Ca showed significant variability between experiments, and the "NO_3^-"×"experiment" interactions were also always

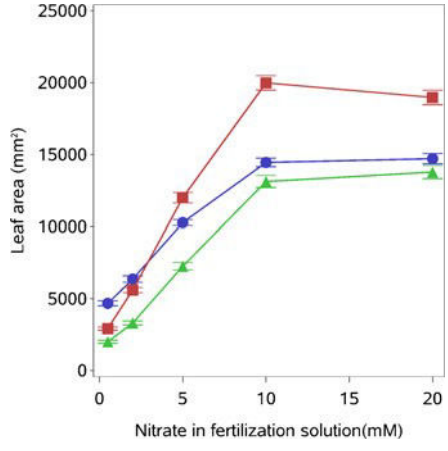

Fig. 1 Leaf area of lettuce plants grown under different NO_3^- fertilization regimes. Each curve represents an independent experiment (blue dots E1; red squares E2; green triangles E3). Horizontal bars represent the standard error of the mean

Table 2 Leaf content (in milligram per gram fresh weight) of lettuce plants grown at five different NO_3^- fertilization regimes, in three independent experiments

NO_3^- concentration in solution (mM)		N (mg/g fw)	NO_3 (mg/g fw)	C (mg/g fw)	SUC (mg/g fw)	GLU (mg/g fw)	FRU (mg/g fw)	P (mg/g fw)	K (mg/g fw)	Ca (mg/g fw)	Mg (mg/g fw)
0.5	E1	1.13	0.02	38.4	4.99	1.67	1.24	0.43	4.51	0.51	0.31
	E2	1.94	0.04	45.6	4.43	2.12	2.25	0.69	3.50	0.92	0.22
	E3	1.37	0.03	45.4	5.71	0.53	0.46	0.71	3.55	1.04	0.37
2	E1	1.48	0.03	33.8	4.73	4.25	4.1	0.46	4.12	0.47	0.34
	E2	2.2	0.09	29.9	4.71	4.17	4.88	0.57	2.95	0.55	0.13
	E3	1.98	0.03	42.1	6.47	1.71	1.51	0.73	3.64	0.54	0.3
5	E1	1.82	0.34	25.8	1.46	5.62	6.71	0.45	3.57	0.38	0.23
	E2	2.29	0.41	24.2	3.09	4.12	5.94	0.51	2.23	0.27	0.11
	E3	2.45	0.15	30.9	5.43	3.25	3.71	0.62	2.89	0.59	0.17
10	E1	2.14	1.51	20.8	0.68	4.07	5.07	0.43	3.43	0.28	0.28
	E2	2.46	0.71	19.6	1.34	1.95	4.31	0.52	2.08	0.22	0.13
	E3	2.68	0.61	25.9	4.45	2.31	4.28	0.53	2.49	0.21	0.08
20	E1	2.62	3.37	20.4	0.61	3.66	4.64	0.45	4.28	0.26	0.18
	E2	2.76	1.07	19.7	1.32	1.34	3.58	0.40	1.76	0.17	0.12
	E3	2.89	0.95	25.1	3.65	1.71	3.67	0.53	2.56	0.33	0.11
p for effects											
NO_3^-		***	***	***	***	***	***	***	***	***	***
Experiment		***	***	***	***	***	***	***	***	ns	***
NO_3^-×experiment		***	***	***	***	***	***	***	***	***	***

Values are the mean of five observations

n.s. not significant

***p<0.0001; significant differences between NO_3 treatments, experiments and their interaction, in a Student Newman–Keuls test

significant. Almost no NO_3^- was found in plants grown at very low NO_3^- concentrations, but the plant NO_3^- content increased exponentially with increasing NO_3^- fertilization regimes. At the 10 and 20 mM NO_3^- regimes, plant NO_3^- content was much higher in E1, with nitrate N accounting for 15 and 30 % of total N respectively as compared to 5–6 % (at 10 mM) and 7–8 % (at 20 mM) in the two other experiments. Similar to plant NO_3^-, total N increased up to 20 mM NO_3^-. In contrast with plant NO_3^-, N accumulation was lower in E1. The highest SUC levels were found at 2 mM NO_3^- and SUC decreased at higher NO_3^- regimes, while GLU and FRU contents increased up to 5 mM NO_3^- (Table 2). The variation in the plant total sugar content (SUC+GLU+FRU) with the fertilization regime was comparable in the three experiments, with an increase from 0.5 to 5 mM NO_3^- and lower values at higher NO_3^- regimes (Fig. 2a). However, the proportion of SUC in the sugar pool decreased sharply with NO_3^- nutrition up to 10 mM, and appeared fairly different from one experiment to another, with a much higher proportion of SUC in E3 (Fig. 2b). Total C, P, K, Ca and Mg contents decreased, on a fresh weight basis, with increasing NO_3^- concentration in the nutrient solution (Table 2). The plant C content decreased exponentially with the NO_3^- regime and was highest in E3. In that experiment, on a dry weight basis, the C content did not change with NO_3^- regime, while it decreased strongly with NO_3^- nutrition in E1. On a dry weight basis, P and K plant content increased with increasing NO_3^- concentrations (data not shown). The increased K^+ concentration in nutrient solutions at 20 mM NO_3^- did not have a systematic effect on plant K accumulation in this regime, as plant K contents were not higher at 20 mM NO_3^- compared to those at 10 mM NO_3^-, in the E2 and E3 experiments. With pooled data from the three experiments, many of the plant components appeared significantly correlated. The most highly correlated components (on a fresh weight basis), were GLU and FRU (r=0.95; 0.75; 0.76 in resp. experiments E1, E2

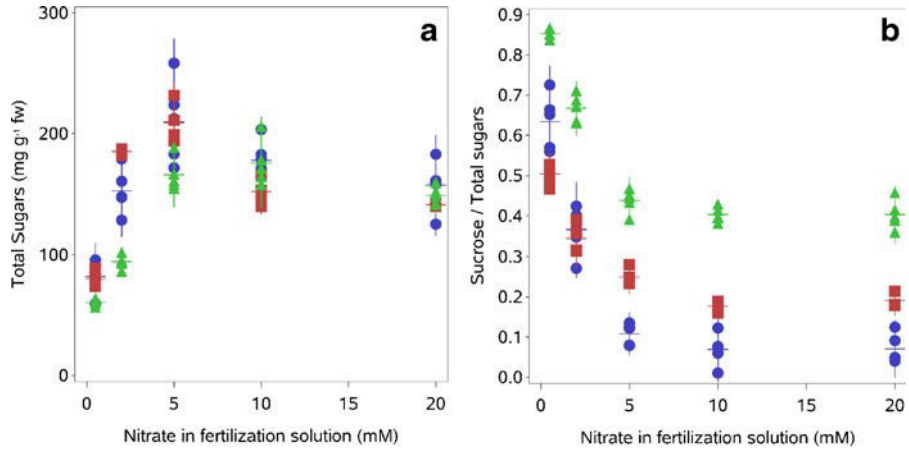

Fig. 2 Total sugars (SUC+GLU+FRU, a) and ratio of SUC/total sugars (b) in leaves of lettuce plants grown under different N regimes in three independent experiments (dots E1; squares E2; trianglesE3). Horizontal bars represent the mean and vertical bars the standard error of the mean

and E3 respectively), Ca and C (r=0.92; 0.93; 0.80), SUC and C (r=0.93; 0.79; 0.81) and Mg and K (r=0.5; 0.79; 0.89). A strong negative correlation was also found between SUC and NO_3^- plant content (r=−0.75; −0.91; −0.87). On pooled data, the plant water content was highly correlated with the C content (r=−0.96) and SUC content (r=−0.81). SUC was highly negatively correlated with FRU (r=−0.64) but only slightly with GLU (r=−0.39).

Disease development at different N nutrition levels

Overall lesion sizes were different for the two fungal pathogens, and for a given pathogen, they were significantly different from one experiment to another (Fig. 3a). Lesion size was greater in E1 for *B. cinerea*. Lesions caused by *S. sclerotiorum* appeared more severe in E2, whereas in that experiment lesions caused by *B. cinerea* were minimal. Necrotic lesions developed usually as an ovoid shape, being slightly wider towards the distal end of the leaf (Fig. 3b,c).

A GLM analysis relating the size of lesions caused by *B. cinerea* to the NO_3^- fertilization and experiment effects, along with their interaction, yielded a highly significant model (r^2=0.41, 1,349 df, F=175, p<0.0001, Fig. 4a). Response curves were best fitted by a logistic function in E1 and a power function in E2 and E3. Whatever the model, the lesion size increased significantly in each experiment between 10 and 20 mM NO_3^- in the nutrient solution. Experimental factors generated a 2.3-fold variation in disease variability. A small but significant (p<0.0001) part of the observed lesion variability was due to a "strain" effect. When added to the GLM model, this third factor improved the model r^2 very slightly (0.44 vs 0.41). The lesions caused by strain BC1 were 34 % greater on average than those caused by the other five strains tested (1,875 mm^2 vs 1,394 mm^2, data not shown). No significant differences were found between the five other strains (data not shown). The "strain×experiment" effect was not statistically significant (p=0.07) and individual tests for each strain all indicated significantly greater lesion sizes in experiment E1. Strain variability from *B. cinerea* was not considered further.

The same GLM model fitting performed on lesions caused by *S. sclerotiorum* yielded comparable results: there was a highly significant effect of N fertilization level on lesion size (r^2=0.58, 224 df, F=58, p<0.0001),

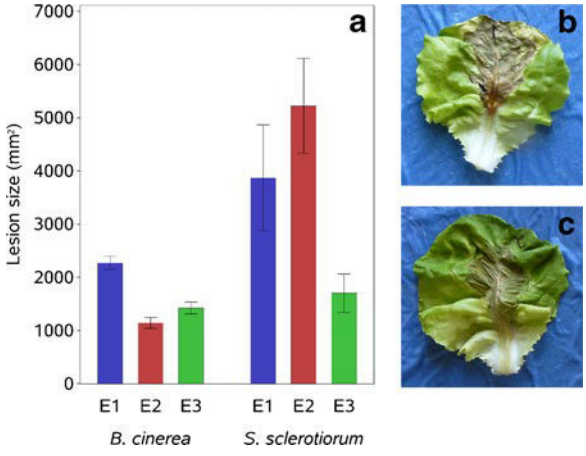

Fig. 3 Overall disease assessment with pooled data, independently of NO_3^- regimes. a Lesion size at 6 days (*Botrytis cinerea*) or 5 days (*Sclerotinia sclerotiorum*) after infection by mycelia disks, in three independent experiments. Bars represent the mean±standard deviation. b Lesions caused by *Botrytis cinerea* on lettuce leaves. c Lesion caused by *Sclerotinia sclerotiorum* on lettuce leaves

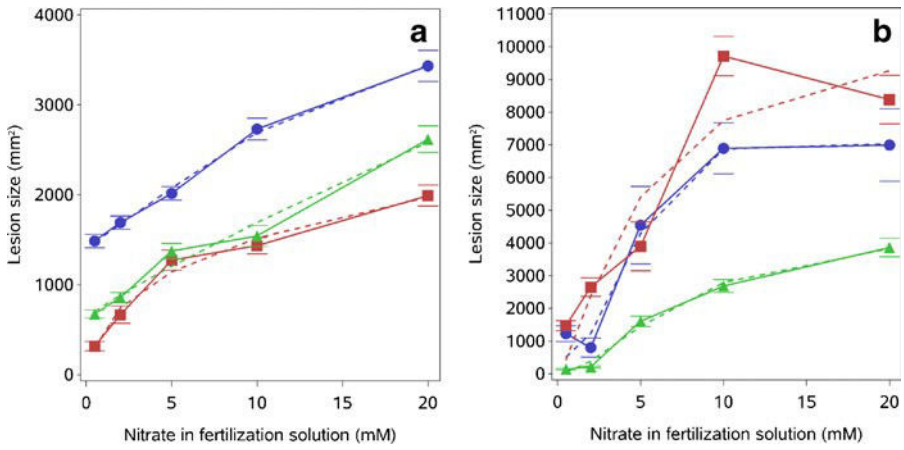

Fig. 4 Size of leaf lesions caused by *Botrytis cinerea* (a; 6 days after inoculation) and *Sclerotinia sclerotiorum* (b; 5 days after inoculation) under different NO_3^- fertilization regimes, in three independent experiments (dots E1; squares E2; triangles E3). Each symbol is the mean of 90 lesion measurements for *B. cinerea* (pooled data for 6 strains) or 15 lesion measurements for S. Sclerotiorum (1 strain). Horizontal bars represent the standard error of the mean. Continuous lines join the symbols, dotted lines are statistical fits by non-linear functions. *Botrytis cinerea* response curves were fitted by a logistic function (E1) and a power function (E2 and E3). *Sclerotinia sclerotiorum* response curves were fitted by a logistic (E1) and a Hill function (E2 and E3)

with also highly significant effects of the individual experiments and the NO_3^-×experiment interaction. Lesion size increased with NO_3^- concentration in the fertigation solution, by a factor of 6.8 from 950 mm^2 at 0.5 mM NO_3^- to 6,500 mm^2 at 20 mM NO_3^- (Fig. 4b). Response curves were best fitted by a logistic function in experiment E1 and a Hill function in the two others. In contrast with what was observed for B. cinerea however, disease reached an asymptote at 10 mM and above: the disease was significantly greater at 20 mM NO_3^- in E3, but not statistically different between the two regimes in the first experiments.

Relationship between plant nutrient content and lesion development

Leaf C and NO_3^- contents (mg g^{-1} fresh weight) were not normally distributed and so were log-transformed. Amongst the plant elements measured, only the N, logC, $logNO_3^-$, SUC contents and the N/C ratio were consistently correlated with lesion areas ($r^2>0.7$) in all three experiments, for the two diseases independently (Table 3). The total sugars, FRU, GLU or hexoses (FRU + GLU) contents, as well as the P, and Mg contents were not significantly correlated with any of the two diseases, while K and Ca were correlated, but not in all experiments, with lesions caused by *B. cinerea* and *S. sclerotiorum*, respectively (Table 3). Additionally, the N/C ratio and the FRU/total sugars ratio also appeared highly significant in regressions for lesion areas for the two pathogens, in all three experiments (data not shown).

As the four identified individual factors (N, logC, $logNO_3^-$, SUC) were correlated, we further analysed the data with type I SS assessment in repeated GLM models (24 possible combinations with 4 variables, see "Materials and methods" section). For *B. cinerea*, the model with these four factors was significant ($r^2=0.70$, 14 df, $F=5.8$, $p=0.01$). The most significant factors were $logNO_3^-$ ($p=0.04$ with type III SS) and N ($p=0.08$ with type III SS). Analyzing the type I SS analysis, the logC and SUC effects appeared not to be independent of the $logNO_3^-$ effect. The N effect was independent from the $logNO_3^-$ effect, and in regression models where both NO_3^- and N were introduced, the parameter related to N was negative. For *S. sclerotiorum*, the model was highly significant ($r^2=0.93$, 14 df, $F=31$, $p<0.0001$). The most significant factors were SUC ($p=0.004$ with type III SS) and N ($p=0.06$ with type III SS). The NO_3^- effect was not independent of the SUC effect, and the N effect was independent of the three other effects. Here again, the parameter associated to N in models was negative when the factor NO_3^- was introduced. The effect of logC was, for the two diseases, in most regressions not independent of the other effects.

Individual regressions are presented for $logNO_3^-$, N and SUC in Fig. 5. In all the experiments, the best model

Table 3 Coefficients of determination r^2 and probabilities of significance of linear models relating lesion size to concentrations of elements in plants (in milligrams per gram fresh weight)

		N		NO3		C		SUC		GLU		FRU		P		K		Mg		Ca		Hexoses		Tot. Sugars	
		r^2	p	r^2	p	r^2	p	r^2	p	r^2	p	r^2	p	r^2	p	r^2	p	r^2	p	r^2	p	r^2	p	r^2	p
B. cinerea	E1	0.96	0.004	0.90	0.014	0.85	0.026	0.74	0.063	0.03	0.75	0.17	0.49	0.01	0.88	0.03	0.76	0.78	0.048	0.91	0.011	0.11	0.59	0.09	0.63
	E2	0.95	0.005	0.96	0.004	0.86	0.024	0.83	0.031	0.16	0.49	0.07	0.65	0.93	0.008	0.96	0.003	0.55	0.15	0.86	0.022	0.01	0.92	0.23	0.41
	E3	0.76	0.051	0.83	0.032	0.77	0.051	0.83	0.033	0.08	0.62	0.52	0.16	0.72	0.067	0.67	0.09	0.66	0.096	0.41	0.24	0.35	0.29	0.05	0.71
S. sclerotiorum	E1	0.84	0.028	0.97	0.002	0.96	0.004	0.96	0.004	0.12	0.57	0.33	0.31	0.01	0.88	0.25	0.38	0.99	3E-04	0.96	0.004	0.23	0.41	0.04	0.75
	E2	0.73	0.064	0.80	0.041	0.78	0.047	0.92	0.009	0.34	0.3	0.01	0.89	0.55	0.15	0.73	0.064	0.28	0.36	0.68	0.089	0.06	0.67	0.44	0.22
	E3	0.83	0.031	0.97	0.002	0.93	0.009	0.92	0.009	0.13	0.55	0.69	0.08	0.92	0.01	0.84	0.019	0.82	0.032	0.51	0.17	0.47	0.19	0.1	0.61

Italic r^2 values indicate r^2 beyond 0.7

for the regression of *B. cinerea* lesions on logNO_3^- was an exponential function, with increasing disease severity at higher plant NO_3^- content (Fig. 5a). However, the fitted parameters were different from one experiment to another, and little was gained by analysing the symptoms on a plant NO_3^- basis rather than on a NO_3^- concentration in solution basis. Although a negative relationship between plant SUC content and lesion area was found in each experiment (Fig. 5b), the best fits were achieved with different functions for the various experiments. Plant N content, isolated from the other plant components, was positively correlated with B. cinerea symptoms (Fig. 5c).

For *S. sclerotiorum*, although the relationships between lesion size and plant NO_3^- were very strong in each experiment, the overall regression showed poor predictive capacity at high plant NO_3^- content (Fig. 5d). As stated earlier, there was a strong negative correlation between NO_3^- and SUC, which was closely correlated with disease intensity, either when considering individual experiments or the pooled data (Fig. 5e). The fitted functions were an exponential function for E1 and E2 experiments, and a linear function for E3. However, the MSEP of the linear and exponential models in this later experiment were very close. The relationship between disease intensity and SUC explains the relatively weak symptoms in 2010, where SUC plant contents were high, regardless of the NO_3^- fertigation regime. As for *B. cinerea*, the plant N content had poor predictive capacity on the pooled data, but total N was positively correlated with disease, when including the nitrate fraction of total N (Fig. 5f). Relating the disease to the proportion of SUC within the total sugars pool did not greatly affect the observed effect of SUC alone on *B. cinerea* or *S. sclerotiorum* (Fig. 6a,b). However, although FRU alone was very poorly correlated with disease intensity, the ratio of FRU on total sugars fitted very well the data, following an exponential function for *S. sclerotiorum* (Fig. 6c,d). Conversely, the ratio of GLU/total sugars did not give any fit to the data (data not shown).

Discussion

Examining the effect of fertilization treatments on the plants, we observed that NO_3^- concentration in the nutrient solution limited growth up to 10 mM NO_3^-, following a typical plateau curve. Doubling the

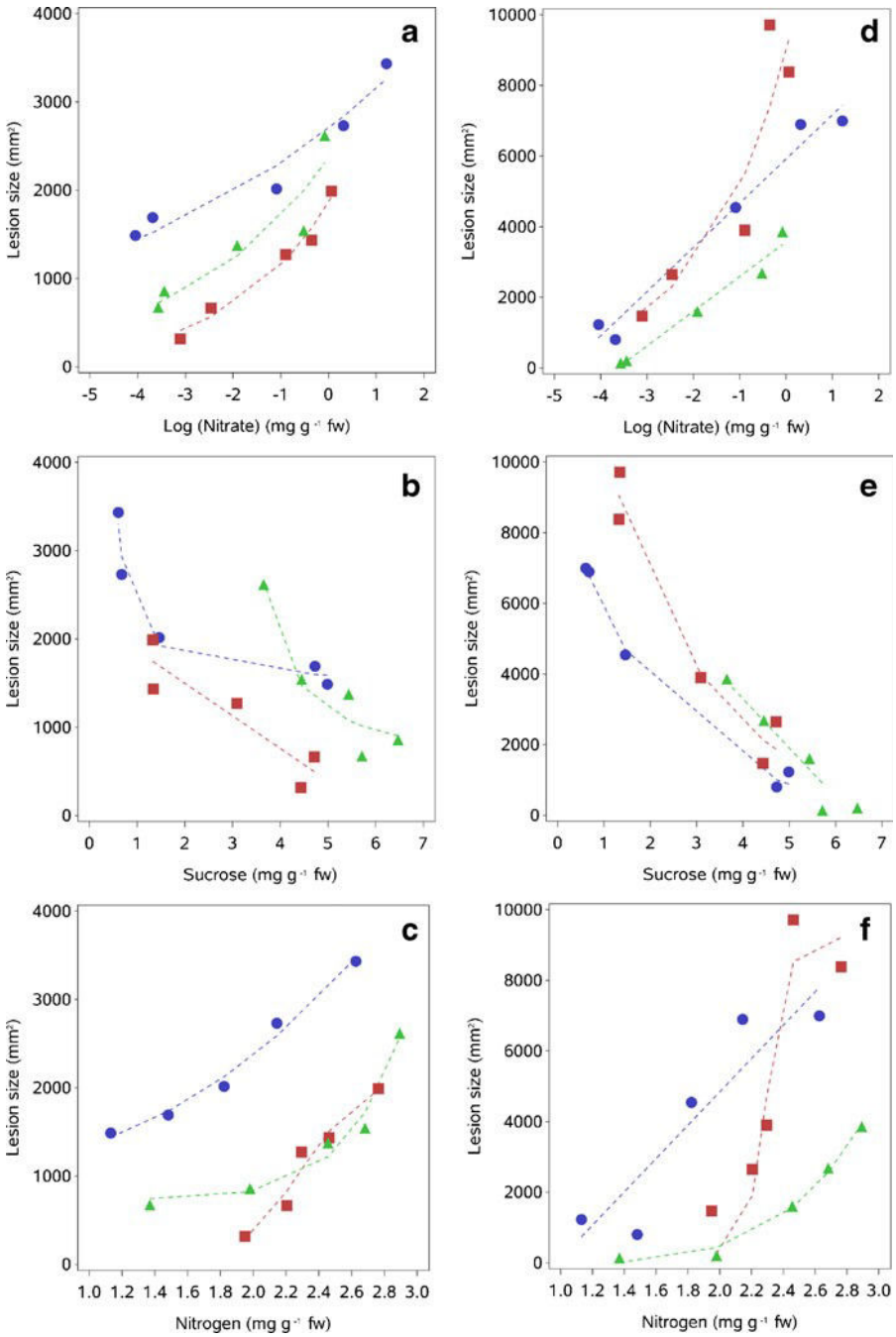

Fig. 5 Size of leaf lesions caused by *Botrytis cinerea* (a, b and c; 6 days after inoculation) and *Sclerotinia sclerotiorum* (d, e and f; 5 days after inoculation) on lettuce plants as a function of leaf contents (in milligram per gram fresh weight). a, d log (NO_3^-); b, e SUC; c, f N. Each symbol relates the mean of five plant analyses to the mean of 90 lesion measurements for *B. cinerea* (pooled data for 6 strains) or 15 lesion measurements for *S. Sclerotiorum* (1 strain). Different symbols and colours represent independent experiments (dots E1; squares E2; triangles E3). Continuous lines join the symbols, dotted lines are statistical adjustments by non-linear functions. Details of fitted functions are given in the text

concentration from 10 to 20 mM NO_3^- did not increase growth, but led to an accumulation of NO_3^- and of reduced N. These results are in line with previous reports showing NO_3^- accumulation in lettuce leaves when its uptake rate is higher than its assimilation rate (Maynard et al. 1976). The observed consequences of N shortage or excess on plant C and dry matter accumulation relative to the plant water content have also been reported before (Seginer 2003; Burns 1994; Cardenas-Navarro et al. 1999). Less is known about the effect of nutrition on lettuce sugar content, but our observations seem consistent with general considerations on the coordination of C and N metabolism (Stitt et al. 2002). In the lowest NO_3^- regime (0.5 mM

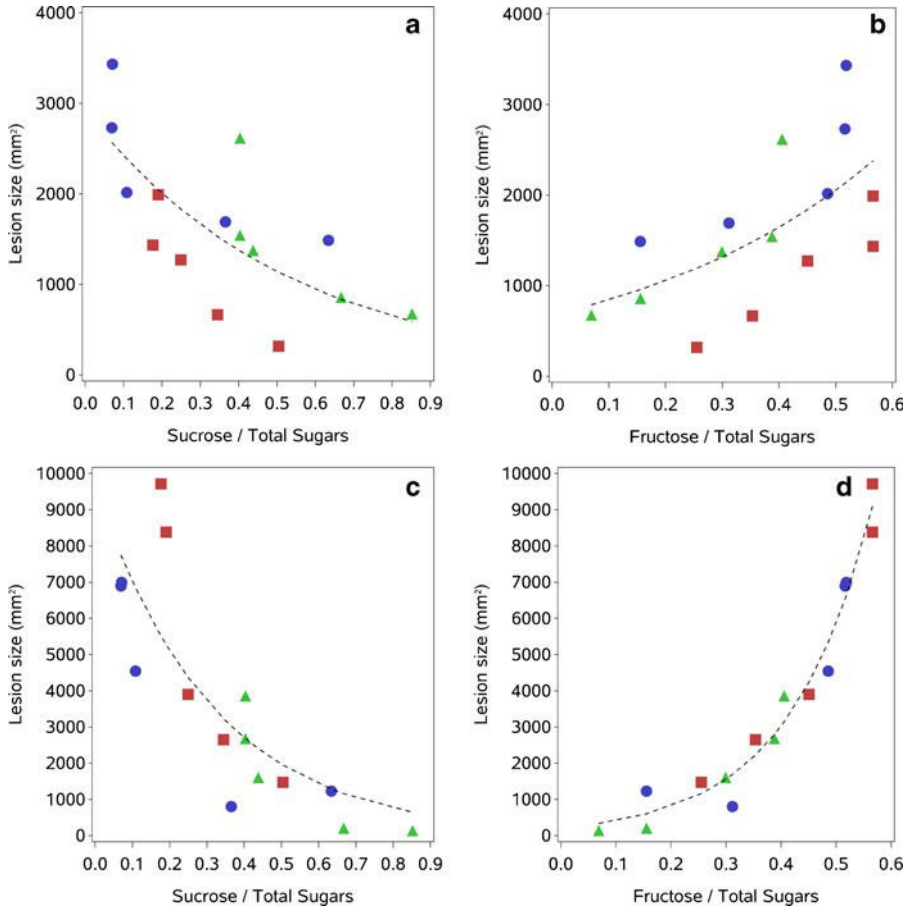

Fig. 6 Fungal lesions caused by *Botrytis cinerea* (a, b) *Sclerotinia sclerotiorum* (c, d) related to the ratio of SUC (a, c) and FRU (b, d) on total sugars (SUC+FRU+GLU). Each symbol relates the mean of five plant analyses to the mean of 90 lesion measurements for *B. cinerea* (pooled data for 6 strains) or 15 lesion measurements for S. Sclerotiorum (1 strain). Different symbols and colours represent independent experiments (dots E1; squares E2; triangles E3). Dotted black lines are fitted exponential functions on the pooled data for the three experiments ($y=k1\times \exp(k2\times X)$, with X=SUC/total sugars in a and c, X=FRU/total sugars in b and d; a k1=2,926, k2=−1.88; F=55.8, $p<0.0001$, 15 df; b k1=676, k2=2.2, F=45.1, $p<0.0001$, 15 df; c k1=9,659, k2=−3.2, F=44.1, $p<0.0001$, 15 df; d k1=214, k2=6.6; F=527, $p<0.0001$, 15 df)

NO_3^-), growth—and probably photosynthesis—were severely restricted, and the available C was stored as SUC as starch accumulation in lettuce is limited (Seginer 2003). For wheat, higher SUC content at low NO_3^- availability has already been reported (Lawlor et al. 1987). At higher regimes (2–5 mM NO_3^-), growth was still N-limited, and the availability of sugars might have reflected the availability of photosynthetic products that were not involved in proteins and organic acids metabolisms. Sugars are also known to maintain osmolarity in lettuce in the absence of NO_3^- (Blomzandstra and Lampe 1985). Significant differences were found between our experiments. Leaf NO_3^- and potassium content at 10 and 20 mM NO_3^- were higher in experiment E1, while the SUC content relative to the total sugar pool was much higher in E3 (Table 2). The high evaporative demand observed during E1 called for increased frequency and duration of irrigation events and, as a consequence of the constant ion concentrations in solutions, the plants were fed with relatively more NO_3^- and potassium in that experiment than in the others. Additionally, we consistently observed in the experiments a strong negative correlation between nitrate and SUC contents. These contrasting environments, in association with the nitrate nutrition treatments, allowed us to obtain a large range of C and N plant contents, which was helpful to assess the influence of primary metabolites on disease intensities.

Several differences were observed between *B. cinerea* and *S. sclerotiorum* infections. First, tissue colonization was much faster for *S. sclerotiorum*, which caused larger lesions in 5 days than *B. cinerea* in 6 days (Fig. 3). As lesions of both fungi appeared at approximately the same time, 24–36 h after inoculation, this was due to a higher mycelial expansion rate of *S. sclerotiorum*. The ability of *S. sclerotiorum* to achieve a quicker colonization of its host than *B. cin- erea* had already been noticed on tomato stems (Gerlagh et al. 1996) and sunflower leaves (Dulermo

et al. 2009), and mirrors the generally higher growth rate of *S. sclerotiorum* in vitro. However, although the experimental methods and environmental conditions in the growth chambers before and after inoculation were similar, the experimental effect on lesion size was not the same for the two pathogens. Maximum lesion development for the two fungi was not found in the same experiments: lesions caused by *S. sclerotiorum* were larger in E2, while those caused by *B. cinerea* were larger—for all the tests on six strains—in "E1". The incubation conditions which conferred the plant more efficient defence against *B. cinerea* were thus not necessarily optimal against *S. sclerotiorum*, and vice versa. Furthermore, response curves of disease severity to host NO_3^- nutrition were not identical: although both diseases were favored by high N fertilization, there was no increase of symptoms between 10 mM and 20 mM NO_3^- for *S. sclerotiorum*, while a significant increase in disease severity was found between 10 and 20 mM NO_3^- for *B. cinerea*. It can therefore be concluded that symptoms of *B. cinerea* and *S. sclerotiorum* on lettuce grown at various N levels differ significantly, although higher N fertilization generally increased the severity of both disease.

Another objective of our study was to assess the effect of plant composition on disease development, regardless of fertilization treatments. Regressions of disease severity on several plant components gave a much better fit to the data than did the NO_3^- treatments. The severity of both diseases was related to the plant's content of NO_3^-, total N, SUC and total C. The regressions on these four components explained 70 and 93 % of overall disease variability in all three experiments for *B. cinerea* and *S. sclerotiorum*, respectively. For *B. cinerea*, the most significant factor was plant NO_3^- content, while SUC and C effects on lesion size could not be separated statistically from the NO_3^- effect. There was an influence of total N independent of that of NO_3^-, illustrated by more severe *B. cinerea* infections in experiments where total N was lower and the nitric fraction of total N higher. Lesion size was negatively correlated with the C/N ratio, which disagree with observations showing that disease incidence after inoculation of *B. cinerea* spores on tomato leaves was positively correlated to C/N (Hoffland et al. 1999). These results as a whole show that total N, total C or their ratio, when considered alone, are not reliable predictors of plant susceptibility to *B. cinerea*. We show here that, in lettuce, NO_3^- accumulation in leaves was associated with higher disease.

In contrast with what was observed for *B. cinerea*, susceptibility of lettuce leaves to *S. sclerotiorum* was more significantly linked to sugars than NO_3^-. We observed a negative exponential relationship between leaf SUC content and lesion size, which was quite similar in the three independent experiments (Fig. 5e). Although FRU and GLU contents were negatively correlated with SUC, we did not observe any positive correlation between hexoses and susceptibility. Thus, either SUC or other metabolites whose variations mirror those of SUC are possibly linked with a decrease of lettuce susceptibility to *S. Sclerotiorum*, or a decreased aggressiveness of the pathogen. Also, ratios representing the proportion of SUC and FRU on the total sugar pool were closely linked to lesion size. It has been shown on sunflower cotyledons that *S. sclerotiorum* depletes the host from its SUC and FRU, but not its GLU (Jobic et al. 2007), during the course of infection. The involvement of sugars in plant defence has been increasingly recognized in recent years. Sugars may act either by providing C skeletons for the synthesis of defensive secondary metabolites, and/or as signals for the induction of defence-related pathways (Berger et al. 2007; Bolton 2009). In several plant–fungus interac tions, SUC is known to be mobilized upon infection via an increase in acid invertase activities of both plant and fungal origins (Berger et al. 2004; Jobic et al. 2007). The use of SUC as a C source for the synthesis by the plant of defensive metabolites first requires its cleavage into FRU and GLU. If a significant involvement of SUC, via its initial transformation into hexoses, was necessary for fuelling the defence, then a lower susceptibility should have been found also at high hexose content; however neither GLU nor FRU were directly correlated to any of the two diseases. SUC is known to be a specific inductor of flavonoid biosynthesis in Arabidopsis (Solfanelli et al. 2006), and SUC-supplemented tissues activate the synthesis and accumulation of flavonoids in lupins challenged with Fusarium oxysporum (Morkunas et al. 2011). An enhanced production of anti-fungal metabolites in plants containing high SUC concentrations is compatible with our observations, SUC playing the role of a signaling molecule rather than that of a C source for polyphenol synthesis. It also appeared that the respective proportions of FRU and SUC in the total sugar pool were even more closely related to the diseases than individual sugars. SUC and FRU were negatively correlated. This suggests that an induction of defence mediated by plant

sugars might be controlled by the relative content in SUC and FRU, rather than their absolute concentration in the infected tissues. Although a negative correlation between SUC content and lesion size was also found for *B. cinerea*, the relationship with SUC gave a poorer fit to the data than NO_3^- did.

The main conclusion of our work is that, at least for one of the two necrotrophs in this study, the effect of N fertilization on susceptibility appeared statistically mediated by the metabolism of sugars. As sugar accumulation is specific in each plant species, and for a given species, in its different organs, it might explain why the variation of plant susceptibility to necrotrophs in response to N fertilization is complex. Genotypes with different sugar accumulation should be useful to test a general relationship between specific sugars and resistance to necrotrophs in lettuce and other species.

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Chapter 4

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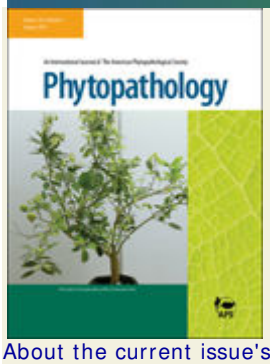

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Nitrogen fertilization of the host plant influences production and pathogenicity of *Botrytis cinerea* secondary inoculum

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The influence of nitrogen (N) nutrition on a plant's susceptibility to *Botrytis* and other pathogens is well documented However, little is known of possible effects on sporulation of the pathogen on diseased tissue and on the pathogenicity of resulting secondary inoculum. To address this question, sporulation by two strains of *Botrytis cinerea* was quantified on tomato plants produced under different N irrigation regimes with inputs of nitrate varying from 0.5 to 45 mmol.L^{-1} (mM). Sporulation decreased significantly ($P<0.05$) with increasing nitrate fertilization up to 15-30 mM. The secondary inoculum was collected and used to inoculate pruning wounds on tomato plants produced under a standard fertilization regime. Pathogenicity of the spores was significantly influenced by the nutritional status of their production substrate. Disease severity was highest with spores produced on plants with very low or very high N fertilization (0.5 or 30 mM nitrate). It was lowest for inoculum from plants with moderate levels of N fertilization. These results suggest that it may be possible to find an optimum level of nitrogen fertilization to reduce the production of secondary inoculum and its pathogenicity to tomato.

Nitrogen fertilization of the host plant influences production and pathogenicity of *Botrytis cinerea* secondary inoculum

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ABSTRACT

The influence of nitrogen (N) nutrition on a plant's susceptibility to *Botrytis* and other pathogens is well documented However, little is known of possible effects on sporulation of the pathogen on diseased tissue and on the pathogenicity of resulting secondary inoculum. To address this question, sporulation by two strains of *Botrytis cinerea* was quantified on tomato plants produced under different N irrigation regimes with inputs varying from 0.5 to 45 mmol $NO_3^-L^{-1}$ (mM). Sporulation decreased significantly ($P<0.05$) with increasing N fertilization up to 15-30 mM $NO_3^-.L^{-1}$. The secondary inoculum was collected and used to inoculate pruning wounds on tomato plants produced under a standard fertilization regime. Pathogenicity of the spores was significantly influenced by the nutritional status of their production substrate. Disease severity was highest with spores produced on plants with very low or very high N fertilization (0.5 or 30 mM NO_3^- L^{-1}). It was lowest for inoculum from plants with moderate levels of N fertilization. These results suggest that it may be possible to find an optimum level of nitrogen fertilization to reduce the production of secondary inoculum and its pathogenicity to tomato.

Additional key words: Gray mold, sporulation, fertilization, Tomato, *Solanum lycopersicum*.

INTRODUCTION

Sporulation is essential for the reproduction and spread of many fungi. For those that are airborne plant pathogens, the kinetics and abundance of spore production play a key role in the development of epidemics (9,33). Fungal sporulation can be influenced by many factors, including the availability of nutrients (7,15,34,60). Some fungi have specific carbon (C) and nitrogen (N) requirements, while for others, sporulation is triggered by nutrient depletion (8,12). For most fungi, the specific substrate also influences spore production. Much information has been generated to optimize substrate composition for mass producing fungi of industrial interest such as biocontrol agents against plant pathogens (17,50) and insects (19,24,26,44). The effects of culture conditions and host substrate on initial spore production have also been documented for certain plant pathogenic fungi (22,32,35,42). Surprisingly little information is available on possible effects of plant fertilization on subsequent sporulation of pathogens although it is known to modify the concentration of nutrients available to plant pathogens and to affect plant susceptibility (23).

The composition and type of nutrient substrate has also been shown to affect the pathogenicity of spores produced *in vitro* by plant pathogenic *Colletotrichum truncate*, *Fusarium avenaceum* and *Phytophthora infestans* (43,54,58); and the entomopathogen *Beauveria bassiana* (4). Aggressiveness may be attenuated when spores are produced under conditions of nutrient stress, as exemplified by *Bipolaris sorokiniana* conidia (2), or increased with increasing availability of key nutrients such as glucose, as exemplified by *Botrytis cinerea* (38). However, to our knowledge no information is available on effects of plant fertilization on the pathogenicity of fungal spores produced on diseased tissues.

Botrytis cinerea, the causal agent of gray mold, is responsible for severe losses in many crops including tomato (36,45,53). In favorable conditions, this fungus can produce a large

quantity of spores on diseased tissue (35) that are easily dispersed and have a key role in the development of gray mold epidemics (10). The control of gray mold is highly dependent on fungicide applications and much research is dedicated to developing alternative control methods (13). A possible alternative could consist in reducing sporulation with the help of biocontrol agents (27) or the use of uv-filtering films (35). Another possibility to reduce sporulation might be through the manipulation of plant fertilization. Several studies have shown that high N nutrition of the host plant promotes the sporulation of several fungi, including *B. cinerea* on sweet basil (55). Although no information is available for the sporulation of *B. cinerea* on tomato, previous studies have shown that high N nutrition reduces the susceptibility of the plant (21,31,52). Furthermore, we were unable to find information regarding the effect of N fertilization of plants on the pathogenicity of resulting secondary inoculum of *B. cinerea*.

The objectives of this study were (1) to assess the effects of N nutrition of tomato on sporulation of *B. cinerea* on diseased tissue and on pathogenicity of the resulting secondary inoculum, and (2) to correlate these effects with modifications in plant tissue composition.

MATERIALS AND METHODS

Production of plants under different N fertilization regimes.

Tomato plants (*Solanum lycopersicum* var. *esculentum* cv Swanson) were grown from seeds in $1cm^3$ rock wool cubes in a heated greenhouse and transferred ten days after sowing to rock wool blocks as previously described (33). The plants were irrigated with a standard commercial greenhouse nutrient solution the first month and then transplanted to 2-L pots filled with a mixture (1:1 V/V) of vermiculite and pozzolana (inert crushed volcanic rock) to start the different N fertilization treatments (40 plants per N level). Five levels of nitrate concentrations (0.5, 5, 15, 30 and 45 $mmol.L^{-1}$ – abbreviated as mM in the rest of this paper) were evaluated to span a wide range from severe N deficiency to heavy excess. To minimize possible complex nutritional effects on the physiology of the plants, two constraints were taken into account when designing the chemical composition of the nutrient solutions: (i) keep other elements (particularly P, K, Ca and Mg) at a constant concentration in the different N treatments, as they may also influence plant susceptibility to pathogens (23) and (ii) keep the total electrical conductivity similar in all treatments, as this is known to influence the physiology of tomato (28). The composition of the 15 mM nitrate solution was adapted from nutrient solutions used by growers in commercial tomato glasshouses. It contained three types of nitrate salts (KNO_3,10 mM; $Ca(NO_3)_2$,1.75 mM; and NH_4NO_3,1.5 mM). The total N level of that solution thus amounted to 15 mM as nitrate plus 1.5 mM as ammonium. For solutions with lower N concentrations, no ammonium was used and Cl^- salts (a combination of KCl, $CaCl_2$ and NaCl) were substituted to nitrate in order to maintain similar cation concentrations. Designing solutions with higher N levels within the two above-mentioned constraints was more complex. To achieve higher N concentrations with minimal consequences for the electrical conductivity, NH_4NO_3 was added to the solutions, bringing the total N levels to 46.5 and 76.5 mM (including 16.5 mM and 31.5 mM as ammonium), respectively, for the solutions containing 30 mM and

45 mM of nitrate. To maintain the electric conductivity at the same level as in other N treatments, the addition of NH_4NO_3 was fully compensated for by the removal of NaCl in the solution with 30 mM nitrate. This was not sufficient for the highest N level. Thus, this last solution had a higher electric charge (59.5 meq of anions and 61.5 meq of cations) than the four others (44.5 meq anions and 46.5 meq of cations). Phosphorous (P, 2 mM), K (12 mM), Ca (5 mM), Mg (4 mM) and minor-elements were kept constant in all solutions.

The plants were fertigated with a drip irrigation system (one dripper per pot with one minute pulses) several times a day depending on the climatic demand. Three pots chosen at random were weighted continuously to evaluate water loss as an indicator of climatic demand. The pH was adjusted to 6 in each treatment by the addition of H_2SO_4. Plants were grown on the respective solutions for four weeks prior to use for experiments on spore production and pathogenicity of secondary inoculum of *B. cinerea*.

Tissue content assessment.

Just before inoculation, five plants were randomly selected from each N regime. For each plant, the stem and leaves were weighed separately and then dried 72h at 70°C. The tissue content was then analysed for calcium, carbon, magnesium, nitrate, potassium, phosphorous and total N; and expressed as mg per gram of dry matter as described previously (31). Soluble sugars (glucose, fructose and sucrose) were determined with an enzymatic method in a microplate reader as proposed by Gomez *et al.* (18).

Evaluation of spore production by *Botrytis cinerea* on differentially fertilized plants.

Strains and preparation of primary inoculum.

For the spore production assay, two strains of *B. cinerea* (BC1 and BC21) were used. They were shown in earlier work to differ in their level of aggressiveness on tomato (31).

Primary inoculum for both strains was produced in Petri dishes on potato dextrose agar (PDA) incubated at 21°C under cool white fluorescent light (14-hour photoperiod; 114 µmole $m^{-2}s^{-1}$). After 3 days of culture, mycelial plugs were excised from the growing margin of the colony with a cork borer and used to inoculate detached organs prepared from the plants grown under the different N fertilization treatments.

Preparation and inoculation of detached plant organs.

The plants were severed at the base and 2 cm-long segments were excised from the stems and petioles (3 each per plant). Leaf discs 2 cm in diameter, were excised from the leaves (3 discs per plant, one from each of leaf number 3, 4 and 5 – starting from the collar upward). Each sample was weighed (mg fresh weight) and then placed individually in the 3-cm diameter cap of a screw-cap tube. Each stem and petiole segment was inoculated in the center with a 5-mm diameter mycelial plug and each leaf disk with a 2-mm diameter plug, ensuring that the mycelium was in contact with plant tissue. The caps were placed in transparent polystyrene boxes over moistened filter paper, covered with lids and placed in a growth chamber under cool white fluorescent light (16-hour photoperiod; 162 µmole $m^{-2}s^{-1}$) at 20°C. For each strain of *B. cinerea*, 9 replicate samples of each type of plant organ were inoculated (3 per plant x 3 plants) and the sporulation experiment was repeated twice.

Quantification of spore production on detached organs.

Ten days after inoculation, the samples were removed from the growth chamber and 5 mL of an alcoholic solution (containing 20 % ethanol V/V and 100 µL of Tween 80 per liter) were added to each cap. A centrifuge tube was then screwed onto each cap and the samples were stored at 4°C until spore counting. Two liquid subsamples were taken from each tube and spores were counted with a Malassez haemocytometer. Spore production on each detached organ was then expressed as number of spores per gram fresh weight.

Assessment of pathogenicity of secondary inoculum

Production of secondary inoculum.

Leaf disks and stem segments excised from plants produced under four different N levels (0.5, 5, 15 and 30 mM nitrate) were inoculated and incubated as described above. Ten days after inoculation, dry spores were collected from the different organs into Eppendorf tubes using a microaspirator connected to a vacuum pump in order to recover spores without plant debris or fluids from macerated tissue. The dry spores were stored at 4°C until use for inoculation trials (4 days maximum). Immediately before plant inoculation, water suspensions were prepared from these dry spores. All inocula were filtered through 30-µm mesh sterile filters (to remove possible mycelial fragments) and adjusted to a concentration of 10^6 spores.mL^{-1}. Two such batches of secondary inoculum were produced independently to assess their pathogenicity. Spores from 14-day old cultures of both strains of *B. cinerea* on PDA were used as a reference (31).

Production of plants under a standard fertilization regime.

The pathogenicity of secondary inoculum was tested on two independent batches of tomato plants (cv Monalbo) produced under a single N regime. The plants were grown in 9 x 9 x 8cm disposable pots containing 450 mL (120g) of a commercial potting mixture (SP533, Klasmann-Deilmann, Germany) composed of blended white and black sphagnum peat (containing 5.25g of 14-16-18 N-P-K fertilizer per kg). The plants were watered daily according to their needs with a solution containing 0.8% (V/V) of a standard nutrient solution prepared by mixing 48g of 16-8-18 NPK fertilizer (EDDHA Optiplan, Duclos, France) per liter of water. They were inoculated after 9 weeks of growth, when they had approximately 9 fully expanded leaves.

Inoculation with secondary inoculum.

Four plants were used for each type of inoculum and each plant was inoculated with both strains of *B. cinerea*. Four leaves were removed on each plant, leaving 10 mm petiole stubs on the stems to foster the establishment of the pathogen, and 10µL aliquots of spore suspension were applied to each pruning wound. Wounded petioles of leaves 5 and 7 were inoculated with strain BC1 and those from leaves 6 and 8 with strain BC21.

Disease assessment.

All plants were incubated in a growth chamber with a 16h (162 µmole $m^{-2}s^{-1}$) photoperiod at 21°C and relative humidity above 90%. During this period, plants were watered once a day. The initiation and length of the resulting stem lesions was monitored daily for 7 days after inoculation. The area under the disease progress curve (AUDPC) was computed as described by Lecompte *et al.* (31) during the period from the third to the seventh day after inoculation and used for statistical analyses. The pathogenicity assessment experiment was repeated twice.

Data analysis.

Statistical analyses were carried out using Statistica software (Statsoft Inc., Tulsa, OK, USA). The ANOVA / MANOVA module was used for analyses of variance and multiple comparisons of means (tests of Neuman and Keuls). A "trial effect" was tested as all experiments were carried out twice independently. No significant effect was detected and the data were pooled for further statistical analyses. Data on spore production were log-transformed prior to the analyses to stabilize the variance (57). Correlation analyses were carried out with the Multiple Regression module.

RESULTS

Influence of plant nutrition on spore production by *Botrytis cinerea* on diseased leaves and stems.

Both strains of *B. cinerea* included in this study sporulated abundantly on the two types of plant tissue tested (Fig. 1). Despite their known difference in aggressiveness on tomato (31), their level of spore production (expressed as number of spores per gram of fresh tissue 10 days after inoculation) did not differ significantly (P=0.36). Sporulation of both strains, was significantly affected by the type of plant tissue colonized (P<0.001), being greater on leaf disks than on stem segments. Spore production was also significantly affected (P<0.001) on both plant tissues by the level of N fertilization provided to the tomato plants during their growth prior to inoculation. For both strains, spore production globally decreased with increasing plant N fertilization levels up to 15-30 mM (Fig. 1).

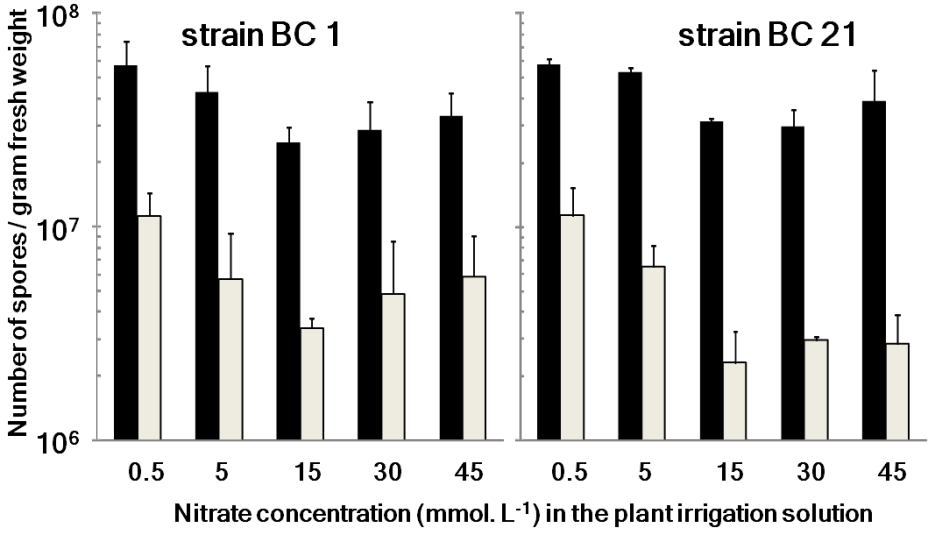

Figure 1: Effect of N nutrition on sporulation of two strains of *Botrytis cinerea* on leaf disks (■) and stem segments (□). The data represent the average number of spores produced in 10 days per gram fresh tissue. Bars indicate the standard error of the mean (two independent tests x 5 replicates per test). For a given strain and type of plant tissue, different letters indicate significant differences among fertilization levels (Test of Newman-Keuls, P<0.05).

Influence of plant nutrition on the weight and composition of leaf and stem tissues.

The fresh and dry weight of both leaves and stems was significantly increased ($P<0.001$ in both cases) by N fertilization up to 15 mM nitrate and then tended to decrease at higher N rates (Table 1). Leaf content of N, C, P, and Mg was greater than that in stems but leaves were lower in NO_3^-, K, and the three sugars (Table 1). Nitrogen fertilization had a very significant effect ($P<0.001$) on leaf tissue concentration of all measured components, while no significant effect was observed for P, K, Mg and Ca in stem tissue. The total N and NO_3^- content of stems and leaves increased with increasing N fertilization. Carbon, P, K, glucose and fructose also tended to increase in leaves but Mg and Ca tended to decrease (Table 1).

Many of the plant components measured were significantly correlated (see supplementary Tables ST1 and ST2). The most highly correlated components in both plant organs were glucose with fructose ($R=0.97$ in leaves and 0.83 in stems; $P<0.0001$), total N with NO_3^- ($R=0.93$ in leaves and 0.97 in stems; $P<0.0001$), and either of the two above sugars with total N or NO_3^- (R from 0.82 to 0.92 in leaves and from 0.47 to 0.82 in stems; $P<0.0001$). A strong negative correlation was found between these four components and Ca in leaves (R from -0.84 to -0.92; $P<0.0001$) but not in stems. A strong negative correlation was also found between sucrose and total N and NO_3^- ($R=-0.76$ and -0.73, respectively; $P<0.0001$) in stems but not in leaves.

Table 1: Effect of N fertilization on the fresh weight, dry matter and tissue content of tomato leaves and stems.

Nitrate fertilization level ($mMol.L^{-1}$)	Fresh weight (g)*	Dry weight (g)*	Tissue content (in mg per g of dry matter)*									
			N	NO_3^-	C	P	K	Mg	Ca	Sucrose	Glucose	Fructose
Leaves												
0.5	12.7 a	2,1 a	16.8 a	0.2 a	371.2 a	6.1 a	21.0 a	5.8 c	13.2 d	6.6 a	3.9 a	4.8 a
5	49.1 b	6,4 b	36.7 b	0.5 a	394.3 b	6.8 b	27.5 b	4.7 b	10.2 c	9.3 b	10.4 b	9.6 b
15	96.0 d	9,3 d	59.9 c	16.6 b	402.6 c	7.6 c	35.0 c	4.6 b	7.5 b	9.3 b	15.3 c	14.3 c
30	86.9 d	8,5 cd	66.1 d	16.6 b	414.5 d	8.6 d	29.7 b	2.7 a	5.6 a	9.0 b	21.6 d	20.7 e
45	73.9 c	7,4 bc	72.6 e	17.6 b	423.3 e	8.5 d	23.8 a	2.4 a	7.4 b	7.2 a	17.2 c	17.8 d
	P<0.001	P<0,001	P<0.001	P<0.001	P<0.001	P<0.001	P<0.001	P<0.001	P<0.001	P<0.001	P<0.001	P<0.001
Stems												
0.5	8.4 a	1,0 a	9.1 a	0.1 a	338.5 b	5.8	55.1	2.5	6.7	24.5 b	71.3 a	9.6 a
5	31.0 b	2,6 b	19.1 b	2.0 a	327.1 a	4.9	44.6	2.0	5.3	18.0 a	127.1 c	27.4 b
15	55.1 d	3,7 d	37.5 c	41.0 b	322.7 a	5.9	53.3	2.3	4.7	14.3 a	114.3 b	38.3 c
30	51.1 d	3,8 d	48.6 d	48.6 c	338.8 b	5.4	51.5	1.9	6.2	13.2 a	125.0 bc	36.1 c
45	40.1 c	3,1 c	50.6 d	55.8 d	334.6 b	8.3	49.1	2.1	8.6	11.8 a	121.3 bc	36.7 c
	P<0.001	P<0,001	P<0.001	P<0.001	P<0.001	P=0.80	P=0.81	P= 0.59	P= 0.343	P<0.001	P<0.001	P<0.001

* Data are means of five replicates per treatment. For a given column and plant tissue , the P value corresponds to a one-way analysis of variance (fertilization effect) and numbers followed by different letters are significantly different (Newman-Keuls tests; P=0.05)

Relationship between tissue content and spore production.

Significant correlations ($P<0.05$) were observed between spore production by *B. cinerea* on detached organs of tomato plants and some of the tissue contents (Table 2). The only significant positive correlations were found between calcium content and sporulation on leaf disks ($R=0.96$) and between sucrose and sporulation on stem segments ($R=0.94$). Significant negative correlations (R values between -0.89 and -0.92) were found between sporulation on leaf disks and several mineral elements as well as glucose, fructose and total sugars. In contrast, fructose and total sugars were the only components having a significant negative correlation ($R=-0.99$ and -0.91, respectively) with sporulation on stem segments.

Table 2: Correlation between tissue content and spore production by *Botrytis cinerea* on leaf disks and stem segments.

Tissue content	Leaf disks		Stem segments	
	R^y	P value	R	P value
N	-0.91	0.033	-0.83	0.080
NO_3^-	-0.92	0.025	-0.78	0.117
C	-0.81	0.095	0.47	0.418
P	-0.87	0.056	-0.19	0.766
K	-0.78	0.123	0.29	0.635
Mg	0.68	0.207	0.59	0.298
Ca	0.96	0.011	0.17	0.780
Sucrose	-0.56	0.322	0.94	0.019
Glucose	-0.92	0.027	-0.85	0.065
Fructose	-0.89	0.042	-0.99	0.001
Total sugars[x]	-0.92	0.026	-0.91	0.030

[x]: sum of glucose, fructose and sucrose

[y]: Correlation analyses were performed on pooled data from two independent sporulation tests, each with two strains of *B. cinerea*. Tissue content was expressed in mg per gram of dry matter and spore production was expressed as spore number per gram of fresh weight

Table 3: Effect of substrate used to produce inoculum of *B. cinerea* on the time needed for symptom development (latency period in days) on the stems of tomato plants. The inoculum was produced either on leaf disks or stem segments from plants subjected to 4 levels of N fertilization.

Nitrate fertilization ($mmol.L^{-1}$)	**BC1**		**BC21**	
	Leaf disks	**Stem segments**	**Leaf disks**	**Stem segments**
0.5	4.00 ± 0.1	3.53 ± 0.1	4.19 ± 0.1	4.18 ± 0.1
5	4.25 ± 0.2	4.06 ± 0.3	4.28 ± 0.0	4.06 ± 0.3
15	4.18 ± 0.0	3.81 ± 0.1	4.00 ± 0.0	4.28 ± 0.2
30	4.12 ± 0.1	3.62 ± 0.2	4.14 ± 0.2	3.87 ± 0.1

Data are means of five replicates per treatment.

Development of stem cankers.

Canker development (AUDPC) was highly affected by the level of N fertilization provided to plants that were used as substrates for the production of inoculum (Fig. 2). The effect of N was more pronounced for the more aggressive strain BC1 ($P<0.001$) than for the less aggressive strain BC21 ($P<0.05$). Interestingly, stem cankers were almost always longer for inoculum produced on PDA than for secondary inoculum produced on plant tissue (Fig. 2).

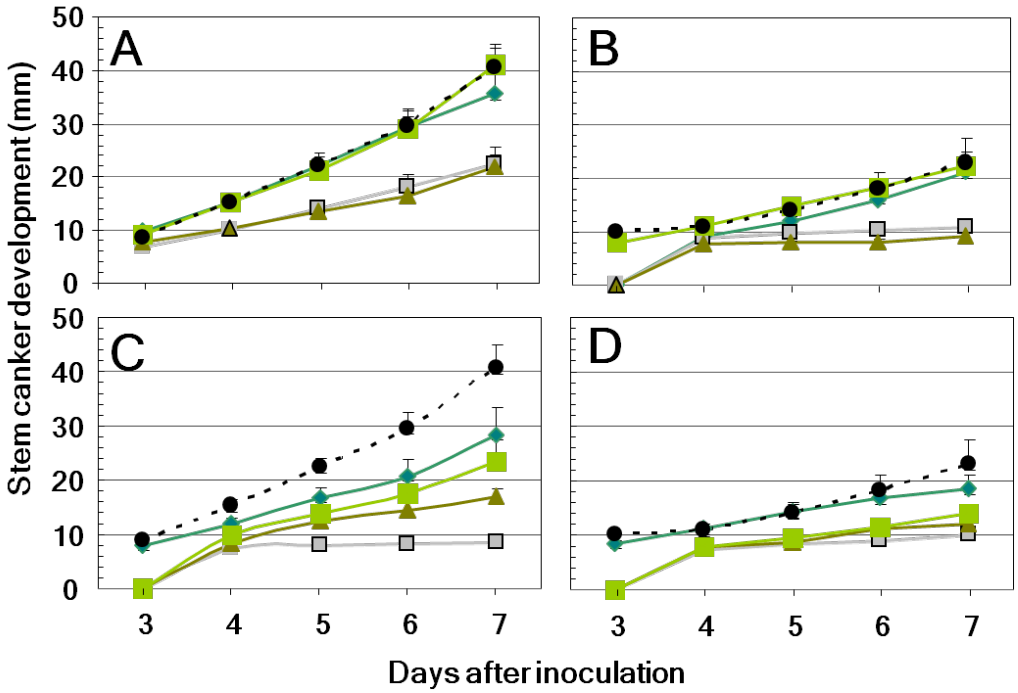

Figure 2: Effect of substrate used to produce the inoculum of two strains of *Botrytis cinerea* on the development of stem cankers on tomato plants cv Monalbo. The inoculum was produced on stem segments (A, B) or leaf disks (C, D) from plants subjected to 4 levels of nitrate fertilization (—◆—: 0. 5, —□—: 5, —▲—: 15 and —■—: 30 mmol.L^{-1}). Inoculum produced on PDA (- -●- -) was also tested for comparison. The bars represent the standard error of the means (2 inoculation sites for each of 5 plants per treatment).

The severity of lesions (AUDPC) was also significantly affected by the type of plant organ (leaf disk vs stem segment) used as substrate for spore production ($P<0.05$). To facilitate the comparison of the effect of plant N fertilization on the pathogenicity of secondary inoculum for both strains of *B. cinerea* from both types of plant substrates relative to inoculum produced on PDA, an index of relative pathogenicity was computed as the ratio:

$$RP = 100 * (AUDPC_{\text{plant substrate X}} / AUDPC_{\text{PDA}}),$$

where $AUDPC_{\text{plant substrate X}}$ assessed the severity of symptoms caused by inoculum produced on plant substrate X and $AUDPC_{\text{PDA}}$ the severity of symptoms caused by inoculum produced on PDA medium. The relative pathogenicity of strain BC1 inoculum produced on stem segments was generally greater than that of inoculum produced on leaf disks (Fig. 3). No clear differences were observed for strain BC21. For both strains, the relative pathogenicity of secondary inoculum was highest when produced on organs from plants that received very low (0.5 mM nitrate) or very high (30 mM nitrate) levels of N fertilization (Fig. 3). Pathogenicity of inoculum was lowest from plants with moderate levels of N.

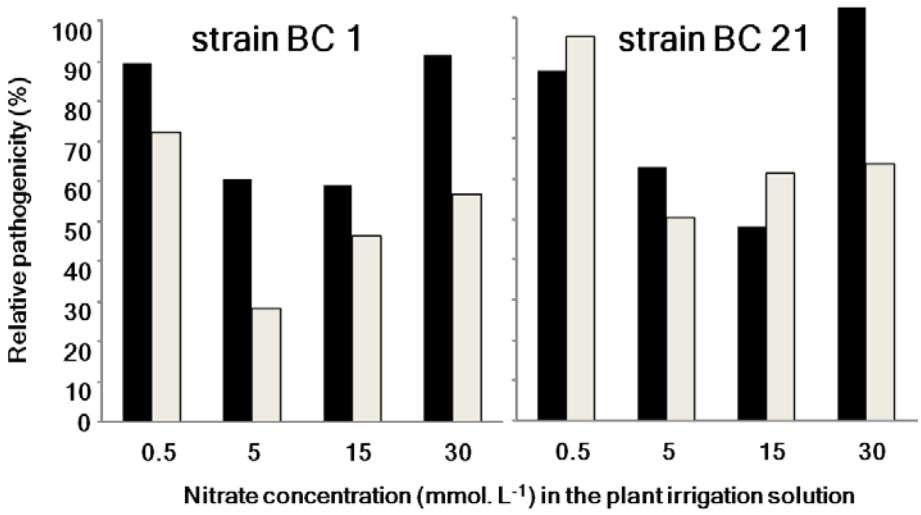

Figure 3: Effect of N nutrition and plant tissue on the pathogenicity of secondary inoculum of *Botrytis cinerea* to stems of tomato plants. Pathogenicity is expressed as the severity of disease (AUDPC) relative to inoculum produced on PDA.

Relationship between tissue constituents and pathogenicity of secondary inoculum.

There was a significantly positive correlation ($P<0.05$) of C content in the stems with pathogenicity of secondary inoculum ($R=0.98$ and $R=0.97$, respectively for strains BC1 and BC21) and a negative correlation with sucrose content in the leaves ($R=-0.97$ for strain BC21) Correlations for other tissue constituents were not statistically significant ($P>0.05$).

Table 4: Correlation between the pathogenicity of secondary inoculum of *Botrytis cinerea* on tomato plants and tissue content for various constituents of detached plant organs (leaf disks and stem segments) used to produce the spores.

Tissue contituent	Strain BC1				Strain BC21			
	Leaf disks		Stem segments		Leaf disks		Stem segments	
	R[y]	P value	R	P value	R	P value	R	P value
N	-0.28	0.712	0.04	0.956	-0.63	0.369	0.15	0.848
NO_3^-	0.02	0.975	0.07	0.923	-0.33	0.669	0.11	0.888
C	-0.40	0.592	0.98	0.018	-0.73	0.262	0.96	0.032
P	-0.14	0.858	0.16	0.835	-0.52	0.476	-0.12	0.880
K	-0.50	0.499	0.49	0.501	-0.70	0.295	0.23	0.768
Mg	0.17	0.821	0.05	0.942	0.54	0.459	-0.23	0.761
Ca	0.27	0.725	0.92	0.074	0.63	0.369	0.87	0.125
Sucrose	-0.82	0.172	0.27	0.727	-0.97	0.026	0.11	0.882
Glucose	-0.23	0.769	-0.45	0.548	-0.59	0.400	-0.19	0.806
Fructose	-0.15	0.842	-0.41	0.586	-0.53	0.462	-0.27	0.724
Total sugars[x]	-0.25	0.745	-0.47	0.523	-0.61	0.383	-0.24	0.757

[x]: sum of glucose, fructose and sucrose

[y]: Correlation analyses were performed on pooled data from two independent tests, each with two strains of *B. cinerea*. Tissue content was expressed in mg. gram of dry matter

DISCUSSION

This study showed that the level of N fertilization of tomato plants had a highly significant impact on the amount of spores produced by *B. cinerea* on diseased tissue as well as on the pathogenicity of this secondary inoculum. Sporulation was also affected by the type of plant tissue used as substrate. While substrate composition has been reported to affect spore production by *B. cinerea* and other fungi (7,16,32,35,54), few studies have addressed the effect of plant fertilization on the production of secondary inoculum on diseased tissue. Infection and spore production of *Blumeria* (syn. *Erysiphe*) *graminis* (powdery mildew of barley) was enhanced with increasing N fertilization (3,25), as was spore production by *Oidium neolycopersici* on tomato leaves (22) and uredospore production per leaf lesion by *Puccinia triticina* on wheat (42). In contrast, our study of *B. cinerea* on tomato shows that spore production was generally highest with low N fertilization (Fig. 1). Overall, this supports the hypothesis that biotrophs (such as the rusts and powdery mildews) and necrotophs (such as *B. cinerea*) may be differentially affected by N fertilization (22,48). However, the plant species might also influence this since increasing N from 1.8 to 28.8 mM in the irrigation solution of basil plants increased the incidence of sporulation by *B. cinerea* on stem segments (from nearly zero to over 75%) three days after inoculation (55). Different strains of a pathogen might also be differently affected, although this was not the case for the two strains of *B. cinerea* used in our study.

Tissue analyses suggested that differences in N fertilization treatments resulted in complex differences in the physiological status of the plants that significantly affected fungal development. As reported in other studies (21,23,55), we also observed that, in addition to total N and nitrate, the tissue content of soluble sugars and several minerals were significantly affected by N fertilization. Earlier work on tomatoes reported a positive correlation between the concentration of total soluble carbohydrates in leaves and the susceptibility of tomato plants

to *B. cinerea* (21). Effects of plant tissue composition on spore production may be more complex since the correlations observed for *B. cinerea* in our study varied depending both on the type of plant tissue and on the type of sugar or mineral (Table 2). For example, total soluble sugars (glucose + fructose + sucrose) in our study were negatively correlated with spore production on both leaf disks and stem segments in contrast to plant susceptibility studied by Hoffland et al. (21). Furthermore, sucrose concentration was positively correlated with spore production on stem segments but negatively (not significant) correlated on leaf disks while glucose was negatively correlated for both types of plant tissues.

Further studies are thus required to understand the mechanisms involved at the level of gene expression. Expression of the sporulation-regulating *brl*A gene of *Aspergillus nidulans* was reported to be induced by either glucose or N starvation, but with different kinetics and with differing phenotypical outcomes (46). Similar studies with *B. cinerea* would be possible, as orthologs of genes known to function upstream of *blr*A (including *fad*A, *flu*G, *flb*C and *flb*D) have been reported in the genome of this fungus (1).

Trophic interactions related to primary metabolism are unlikely to be the only mechanisms implicated. Other compounds related to plant defense mechanisms were not examined in our study but would merit further attention as they could influence sporulation indirectly through their effect on plant tissue colonization by the pathogen. N nutrition has a large effect on the amino acid content (30) and constitutive phenolics (51) involved in various stages of the infection process in tomatoes. In other plant models, peroxidase and chitinase (two enzymes implicated in plant defense) were lower when N was limiting (11). Further work could also include the effect of N (and resulting differences in plant tissue constituents) on the expression of pathogenicity-related genes of *B. cinerea*. Nitrogen-limiting conditions can influence the expression of such genes for several plant pathogenic fungi (47,49) and possibly for human pathogens such as *Aspergillus fumigatus* (37).

Beyond an effect on sporulation, we also observed a highly significant effect of N fertilization and type of plant tissue on the pathogenicity of secondary inoculum produced on diseased tissue. Since the plants used for assessing pathogenicity were all produced under identical conditions and were inoculated with the same number of spores free of plant debris or maceration fluids from their initial production substrates, we hypothesize that the observed effects arose from actual differences in the spores themselves.

The effect of the growth medium on the composition of spores is well documented. Studies of the entomopathogenic fungi *Beauveria bassiana*, *Metarhizium anisopliae* and *Paecilomyees fumosoroseus* showed that the composition of the growth medium influenced endogenous reserves of total carbohydrates, glycogen and lipids (29), trehalose and mannitol (39), erythritol (56) and proteins (6,56) in spores. These effects were sometimes accompanied by differences in the behaviour of the spores, notably their ability to survive various stress conditions and the kinetics of germination (6,20,40,41). It would be interesting to analyse possible differences in various metabolites in *B. cinerea* spores affected by fertilization of the host plant. However, it is not clear whether a putative difference in the endogenous reserves of the secondary inoculum could account fully for the differences in pathogenicity observed in our study. At the early stage of infection, when endogenous reserves of the spores would likely have the most effect, only small and often non statistically significant differences were observed in the time needed for initiation of stem cankers (Table 3). In contrast, the subsequent phase of disease development and lesion expansion were strongly affected (Fig. 2), suggesting that the effect on the fungus long outlasted the possible mobilization of different endogenous reserves contained in the initial conidia. The implication of epigenetic phenomena reported for several other organisms subjected to different nutritional environments would merit more attention (5,14,59).

In conclusion, the nutritional status of the host plant had a significant impact on the abundance and pathogenicity of secondary inoculum produced by plant pathogenic *B. cinerea*. This information could lead to exciting new studies to elucidate cellular mechanisms involved and suggests that fertilization can influence the development of epidemics on a field scale. Conversely, it opens the possibility of manipulating crop fertilization not only to reduce plant susceptibility (31) but also to limit the impact of secondary inoculum and minimize the need for pesticide use.

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Chapter 5

Publication 4

Manzoor A. Abro[1], François Lecompte[2], Marc Bardin[1] and Philippe C. Nicot[1(*)] 2012.

Enhancement of biocontrol efficacy against *Botrytis cinerea* through the manipulation of nitrogen fertilization of tomato plants. Agronomy for Sustainable Development (Submitted on 19th December 2012)

Enhancement of biocontrol efficacy against *Botrytis cinerea* through the manipulation of nitrogen fertilization of tomato plants

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Abstract: Gray mold, caused by *Botrytis cinerea*, constitutes a recurrent menace for greenhouse production of tomatoes. Its management can be difficult despite many chemical treatments and much effort is dedicated to developing alternative control methods and enhancing their efficacy. Although nitrogen (N) fertilization is known to affect plant susceptibility to certain pathogens, little is known on its possible effect on the efficacy of biological control. In the present study we examined the effect of five levels of N nutrition on the efficacy of two biocontrol agents *(Trichoderma atroviride* and *Microdochium dimerum)* to protect pruning wounds of tomato against *B. cinerea*. Plants were grown for two months in a greenhouse with a soil-less drip-irrigation system. Differential N nutrition was applied for the last four weeks prior to leaf pruning, treatment of wounds with the biocontrol agents and inoculation with two strains of *B. cinerea*. They were then incubated in conditions conducive to disease development. Plant fertilization strongly affected disease development for both strains of the pathogen and it influenced the efficacy of both biocontrol agents. Disease severity decreased with increasing N fertilization up to 10 mmol of nitrate per liter of nutrient solution. High nitrogen fertilization also increased biocontrol efficacy by delaying symptom development on the stems and slowing down lesion expansion. The extent of this effect

depended on the biocontrol agent and on the strain of the pathogen. To our knowledge this is the first report of an effect of N fertilization on the efficacy of biocontrol against an airborne disease. It suggests that manipulating N fertilization could be a new useful tool for integrated protection of greenhouse tomato, not only through reducing plant susceptibility to gray mold, but also by enhancing the efficacy of biocontrol agents.

Key words: Biological control, Tomato, *Trichoderma atroviride*, *Microdochium dimerum*, *Botrytis cinerea*, Nitrogen fertilization.

1. INTRODUCTION

Reducing the reliance on pesticide use for crop protection is a generally acclaimed goal. Its achievement, however, requires the availability of efficacious alternative methods for growers to limit the risk of yield losses from pests and diseases. Much hope is placed on biological control and commercial products are increasingly available against arthropod pests and more recently against diseases (van Lenteren 2012; Nicot 2011). For disease control, a lower and less regular level of efficacy, compared to pesticides, is often cited as one of the obstacles to the commercial development of biological control agents (BCAs) and their wide adoption by growers (Nicot 2011). Much research has been dedicated to enhancing the efficacy and reliability of BCAs. One approach has consisted in improving the performance of the microbial BCA strains through mutagenesis or genetic manipulation (Clermont et al. 2011) or through optimizing nutrient substrate composition and conditions applied during biomass production (Jackson et al. 1991). Another approach may be to apply various compounds (including calcium salts, chitosan, amino acids and carbohydrates) on the plants together with the BCA (El-Ghaouth et al. 2002; Guetsky et al. 2002; Janisiewicz et al. 1992). Soil amendments with various organic compounds, including chitin, have also been shown to improve biocontrol efficacy of soilborne diseases (Barakat 2008; Lopez-Mondejar et al. 2012). Surprisingly, little information is available on the possible role of mineral plant fertilization on the efficacy of BCAs, although its effect on the susceptibility of the host plant to pathogens is well documented for a variety of crops and diseases (Datnoff et al. 2007).

In a recent study, we showed that the susceptibility of tomato to *Botrytis cinerea*, a fungus responsible for economically important diseases of a variety of crops, was significantly influenced by nitrogen (N) fertilization (Lecompte et al. 2010). N fertilization also had an impact on the abundance of spore production by *B. cinerea* on diseased plants and on the pathogenicity of that secondary inoculum (Abro et al., 2012). For greenhouse tomato

production, this fungus constitutes a recurrent menace. Its management can be difficult despite many chemical treatments and much effort is dedicated to developing alternative control methods and enhancing their efficacy.

The objectives of the present study were thus to investigate a possible effect of N fertilization on the efficacy of two beneficial fungi shown in previous work to provide protection of tomato pruning wounds against *B. cinerea* (Nicot et al. 2003; Bardin et al. 2008). For both BCAs, we observed enhanced biocontrol at high N fertilization levels, even in the presence of high inoculum levels of the pathogen.

2. MATERIALS AND METHODS

2.1 Plant production

Tomato plants were produced under five differential N nutrition regimes as described before (Lecompte et al. 2010). In short, seeds of cv Swanson were sown in 1 cm^3 rockwool plugs in a greenhouse. Ten days after sowing, the plugs were transferred onto rockwool blocks and the plantlets (one per block) were fertigated twice a day with a standard commercial nutrient solution (Duclos international Lunel, France). After one month, the plants (bearing 3-4 leaves) were placed on the top of 2 liter pots filled with a mixture (1:1 V/V) of vermiculite and pozzolana to start the nutrition treatments. Five levels of nitrate concentration were tested in the fertilization solution: 0.5, 2, 5, 10 and 20 $mmol.L^{-1}$ (abbreviated as mM in this paper). The plants were fertigated with a drip irrigation system (one dripper per pot) at a frequency adapted to the climatic demand. Two batches of 200 plants (40 for each fertilization regime) were produced independently in 2009 and 2010 to provide independent repetitions of the whole study.

2.2 Plant tissue analysis

From each batch of 200 plants, five were randomly selected for each N nutrition regime just before the inoculation assays. These samples were used to measure fresh and dry weight of aerial parts and for analyses of mineral content of stem tissues as described before (Lecompte et al., 2010). Seven tissue components were measured: total nitrogen (N), total carbon (C), nitrate (NO_3^-), phosphorous (P), potassium (K), magnesium (Mg) and calcium (Ca). In addition, glucose, fructose and sucrose contents were determined with an enzymatic method, as proposed by Gomez et al. (2007).

2.3 Biocontrol agents

We used two fungal species known from previous work in our laboratory for their protective effect against *B. cinerea* on tomato. Strain L13 of *Microdochium dimerum*, isolated from pruning wounds on tomato (Nicot et al., 2002) was grown on yeast peptone glucose agar (YPGA) containing per liter: 7g Bacto™ yeast extract, 7g BBL™ Gelysate™ peptone, 7g glucose and 15g agar. Strain T1 of *T. atroviride*, kindly provided by Agrauxine, was produced on Difco™ potato dextrose agar (PDA). Both fungi were grown in a growth chamber (21°C and 16-hour photoperiod; 162 µmole $m^{-2}s^{-1}$). Spores were collected in sterile distilled water from the surface of 14-day old cultures and suspensions adjusted to 10^7 spores.mL^{-1} were prepared for each strain.

2.3 Plant treatments with biocontrol agents

From each regime of N fertilization, two groups of 10 plants were treated with either biocontrol agent and one group was used as control. On each plant, four leaves were removed, leaving 5-10 mm petiole stubs on the stems. The pruning wounds were either left untreated (control plants) or treated with 10µL aliquots of a spore suspension of *T. atroviride* or *M. dimerum*. Immediately after treatment, the plants were inoculated with *B. cinerea*.

2.4 Production of **Botrytis cinerea** *inoculum*

We used two strains of *B. cinerea* with contrasted aggressiveness on tomato. From previous work of the laboratory, strain BC1 was known to have a high level of aggressiveness on tomato, while strain BC21 had a medium to low level of aggressiveness (Lecompte et al., 2010). Inoculum was produced on PDA in a growth chamber as described above. Spores

were collected in sterile distilled water from the surface of 14-day old cultures and suspensions were prepared and adjusted to 10^6 and 10^7 spores.mL^{-1} for each strain. As the objective of the study was to investigate the possibility of manipulating N fertilization to improve the efficiency of biocontrol, high inoculum concentrations of *B. cinerea* were used purposefully to put the BCAs in a difficult situation.

2.5 Inoculations

For each treatment and N fertilization level, five plants were inoculated with strain BC1 and five with strain BC21. On a given plant, the pruning wounds of the third and fifth leaves (numbered from the bottom up) were inoculated with 10 µL aliquots of suspension containing 10^7 spores.mL^{-1} and the fourth and sixth leaves with suspensions containing 10^6 spores.mL^{-1}. Following inoculation, the plants were incubated for 7 days in controlled conditions at 21°C, 90% relative humidity under 14 hours of photoperiod as described before (Lecompte et al., 2010). During this period, they were irrigated manually, using the same fertilization solutions as those used before inoculation.

2.6 Disease assessment and data analysis

The plants were examined daily from the 3rd to the 7th day after inoculation and the infection of petiole stubs, the initiation and length of resulting stem lesions were monitored. The latency period was calculated as the number of days between inoculation and lesion appearance on stems. If a lesion was visible on the third day after inoculation, the latency period was a given a value of 3 days; if no lesion was visible on the last day of disease assessment, it was a given a value of 8 days. On each inoculation site, the rate of lesion expansion (mm per day) was calculated as the average daily increase in the length of the

lesions between their appearance on the stem and the last day of measurement. It was thus estimated based on 1 to 5 measurements of lesion size depending on the day of lesion appearance. The area under the disease progress curve (AUDPC) was computed over the 5 days of disease rating as described by Lecompte et al. (2010). The whole experiment was conducted twice on independently produced batches of plants. Analysis of variance was used to compare the two trials and to test for an effect of fertilization on disease development. When appropriate, the means were compared with the test of Newman and Keuls. To compare the efficacy of the BCAs, a protection index was computed as 100 * (AUDPC $_{untreated}$ – AUDPC $_{biocontrol}$)/AUDPC $_{untreated}$, where AUDPC $_{biocontrol}$ and AUDPC $_{untreated}$ were the average AUDPC (for plants treated with a BCA or for control plants left untreated, respectively) computed for a given BCA and N fertilization level combination. The correlation between disease severity (AUDPC) and tissue content in the tomato stems for different minerals and sugars was investigated by using Pearson's correlation coefficients. The Statistica software package was used for all data analyses.

3 RESULTS AND DISCUSSION

3.1 Effect of nitrogen fertilization on disease severity

On the untreated control plants, the pathogen colonized the petiole stubs and eventually developed stem cankers on all inoculation sites in both replicate experiments. In contrast, some of the wounds treated with either biocontrol agent were protected (despite the purposefully high level of *B. cinerea* inoculum used in the study), resulting in no or reduced disease development. As expected from previous work (Lecompte et al., 2010), disease severity was significantly higher on plants inoculated with strain BC1 than BC21 of *B. cinerea*, regardless of inoculum concentration (Fig. 1). A global analysis of variance on AUDPC (r^2=0.78, F=108, p<0.0001, 1199 DF), accounting for five classification variables (*B. cinerea* strain, inoculum concentration, biocontrol, N fertilization level and experiment) and their interactions, yielded significant effects for all variables and interactions, except the "concentration x experiment" interaction (Table 1). Although significant differences between experiments were observed, the contribution of this factor to the global model variance was low as compared to other ones. Therefore, for the sake of conciseness and clarity, the major effects described below will be presented with mixed data from the two experiments; however differences between experiments will be pointed out when necessary .

Table 1: Global analysis of variance of the AUDPC observed in two repeated tests on tomato plants grown at various regimes of nitrogen fertilization and inoculated with *Botrytis cinerea* with or without bioncontrol agents**.**

Variable	Values	Degrees of freedom	Type III sum of squares	Mean Square	F Value	Pr > F
Strain (1)	BC1 ; BC21	1	612572	612572	1556.26	<.0001
Nitrate rate (2)	0.5 ; 2 ; 5 ; 10 ; 20 (mM)	4	407244	101811	258.65	<.0001
Inoculum concentration (3)	10^6 ; 10^7 (spores.mL^{-1})	1	98967	98967	251.43	<.0001
Biocontrol (4)	Control ; T. atroviride ; M. dimerum	2	195752	97876	248.66	<.0001
Experiment (5)	2009 ; 2010	1	22965	22965	58.34	<.0001
(1)*(2)		4	98521	24630	62.57	<.0001
(1)*(4)		2	46967	23483	59.66	<.0001
(1)*(3)		1	20180	20180	51.27	<.0001
(1)*(5)		1	12195	12195	30.98	<.0001
(2)*(5)		4	39345	9836	24.99	<.0001
(4)*(5)		2	15474	7737	19.66	<.0001
(3)*(4)		2	8751	4376	11.12	<.0001
(2)*(3)		4	7814	1953	4.96	0.0006
(3)*(5)		1	922	922	2.34	0.1262
(2)*(4)		8	6776	847	2.15	0.0288

For both strains of *B. cinerea* and both inoculum concentrations, in the two experiments, disease severity was significantly affected by the level of N fertilization provided to the plants. On control plants with no BCA, severity decreased significantly (P values < 0.0001 in all situations) with increasing N fertilization level up to 5 mM, and in some cases at higher N rates (Fig. 1). These results are in agreement with those of earlier work showing that high N fertilization reduced the severity of *B. cinerea* on tomato (Hoffland et al., 1999; Lecompte et al., 2010). In the presence of either BCA (*M. dimerum* and *T. atroviride*), the AUDPC also decreased significantly with increasing N fertilization (up to 5-20 mM, Fig. 1). For plants treated with *M. dimerum*, differences between experiments were observed. In the 2010 experiment, disease severity was already somewhat low at low N levels and decreased only slightly with increasing N fertilization. In contrast, the decrease of disease severity with increasing N levels was more pronounced in 2009.

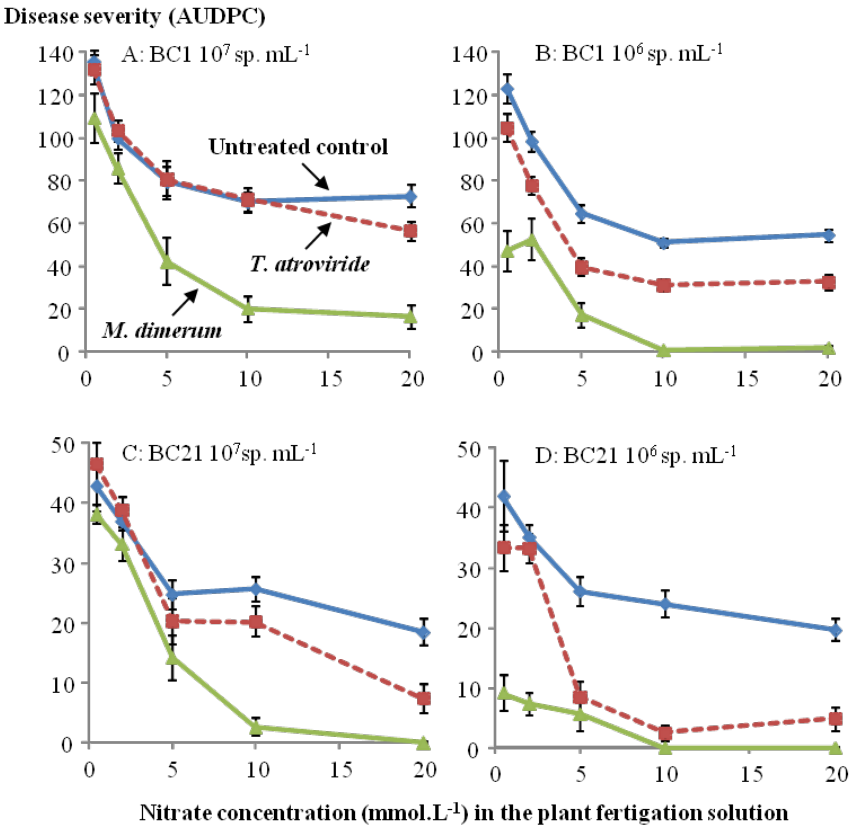

Fig. 1 Effect of nitrogen fertilization on the severity of disease caused by two strains of *B. cinerea* (highly aggressive strain BC1 and mildly aggressive strain BC21) on stems of tomato plants. The plants were inoculated with the pathogen alone (◆) at two different spore concentrations, or together with biocontrol agent *Trichoderma atroviride* (■) or *Microdochium dimerum* (▲). Disease severity (represented by the area under the disease progress curve) was differentially reduced in the presence of the biocontrol agents. In all cases, severity of disease decreased with increasing N fertilization levels. The bars represent the standard error of the means.

3.2 Effect of nitrogen fertilization on the efficacy of biocontrol

The efficacy of biocontrol globally increased with increasing N fertilization; however variations depending on the strain, the concentration of the pathogen's inoculum and the species of BCA were observed (Fig. 2). *M. dimerum* provided higher levels of protection than *T. atroviride* and the efficacy of either BCA was higher against the mildly aggressive than against the highly aggressive strain of *B. cinerea*. In the presence of high *B. cinerea* inoculum concentration, the efficacy of both BCA was low for plants grown at low N (Fig. 2A). The protection index of *M. dimerum* increased sharply with increasing N fertilization, to reach nearly 80% and 100%, respectively, against the highly and the mildly aggressive strains of *B. cinerea*, while that of *T. atroviride* increased only moderately. At the lower inoculum concentration (Fig. 2B), *M. dimerum* achieved good disease control at low N levels, and almost fully protected the plant against both strains of *B. cinerea* at high N. Increasing N also improved the protective effect of *T. atroviride* against mildly aggressive strain BC21, but only limited protection was achieved against strain BC1, even at high N fertilization.

Few studies have examined the effect of soil fertilization on biocontrol. Khattabi et al. (2004) reported that soil amendment with four types of N fertilizers resulted in decreased survival of sclerotia of soilborne pathogen *Sclerotium rolfsii* and that the antagonistic activity of biocontrol fungus *T. harzianum* against those sclerotia increased with increasing N doses. N amendments in nutrient agar media have also been shown to increase *in vitro* biocontrol activity of certain microorganisms.

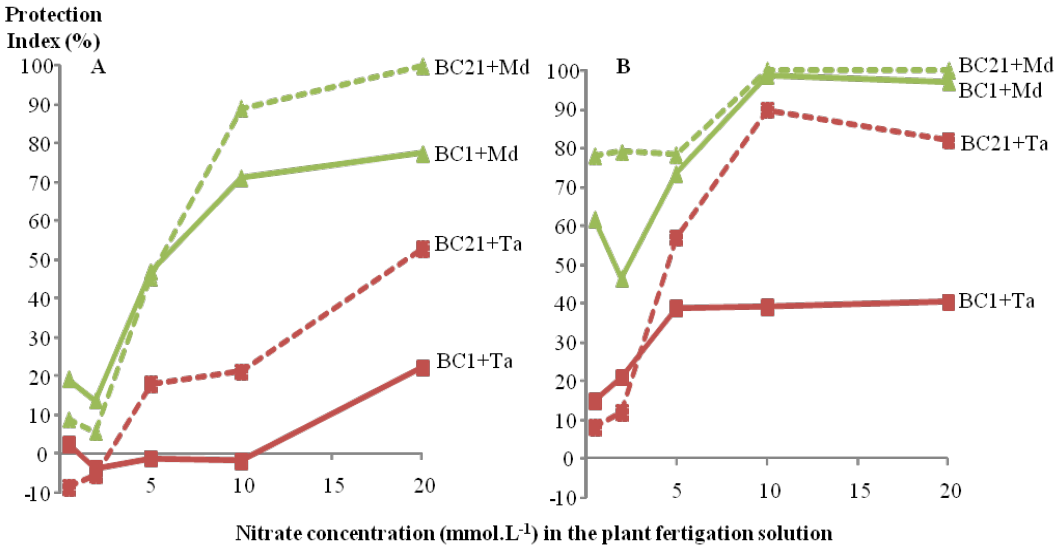

Fig. 2 Effect of nitrogen fertilization of tomato plants on the efficacy of protection provided by two biocontrol agents against two strains of *B. cinerea* (BC1 and BC21). The plants were inoculated with the pathogen alone at two different spore concentrations (A: 10^7 or B: 10^6 spores.mL^{-1}), or together with biocontrol agent *Trichoderma atroviride* (Ta: ■) or *Microdochium dimerum* (Md: ▲). The efficacy of biocontrol was enhanced by high N fertilization levels. The bars represent the standard error of the means.

To gain a better understanding on the possible phenomena implicated in the similar effect observed in our study, two key steps of pathogenesis were examined more closely, one (the time needed for symptom development) reflecting the first interactions between the plant, the pathogen and the BCA, and the second (the rate of stem lesion expansion) reflecting longer interactions over several days between the three organisms.

3.3 Effect of nitrogen fertilization on the latency period

On untreated control plants the latency period increased significantly with increasing N fertilization, slightly for BC1 and more importantly for BC21 (Fig. 3, P values < 0.0001 in all situations). This confirmed observations from our earlier work (Lecompte et al., 2010).

On plants treated with either BCA, the latent period was also significantly affected by N fertilization (Fig. 3, P values < 0.002 in all situations). In the presence of *M. dimerum*, the latency for both strains of *B. cinerea* was significantly higher than on control plants at N levels of 5 mM and above when the inoculum concentration was high and at all N levels when the inoculum concentration was low. In the presence of *T. atroviride*, the latency of *B. cinerea* was similar to the control at 0.5 and 2 mM NO_3^- in all situations, and significantly higher at N levels of 5mM and above, except for highly aggressive strain BC1 at high inoculum concentration.

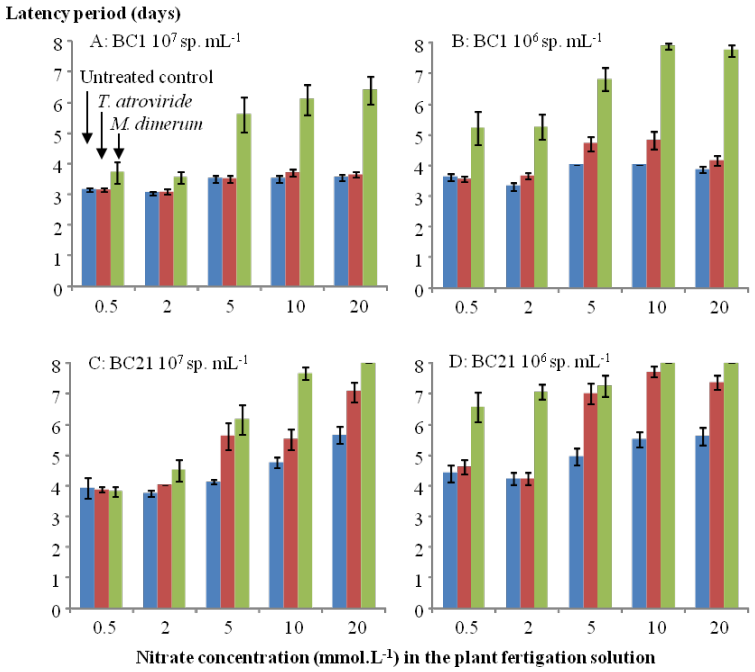

Fig. 3 Effect of nitrogen fertilization on the latency period before symptom appearance on stems of tomato plants, following inoculation by two strains of *B. cinerea* (highly aggressive strain BC1 and mildly aggressive strain BC21). The plants were inoculated with the pathogen alone (■) at two different spore concentrations, or together with biocontrol agent *Trichoderma atroviride* (■) or *Microdochium dimerum* (■). The latency period was differentially affected depending on the strain of the pathogen and the biocontrol agent. In all cases the latency period was higher at high N fertilization levels. The bars represent the standard error of the means .

This differential effect of N fertilization on the latent period, dependant both on the strain of *B. cinerea* and on the strain of BCA, reflects the outcome of early interactions between the three organisms, leading to the emergence of stem symptoms. Overall, for both BCA, the increase in the latency period with increasing N level paralleled that of overall efficacy of biocontrol. This suggests that the early interactions between the BCA, the plant and the pathogen were important for the outcome of wound protection. It is also compatible with the observation by O'Neil et al. (1997), on tomato plants in absence of any protective treatment that the susceptibility of stems to infection by *B. cinerea* declined sharply with wound age within the first day after leaf removal.

3.4 Effect of nitrogen fertilization on lesion expansion

On untreated control plants inoculated with highly aggressive strain BC1, the daily rate of lesion expansion decreased significantly with increasing N fertilization regardless of inoculum concentration ($p<0.001$ in both cases; no effect of inoculum concentration, $p>0.58$; averaged data shown in Table 2). Conversely, lesion expansion increased slightly with increasing N levels above 2 mM for strain BC21 (Table 2), as observed in earlier work contrasting highly and mildly aggressive strains of *B. cinerea* (Lecompte et al 2010).

On plants treated with either BCA, lesion expansion was also significantly affected by N fertilization and the strain of *B. cinerea*, but not by the concentration of inoculum ($P > 0.80$).

Table 2: Effect of nitrogen fertilization of tomato plants on expansion of stem lesions caused by *Botrytis cinerea*. The plants were inoculated with the pathogen alone (highly aggressive strain BC1 or mildly aggressive strain BC21), or together with biocontrol agent *Trichoderma atroviride* or *Microdochium dimerum.* Lesion expansion (in mm per day) was differentially affected depending on the strain of *B. cinerea* and the biocontrol agent. High N levels reduced lesion expansion and thus enhanced the protective effect of the biocontrol agents.

Strain of *B. cinerea* and	**Nitrate concentration (mmol.L^{-1}) in the plant fertigation solution**									
Plant treatment	0.5		2		5	10		20		
Strain BC1										
Untreated Control	12.14	a[C]	9.33		5.89	3.44	a	3.35	a	
Trichoderma atroviride	10.33	b	8.40		4.19	3.48	a	1.77	b	
(% reduction)[A]	(15)		(10)		(29)	(-1)		(47)		
Microdochium dimerum	10.44	b	7.61		6.06	1.21[D]	b	0.91[D]	b	
(% reduction)	(14)		(18)		(-3)	(65)		(73)		
P value[B]	0.015		0.095		0.085	0.035		0.000		
Strain BC21										
Untreated Control	2.48		1.35		1.54	2.00		3.24		
Trichoderma atroviride	2.36		1.54		1.54	1.90		0.88[D]		
(% reduction)	(5)		(-14)		(0)	(5)		(73)		
Microdochium dimerum	1.62		1.70		0.99	*		*		
(% reduction)	(35)		(-26)		(35)					
P value	0.068		0.165		0.227	0.728		0.000		

[A] % reduction in lesion expansion relative to the untreated control

[B] Test of biocontrol treatment effect

[C] For a given strain of *B. cinerea* and nitrate fertilization level, numbers followed by different letters were significantly different (Newman and Keuls test; P=0.05)

[D] Data for 2009 only (no stem lesion development in 2010)

* No stem lesion development, due to full protection of the pruning wound.

For plants inoculated with highly aggressive strain BC1, significant differences between control and BCA-treated plants were mostly observed at high N levels, with reductions in lesion expansion amounting to 65-73 % for *M. dimerum* and nearly 50% for *T. atroviride* (Table 1). Analyzing the influence of N fertilization on the reduction in lesion expansion by strain BC21 was complicated by the fact that no stem lesions developed at high N levels in the presence of *M. dimerum* and only in 2009 for *T. atroviride*. A significant reduction in lesion expansion by BC21 was only observed in the presence of *T. atroviride* on plants grown at the highest N level (Table 2).

Thus, even if the major effect of plant N nutrition on biocontrol efficacy mostly occurred at the early infection phase, marked by a delay in symptom development, a reduction in lesion expansion was able, in some cases, to contribute to increased biocontrol at high N level when stems became infected.

3.5 *Relationship between stem tissue content and the disease severity*

A difference in N fertilization levels resulted in significant differences in fresh and dry weight (g) as well as in tissue content of eight of the ten components quantified in the tomato stems (Table 3). Dry and fresh weight, total N, and NO_3^- increased with increasing nitrate level up to 20 mM in the irrigation solution, while fructose increased significantly up to 5-10 mM, and C was not significantly influenced, confirming some of the results of earlier work (Lecompte et al., 2010).

Highly significant correlations ($P<0.001$) were observed between disease severity (AUDPC) and the concentration in the stems (mg per gram dry matter) of some of the tissue components: negative correlations with total N, NO_3^-, and fructose, and positive with sucrose (Table 4). However, these four tissue components were significantly auto correlated, with

highly positive correlations between N, NO_3^- and fructose content, and highly negative correlations between sucrose and the other three components, making it difficult to point to putative causal effects. Ccorrelations between susceptibility or resistance to diseases and various indicators of nitrogen status of the host plant (amino acids, nucleic acids, phenols, total N, protein content, C / N, etc) have been reported before in other plant-pathogen systems (Huber and Thompson, 2007). Also, a relationship between plant sugar content and

Table 3: Effect of nitrogen fertilization on the fresh and dry weight and on tissue content of tomato stems. Increasing N fertilization resulted in significant increase in fresh and dry weight, as well as tissue content of total N, NO_3^- and fructose, and decrease in sucrose.

NO_3- ($mM.L^{-1}$)	Fresh weight(g)	Dry weight (g)	Tissue content (in mg per gram of dry matter)									
			N	NO_3^-	C	P	K	Mg	Ca	Sucrose	Glucose	Fructose
0.5	21.5 a	4.1 a	7.5 a	0.1 a	369.7	7.2	54.6	3.2	6.1	32.8 c	83. 6a	7.9 a
2	33.6 a	5.6 a	11.3 a	0.2 a	374.5	6.8	53.3	3.5	5.9	29.6 c	110.8 b	9.7 a
5	66.1 b	8.1 ab	25.0 b	7.7 a	369.7	8.6	62.4	4.4	11.4	18.5 b	107.2 b	26.8 b
10	111.2 c	10.7 b	32.2 c	27.0 b	365.2	6.5	57.7	3.6	10.6	18.7 ab	104.2 b	33.9 c
20	126.6 c	11.9 b	34.7 c	41.9 c	350.9	6.2	57.5	3.4	14.8	13.7 a	73.1 a	26.4 b

* Data are means of five replicates per treatment. For a given column the numbers followed by different letters are significantly different (Newman-Keuls tests; P=0.05) .

Table 4: Correlation between the tissue content of tomato stems and the severity of disease on plants produced at various concentrations of nitrate in the irrigation solution. The tomato plants were inoculated with two strains of *Botrytis cinerea* alone or co-inoculated with biocontrol agents *Trichoderma atroviride* or *Microdochium dimerum.* Among tissue components, total N, NO_3^- and fructose were consistently negatively correlated and sucrose positively correlated to disease severity.

Tissue component	**BC1 (control)**		**BC1+*T. atroviride***		**BC1+*M. dimerum***		**BC21 (control)**		**BC21+*T. atroviride***		**BC21+*M. dimerum***	
	R[A]	P value	R	P value	R	P value	R	P value	R	P value	R	P value
N total	-0.81	0.000	-0.74	0.000	-0.76	0.000	-0.86	0.000	-0.81	0.000	-0.65	0.002
NO_3^-	-0.69	0.001	-0.66	0.001	-0.71	0.000	-0.80	0.000	-0.74	0.000	-0.63	0.002
C	0. 39	0.088	0.40	0.074	0.45	0.046	0.53	0.014	0.49	0.026	0.41	0.071
P	0.09	0.677	0.10	0.659	-0.13	0.583	0.11	0.618	0.08	0.724	0.16	0.476
K	-0.30	0.185	-0.27	0.234	-0.28	0.229	-0.33	0.154	-0.33	0.150	-0.20	0.381
Mg	-0.30	0.192	-0.24	0.299	-0.17	0.451	-0.25	0.278	-0.23	0.316	-0.09	0.682
Ca	-0.39	0.084	-0.37	0.099	-0.38	0.092	-0.46	0.038	-0.43	0.055	-0.32	0.157
Sucrose	0.82	0.000	0.76	0.000	0.76	0.000	0.90	0.000	0.83	0.000	0.64	0.002
Glucose	-0.06	0.790	0.00	0.979	0.07	0.744	0.09	0.693	0.66	0.669	0.09	0.697
Fructose	-0.84	0.000	-0.74	0.000	-0.77	0.000	-0.82	0.000	-0.81	0.000	-0.64	0.002
Total sugars	0.03	0.875	0.05	0.817	0.04	0.861	0.09	0.703	0.05	0.806	0.04	0.844

[A] Correlation analysis were performed on pooled data from two independent tests (2x5 replicates per modality). Tissue content was expressed in mg per gram of dry matter and disease severity was represented by the area under the disease progress curve

susceptibility has been suggested by Bolton, (2009). Interestingly, no correlation was observed between disease severity and total soluble sugars, contrarily to the results of Hoffland et al. (1999) with total soluble carbohydrates, suggesting that other compounds than those measured in our study (glucose, fructose, sucrose) may be implicated, or that only some of the soluble sugars, fructose and sucrose, are implicated in the response to infection. The recent description of a mutant of *B. cinerea* incapable of using fructose as a sole carbon source (Aguayo et al., 2011) should provide a useful tool to investigate this subject further.

Competition for nutrients is a common mode of action of biocontrol agents on plant surfaces. Although no information is available for the two BCAs included in our study, competition for fructose and nitrate has been identified as a factor in the mode of action of certain BCAs (Jacobsen, 2006). However, in our study, repeated analyses of covariance on disease severity showed that there were no interactions between plant treatments (with either BCA or untreated controls) and the various plant components identified, as the slopes relating AUDPC to each of the individual plant components were similar for plants inoculated with *B. cinerea* alone or with *B. cinerea* and a BCA (graphs not shown). This indicated that the effects of these components were not different for plants inoculated with *B. cinerea* alone or with *B. cinerea* plus a BCA, and conflicted with the hypothesis of a direct effect of the plant components on biocontrol. Rather, the results suggest that the tissue content affected the capacity of the pathogen to infect the plant, and that the outcome of the biocontrol was in turn affected by this modified interaction between the host and the pathogen. This hypothesis is in accordance with the observation that at each N regime, the efficacy of biocontrol was directly related to the pressure exerted by the pathogen, in terms of inoculum concentration and strain aggressiveness. Increasing the plant N content would either enhance plant defenses or reduce the ability of the fungus to colonize the tissue, and by this direct reduction of fungal growth, indirectly facilitate the action of the BCAs and hence their efficacy. Thus, the novel results

presented here on the effect of N fertilization on the efficacy of two biocontrol agents against *B. cinerea* should stimulate further work to better understand which mechanisms are involved at the cellular and molecular level.

4 CONCLUSIONS:

The results of this study clearly indicated that the level of N fertilization provided to the plant can influence not only the susceptibility of tomato stems to *B. cinerea* but also the efficacy of biocontrol. High N fertilization enhanced the efficacy of both biocontrol agents tested against *B. cinerea*. To our knowledge this is the first report of an effect of N fertilization on the efficacy of biocontrol against an airborne disease. It suggests that the manipulation of N fertilization could provide a new useful tool for integrated protection of greenhouse tomato. To devise specific recommendations for growers, further work is needed to validate the present results in conditions of commercial production and to evaluate possible side effects on other diseases or pests in the tomato greenhouse.

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Chapter 6

Publication 5

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Cyto-histological evidences for the influence of nitrogen fertilization on the efficacy of biological control against *Botrytis cinerea* in tomato

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Abstract.

Tomato stem cankers due to *Botrytis cinerea* is a serious problem causing plant death and decreased yields in greenhouses. The disease control by fungicides encounters some failures due to pathogen resistance. The use of biological control agents may be a good alternative to overcome this problem but the success of this method is thought to depend on numerous factors especially the nutritional status of the host plant. The present study was conducted to assess at histological levels the interaction between nitrogen fertilization of tomato with *Botrytis* infection and the efficacy of biological control with *Trichoderma atroviride* and *Microdochium dimerum*. Tomato plants were fertigated with three N regimes (0.5, 5, and 20 mM NO_3^-) and inoculated with *Botrytis* alone or in combination with the biological control agents. The performed cytological studies using light microscopy showed that at high N fertilization the severity of disease decreased due to structural alteration of *Botrytis* mycelial cells. Biological control with *T. atroviride* also decreased the disease severity compared to control especially at high N fertilization. At cellular levels this was associated with profound mycelial cell destructions in form of vacuolisation, glycogen deposition and mycelial cell

death. Similar cyto-histological alterations occurred with *M. dimerum*. However, this biological control agent proved to be more effective in comparison with *T. atroviride.* Taken together the results suggested that not only high N levels interfere with mycelial development of *B. cinerea* but also improve the effectiveness of biological control agents.

Key words: *Botrytis cinerea*, tomato, nitrogen fertilization, biological control, cytology, mycelial structure

Introduction

Grey mould caused by *Botrytis cinerea* is a major problem in greenhouse tomato (*Solanum lycopersicum*) production causing serious economic losses (Jarvis 1989). It is able to invade the plant tissues directly or through pruning wounds that results in stem cankers and rapid plant death (Decognet et al. 2010). The disease in mainly controlled *via* chemical treatments but many classes of synthetic fungicides have failed due to the genetic variability of the pathogen (Williamson et al. 2007). Another alternative method to control *B. cinerea* on tomato is by means of biological control agents (Eden et al. 1996; Elad et al. 1995; Nicot et al. 2003b). Biological control agents significantly reduced the incidence of stem lesions on tomatoes (Decognet et al. 1999a). For example, the efficacy of *Microdochium dimerum* has been shown in earlier studies to provide high levels of protection on pruning wounds of tomato in green house conditions (Nicot et al. 2003b). *Trichoderma harzianum* controls various plant pathogens including *B. cinerea* in tomato (Eden et al. 1996; Elad et al. 1999b) (Harman et al. 2004).

It is well known that the nutritional status of the host plants affects their susceptibility to pathogens (Datnoff et al. 2007). However, the effect of nitrogen (abbreviated as N in this paper) fertilization on susceptibility to *Botrytis* is controversial (Dik and Wubben 2004; Yermiyahu et al. 2006). For example, elevated N concentrations in the fertilizer solution increased disease severity of *Botrytis* on strawberry (Nam et al. 2006), in *Begonia* (Pitchay et al. 2007), and in sweet basil (Yermiyahu et al. 2006). In contrast to high susceptibility at higher N in tomato-*B. cinerea* pathosystem (Lecompte et al. 2010) found that high N fertilization lowers the level of disease severity. Similarly, (Hoffland et al. 1999) revealed that leaves of tomato plants grown at low N availability were about 2.5 times more susceptible to primary lesion formation by *B. cinerea* compared to plant grown at high N availability. In sweet pepper results indicate that the increasing nitrate in nutrient solution

positively affected vegetative growth of the plants and also decreased the susceptibility against *B. cinerea* (Yoon et al. 2010).

Several histological studies have been devoted to the interaction between *B. cinerea* and different host plants such as faba bean leaves (Cole et al. 1998), wax flowers (Dinh et al. 2011), nectarines and plum (Fourie and Holz 1995), lily leaves (Hsieh et al. 2001), grapes (Pezet and Pont 1992), rose flowers (Pie and Deleeuw 1991), tomato plants (Rijkenberg et al. 1980), gerbera flowers (Salinas and Verhoeff 1995), Arabidopsis (van Baarlen et al. 2007). They all showed that the mycelium of *B. cinerea* developed intercellularly and it propagates by degrading cell walls. In tomato *Botrytis* patho-system we previously demonstrated that the conidia germinated on inoculated petioles giving raise to mycelium that attained rapidly stem tissues 72 hours post inoculation (Ajouz et al. 2011). But histological data on the initial interaction and colonisation of tomato stem grown with different levels of N fertilization after inoculation with *B. cinerea* and on the efficacy of biological control is largely lacking. A thorough knowledge of the histology of the interaction between tomato, N fertilization, *B. cinerea* and antagonists can provide useful information for the efficacy of biological control agents against this pathogen.

The objective of the present study was to examine the effects of N fertilization of the tomato plants on: (1) *B. cinera* mycelim development (2) the efficacy of biological control by using *T. atroviride* and *M. dimerum* through cyto-histological techniques by means of light microscopy.

Materials and methods

Production of plant material and nitrogen treatments

The used tomato (*Solanum lycopersicum*, cv Swanson) plants were produced as previously described by (Lecompte et al. 2010) and maintained under greenhouse conditions until used for experimentations. During this period, the plants were fertigated twice a day with a standard commercial nutrient solution (Duclos International Lunel, France). After this period, the plants (bearing 3-4 leaves) were transplanted in 2 L pots filled with a mixture (1:1, V/V) of vermiculate and pozzolana (inert crushed volcanic rock) to start the nutrition treatments. Three levels of N fertilization concentrations were tested in the fertilization solution: 0.5, 5, and 20 mM. L^{-1} NO_3^- using a drip irrigation system (one dripper per pot) up to 6 times a day depending on the climatic demand, with one minute pulses. For each N level, sixty plants were used. The pH of the nutrient solution was adjusted to 6 in each treatment by addition of H_2SO_4. To evaluate the climatic demand for irrigation adjustment three pots chosen at random were weighted continuously to evaluate their loss of water, thus the climatic demand in the green house.

Inoculum production

Strain BC1 of *B. cinerea* was used in this study. From a previous work of our laboratory, BC1 was shown to have a high level of aggressiveness on tomato (Ajouz et al. 2010). The strain was grown on PDA (Potato Dextrose Agar Medium) (Difco, Detroit, USA) in a growth chamber (21°C, 14h photoperiod). For two BCAs *T. atroviride* strains T1 and *M. dimerum* L13 were tested in this study. They were selected for (1) their known protective effects of leaf pruning wounds on tomatoes against *B. cinerea* and (2) their difference in their presumed mode of action: nutrient competition for *M. dimerum* (Nicot et al. 2003a) and antibiosis for *T. atroviride* (Dipietro 1995; Elad et al. 1999a). Both strains of BCAs were grown on PDA

medium. For BCAs and *B. cinerea* spores were collected in sterile distilled water from the surface of 14 day-old cultures. Each suspension was filtered through a 30 µm mesh sterile filter to remove mycelium fragments and adjusted to the desired concentrations using a haemocytometer. For inoculation of *B. cinerea* on tomato plants the inoculum as spore suspension was adjusted to 10^6 spores. mL^{-1} and for the two BCAs tested the suspensions were adjusted to 10^7 spores.mL^{-1}.

Inoculations

In these experiments, set of 15 plants were randomly chosen from each N treatment and were inoculated with a virulent strain of *B. cinerea* (BC 1). For each plant, four leaves were excised conserving 5-10 mm petioles that were inoculated with 10 µL of spore suspension. The BCAs were applied five minutes after the inoculation with *B. cinerea*. On each inoculated pruning wound, 10 µL of spore suspension of either *M. dimerum* or *T. atroviride* were deposited. For each set of 15 inoculated plants per fertilization regime, 5 plants were used as control (inoculated with sterile distilled water) and 10 plants were further inoculated with BCAs (5 plants with *M. dimerum* and 5 with *T. atroviride*).

Incubation and disease assessment

Following inoculation, the plants were transferred to a growth chamber in conditions conducive to disease development (21°C, RH above 85%) with a 14 h photoperiod. During this period, the plants were irrigated manually using the same fertilization solutions as those used before inoculation. Each inoculated wound was examined daily, between day 3 and day 7 after inoculation to evaluate infection level of the petiole stubs by *B. cinerea* and subsequent development of stem lesions. The incidence of stem lesions and the length of developing cankers (mm) were recorded daily for all treatments. The area under the disease progress

curve (AUDPC) was computed as described by (Lecompte et al. 2010) during the period from the third to the seventh day after inoculation.

Data analysis

Significant differences between treatments were assessed by analysis of variance. When significantly different, results from different treatments were treated separately. The AUDPC (computed between day 3 and 7) on day 7 after inoculation of *B. cinerea* was analysed. ANOVA module of statistica software was used to analyse all the data.

Cyto-histology

Petiole samples were excised from five tomato plants from each treatment 48 h after inoculation (HAI) and structurally compared to healthy petioles. We have previously showed that 48 HAI *Botrytis* mycelium is well established toward petiole tissues (Ajouz et al. 2011). The samples were immersed for 12 h at 4°C in a FAA fixative solution (formaldehyde, glacial acetic acid, alcohol: 1/1/8: v/v/v). Fixed specimens were rinsed in distilled water, dehydrated in a graded ethanol series (70–100%) and embedded in methacrylate resin (Technovit Kulzer 7100, Heraeus Kulzer, Wehrheim, Germany).

The specimens were orientated to obtain longitudinal sections. After resin polymerization at room temperature, the blocks were cut into 3 µm thick ribbons using an automatic retraction microtome (Supercut 2065; Reichert-Jung, Leica Intruments, Nussloch, Germany) equipped with disposable knives (Histoknife H; Heraeus-Kulzer). Sections were mounted on microscope slides and stained using the periodic acid Schiff's reagent (PAS) procedure to visualize polysaccharides (pink) and naphthol blue black to visualize proteins (dark blue) (El Maataoui and Pichot 1999). Observations were performed using a Leica DMR

light microscope. Images were captured using a Leica DFC 300 FX digital camera and analyzed using LAS software (Leica). At least three samples were analyzed for each treatment. Attention was paid to spore germination, mycelium structure and progression in petiole tissues, and cyto-pathological effects.

Results

Effect of N plant fertilization on disease severity

Disease severity assessed by AUDPC seven days after inoculation showed that N fertilization induced noticeable alterations of *Botrytis* aggressiveness when inoculated alone on tomato (Fig. 1). For example, at very low N level (0.5 mM) the obtained AUDPC was ~123 whereas at high N level (20mM) the AUDPC was ~54, i.e. 50% reduction. Similar alterations have been observed in the case of co-inoculations with BCAs. Both biological control agents reduced the severity of the disease as compared to control (*Botrytis* alone) (Fig 1). However this reduction was nitrogen and species dependent. Indeed, co-inoculation with *T. atroviride* produced ~104, 39 and 34 mm AUDPC at 0.5, 5 and 20 mM N respectively. The AUDPC obtained with *M. dimerum* was ~48, 19 and 5 mm at 0.5, 5 and 20 mM N respectively. At all N levels ggenerally *M. dimerum* was more effective as compared to *T. atroviride* against *B. cinerea* for the protection of the pruning wounds on tomato stems.

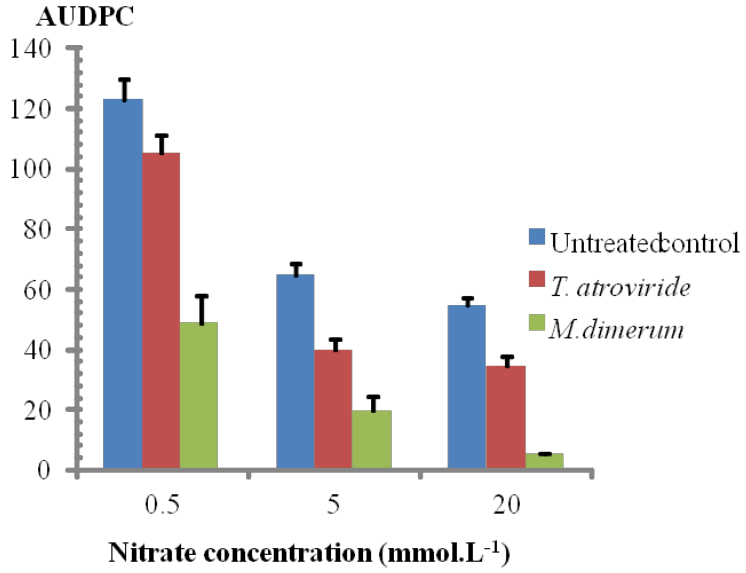

Fig. 1: Effects of N fertilization on disease severity and on efficacy of biological control as accessed *via* AUDPC seven days after inoculation with *B. cinerea* alone and when co inoculated with *T. atroviride* or *M. dimerum*. (See text for explanations)

Cyto-histology

Sections from control samples (water treated) showed that the tissular organisation of tomato petiole is composed (from exterior to interior) (Fig. 2A) of a protective epidermal layer, a cortical parenchyma with chloroplasts, a collenchymas exhibiting thick-walled, empty cells and vascular tissues embedded in a massive medullar parenchyma. At cyto-histological level, minor alterations have been observed between the tested levels of N. For example, it has been noted that at low concentrations, the parenchymatous cells showed important starch accumulation as attested by the presence of numerous, PAS-positive inclusions (Fig. 2B).

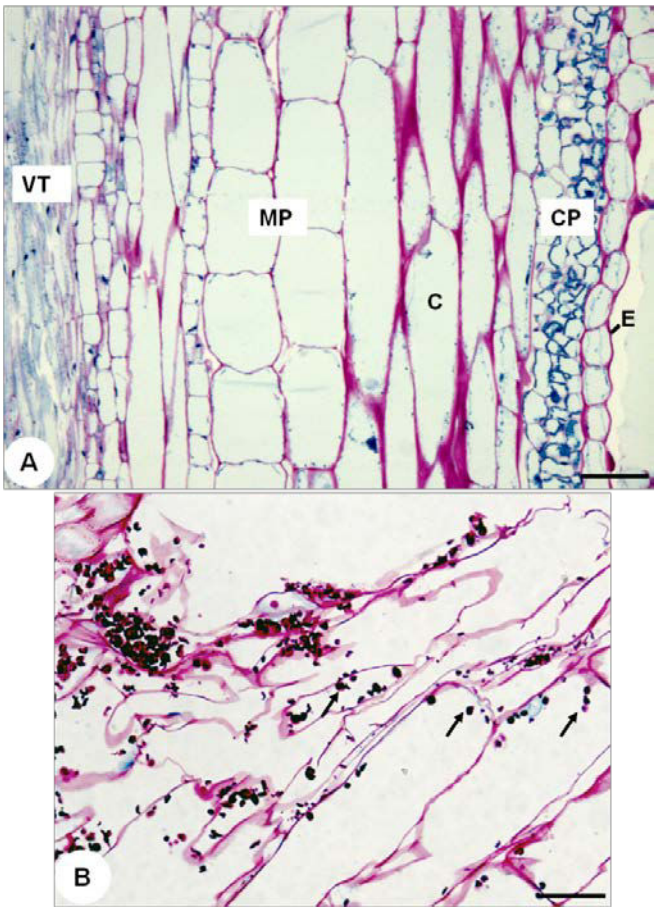

Fig. 2: Tissular organization of tomato petiole (control). **A**, Longitudinal section showing the epidermis (E), the cortical parenchyma (CP), the collenchymas with thick walls (C), the medullar parenchyma (MP) and the vascular tissues (VT). **B**, Micrograph of the sectioned zone of petiole showing starch accumulation (red granules, arrows) 48 hours after water deposition. Bars = 50 µm, **A** and **B**.

Effects of N fertilization on infection by B. cinerea

Sections from petioles inoculated with *B. cinerea* (BC1) alone showed that spores germination occurred in all N treatments and initiated a dense network of hyphae invading all tissues with an intercellular progression due to middle lamella and cell wall lyses (Fig. 3A). Parenchymatous tissues were more intensively affected as attested by the presence of numerous cells exhibiting cytological traits characteristic of programmed cell death (Fig. 3B). Thorough microscopic observations showed that the three tested N levels induced different alterations of mycelium structure and development. Indeed, sections of petioles from 0.5 mM and 5 mM N-treated tomato plants showed that the hyphal cells exhibited normally structured, multinucleate, protein-rich cytoplasm with small vacuoles (Fig. 3B). In contrast, sections of petioles taken from 20 mM treated plants showed profound alterations in the hyphal cell structure. These consisted of the presence of large vacuoles and prominent PAS-positive granules corresponding to glycogen accumulation (Fig. 3C).

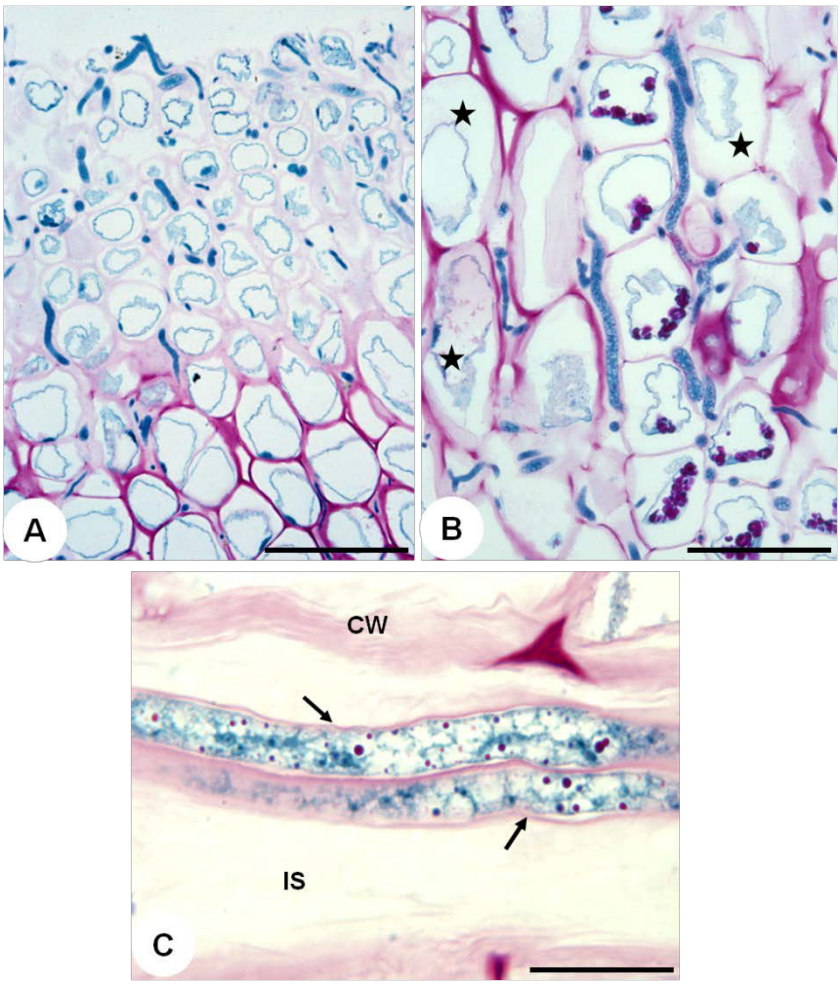

Fig. 3: Effects N fertilization on the colonization of tomato petiole tissues by *B. cinerea* alone. **A**, Section of a petiole taken from 0.5 mM treated plants showing numerous hyphae (arrows) that developed intercellularly and colonized all tissues particularly the medullar parenchyma (MP) by degrading the middle lamella. **B**, High magnification showing well structured mycelial cell compartments (arrows) and programmed cell death of host cells (stars) in the presence of 5mM N. **C**, Section of a petiole taken from 20 mM treated plants showing cytological alterations of mycelial cell compartments. Note the presence of numerous vacuoles and glycogen accumulation (red granules). CW = cell wall, IS = intercellular space. Bars = 50 **A**, **B** and **C**.

Effects of N fertilization on the interaction between M. dimerum and B. cinerea

Petioles co-inoculated with *B. cinerea* and *M. dimerum* strain (L13) showed that independently of nitrogen level both fungi (pathogen and BCA) developed mycelium intercellularly within the tissues (Fig. 4A). The mycelium of both fungi is easily recognized: multinucleated, large compartments for *B. cinerea vs* bi nucleated, thin compartments for *M. dimerum* (not shown). However it was noted that *Botrytis* mycelium exhibited cytological features indicating antagonistic interaction with L 13. Indeed, in all observed samples mycelial cells exhibited highly vacuolated cytoplasm with glycogen deposition. The extent of these alterations appeared to be nitrogen dependent. For example they were moderated with 0.5 and 5 mM N (Fig. 4B) and intensive with 20 mM N leading to mycelium degeneration (Fig. 4C). In this case, mycelial compartment (cells) loosed their organelles except the cellulosic cell walls (Fig. 4D) that exhibited a loosened appearance and desegregation in places. Additionally, it was observed that mycelial compartments were shorter than BC1 alone suggesting an initiation of chlamydospore formation (Fig. 4D). In some samples it was also noted the formation of conidia typical of the genus *Microdochium* (Fig. 4D).

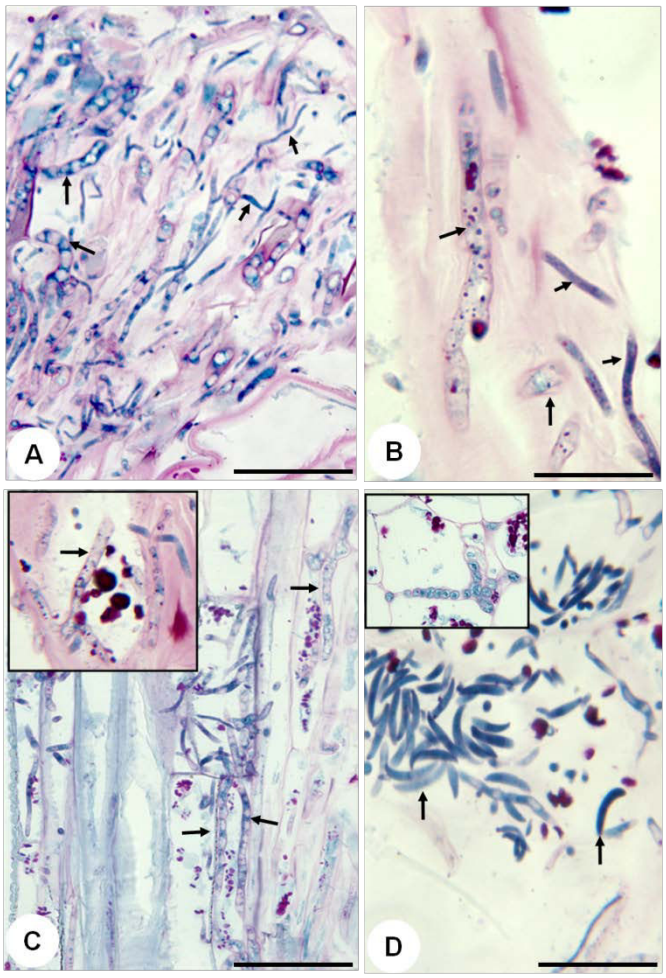

Fig. 4: Cyto-histological aspects of the effects of N fertilization on the interaction between *B. cinerea* and *M. dimerum*. **A**, Section of a petiole co-inoculated with spores of the two fungi showing that mycelial development took place concomitantly: large compartments for *B. cinerea* (arrows), uni-nucleated, thin compartments for *M. dimerum* (arrowheads). **B**, Section from 5 mM N treated plant illustrating the degeneration of *B. cinerea* mycelium (arrows). Note the presence of glycogen (red granules) and vacuoles in the mycelial compartments. Arrowheads indicate the *M. dimerum* hyphae. **C**, In the presence of 20 mM N *Botrytis* mycelium (arrows) exhibited intense vacuolization and cytoplasm condensation as a prelude of death (inset). **D**, Micrograph showing abundant, crescents-like conidia of *M. dimerum* in petiole tissues (arrows). Bars = 50 **A**, **B**, **C** and **D**.

Effects of N fertilization on the interaction between T. atroviride and B. cinerea

Co-inoculation with T1 strain of *T. atroviride* resulted in cytological disruption of *Botrytis* hyphal cells the extent of which was nitrogen-dependant. Microscopic observations showed that T1 mycelium developed with the three levels of N fertilization inducing vacuolisation, glycogen accumulation and apical growth arrest of the pathogen mycelium (Fig. 5A and B). With all three N levels used it was observed that hyphae of *Trichoderma* establish close contact with *Botrytis* hyphae suggesting a hyper parasitic behaviour (Fig. 5C). Here again the intensity of antagonistic effect appeared to be modulated by nitrogen levels (compare Figs. 5A and B). Increasing the N concentration resulted in an increase of T1 effectiveness. It was also observed that *Trichoderma* sporulates in plant tissues particularly in xylem vessels (Fig. 5D).

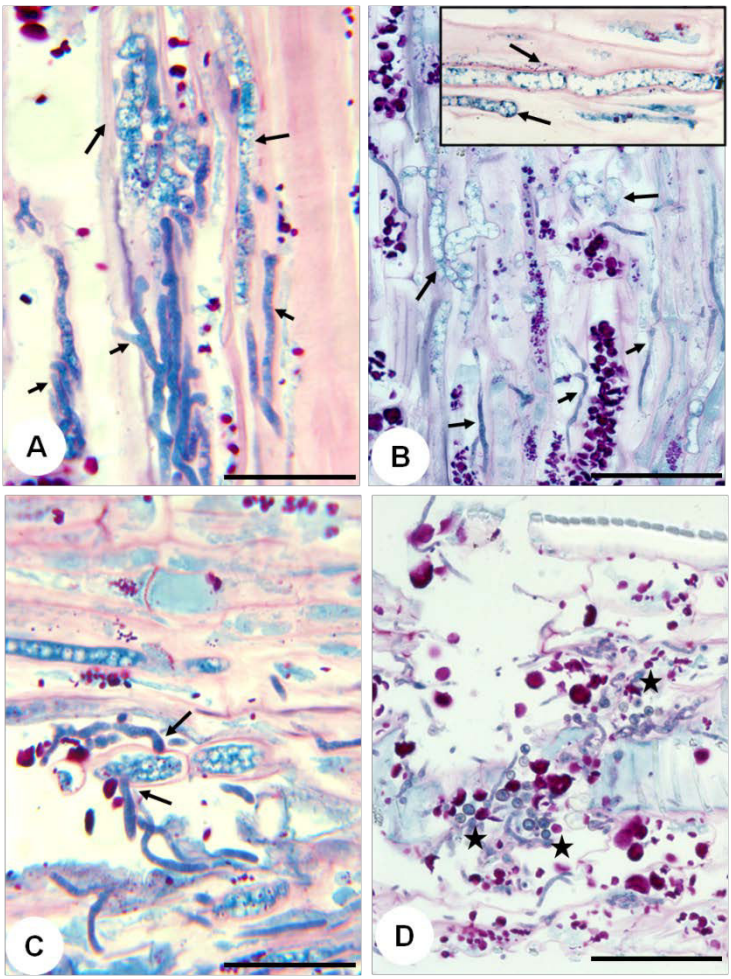

Fig. 5: Cyto-histological interaction between *B. cinerea* and *T. atroviride* T1 according to N fertilization. **A**, Section of a petiole from 0.5 mM N treatment showing the development of both *B. cinerea* (arrows) *and T. atroviride* (arrowheads) mycelia. Note the vacuolated aspect of *Botrytis* hyphae. **B**, Section from 5 mM N treated plant showing cytological alteration of *B. cinerea* mycelium (arrows) in the presence of *Trichoderma* (arrowheads). Vacuolization and hyphal cell destruction are evident and more pronounced with 20 mM N fertilization (**B**, inset). **C**, Section showing *Trichoderma* hyphae in close contact with *Botrytis* mycelium illustrating hyper parasitism (arrow). **D**, Micrograph showing sporulation *Trichoderma* in xylem vessels (stars). Bars = 50 **A**, **B**, **C** and **D**.

Discussion

The results of this study demonstrate that the over all severity of disease (AUDPC) was consistently decreased with increasing N fertilization. Similarly, it also enhanced the efficacy of the two BCAs tested (*T. atroviride* and *M. dimerum*) against *B. cinerea* in tomato. Our results are in confirmation with the earlier results showing that high N fertilization reduced the disease severity of tomato against *B. cinerea in planta* (Hoffland et al 1999, Lecompte et al 2010). In addition we recently reported that (Lecompte et al. 2010) in-vitro conditions high N fertilization decreases the mycelial growth of *B. cinerea*. Our cytological observations clearly showed that when *B. cinera* is inoculated alone high N fertilization levels induced profound hyphal cell destructions that provide evidence of the observed infection arrest.

Concerning effects of N fertilization on efficacy of biological control our results also show that high N also enhances the efficacy of the two BCAs against *B. cinerea*. Mineral nutrients are one possible factor that can be manipulated to enhance efficacy of biological control. A wide range of supplements were used previously to enhance the efficacy of biological control agents against various plant pathogens (Cabrefiga et al. 2011; Cao et al. 2008; Conway et al. 2004; Dawson-Andoh and Lovell 2000; Droby 2001). Increasing concentrations of $CaCl_2$ resulted in decreased spore germination and germ-tube growth of *B. cinerea*. The biological control activity of *Candida oleophila* against *B. cinerea* was also enhanced with increasing $CaCl_2$ concentrations (Wisniewski et al. 1995).

The effectiveness of *M. dimerum* (strain L 13) to protect pruning wounds of tomato at the crop level against *B. cinerea* has been demonstrated in earlier studies (Bardin et al. 2008; Decognet et al. 1999b). But the involved mode of action has not been well documented. Since no hyper parasitism vis-à-vis *B. cinerea* and no toxin production have been observed it was hypothesized that nutrient competition and pH modification could be involved. Our

results indicate that the extant of *M. dimerum* effectiveness against *B. cinerea* is modulated by N fertilization of tomato. As possible mechanisms to explain this phenomenon we could hypothesize that high N fertilization has: (1) a direct effect on the pathogen development by reducing the growth and aggressiveness. (2) a direct effect on antagonistic activity of BCAs by increasing its growth and development (3) indirect effect on the defense of the plant.

Concerning nutrient competition, *B. cinerea* needs an exogenous carbohydrate source (such as glucose and fructose) for spore germinations (Blakeman, 1975). This nutritional dependence suggests that microorganisms that use nutrients efficiently may represent good competitors against this pathogen (Elad and Stewart 2004). The cytohistological study performed here clearly shows that in the presence of *M. dimerum*, the mycelium of *B. cinerea* exhibited cytological alterations and developmental failures (cell wall desegregation and rupture). These antagonistic effects could be attributed to both nutrient deprivation and pH alkalinization or both. These observations suggest that the antagonistic effect of L 13 is more efficient with high N fertilization levels probably due to the negative effects of high N fertilization on the mycelium development of *B. cinerea*.

In the case of *B. cinerea*-tomato pathosystem we have already reported that high N fertilization reduced the severity of disease (Lecompte et al. 2010). Here we additionally show that the high N plant nutrition also enhance the effectiveness of BCAs. Here we hypothesized that L13 hinders the mycelial development of *B.cinerea* probably by nutrient competition. *M. dimerum* seems to be capable of metabolizing the nutrient compounds faster than pathogen thus interfering with mycelial development of the pathogen. Another putative hypothesis is that the alkalinization of the medium which is a consequence of the metabolism of *M. dimerum* could affect the pathogenicity of *B. cinerea* and thus ensure better protection of pruning wounds. Indeed the activity of enzymes responsible for pathogenicity of *B. cinerea*, such as endopolygalacturonases that degrade pectin in cell walls is even lower than

the pH tends to be basic (Prins et al. 2000). The increasing of pH in the presence of *M. dimerum* could therefore hinder the progress of *B. cinerea* in tissues of the host plant and also increase the efficacy of *M. dimerum* against *B. cinerea*.

Our results also indicate that *T. atroviride* exhibited an inhibitory effect against *B. cinerea* at high N fertilization *in planta*. The interaction between the antagonistic activity of *Trichoderma* spp. against plant pathogens and mineral nutrition has been studied previously in *in vitro* conditions. For example (Khattabi et al. 2004) reported that the antagonistic activity of *T .harzianum* against *Sclerotium rolfsii* was enhanced by increasing nitrate levels. Similarly (Duzniewska 2008) showed that the increasing levels of mineral nutrients, including nitrogen, resulted in significant increase of the growth rate of *T. harzianum* mycelium and favorably affects the antagonism of *Trichoderma* against *B. cinerea*. This suggests that high N levels increase the ability of the *T. atroviride* to inhibit the development of the pathogen. It is generally thought that *Trichoderma* interfere with phytopathogenic fungi *via* an antibiosis mechanism (Dipietro et al. 1993; Schirmbock et al. 1994). Some studies have shown that *Trichoderma* spp. gave promised results as biological control agents against *B. cinerea* for the protection of pruning wounds of tomato (Elad et al. 2004). The microscopic observations of the present study suggest that this effect may be due to cytological alterations of the mycelial cells. These alterations could be attributed to the lytic effect of antibiotic substances secreted by *T. atroviride* (Schirmbock et al. 1994) leading to the arrest of mycelium development. The fact that high N fertilization increases the effectiveness of *T. atroviride* may reflect the direct effect of N on *B. cinerea* mycelium.

In conclusion this study showed that at cyto-histological levels high N fertilization greatly influences *B. cinerea* mycelial development in tomato tissues. It is also enhanced the efficacy of fungal biological control agents. Thus the manipulation of N fertilization makes it possible not only to reduce the disease severity but also improve the efficacy of biological

control against *B. cinerea* in green house tomato. To our knowledge, this is the first *in planta* observation of the interaction between N fertilization and biological control agents with *B. cinerea*.

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General discussion

Mineral nutrients are routinely used to increase crop yield and improve overall plant health and quality. For sustainable eco-system and improved production efficiency their judicious use is very critical. Nutrients are important for growth and development of plants and also for microorganisms, and they are important factors in disease control. All the nutrients can affect disease severity (Huber 1999). However, there is no general rule, as a particular nutrient can decrease the severity of one disease but at the same time can also increase the severity of other disease or have a completely opposite effect in a different environment (Huber 1980). The nutritional status of a plant has a major impact on disease susceptibility, and this has been exploited for suppressing a variety of diseases (Engelhard, 1989).

In all mineral elements nitrogen is by far the most extensively reported element affecting plant disease. Several results at field and green house experiments level suggest that high N fertilization can either reduce or increase disease development depending upon the host crop and the pathogen involved. Some times for the same pathogen effect of N is differentially affected by different host crop. For example, Pitchay et al (2007) have reported a much higher incidence of *B. cinerea* lesions in the begonia at high fertilization. But in the tomato, it has been consistently reported a negative relationship between high N nutrition and susceptibility to *B. cinerea* (Lecompte et al 2010).

During the 1st part of this study we have tested the effect of different levels of N fertilization on the susceptibility of tomato to six strains of *B. cinerea* widely different in their aggressiveness (three are mildly aggressive and three are highly aggressive). The results of this study show that over all high N fertilization between 10-15 mM reduced the susceptibility of tomato to *B. cinerea*, for all six strains high N decreased the severity of disease. However,

we have found that the contrasted effects between the six strains of *B. cinerea* tested. For more aggressive strains high N fertilization reduced the lesion expansion on the tomato stems, in contrast for mildly aggressive strains high N fertilization slightly increase the daily lesion expansion.

Nutritional status of the host plant can affect the response of the pathogen. Contrasted intra-specific pathogen responses to plant nutritional status has already been reported in other pathosystems. However, the correlation between nutritional status of the host plants and plant susceptibility for the various strains tested, always either positive or negative, but not opposite as we have observed here. For example, when different isolates of *Blumeria* (*Erysiphe graminis*) was tested on barley the infection efficiency of the pathogen was mostly dependent on the isolates used, it was 2.5 to 7.8 times higher at 240 mg N per plant compared to 30 mg N per plant (Jensen and Munk, 1997). In other study the inhibitory effect of calcium on *B. cinerea* has also been shown to depend on the isolate used, but high calcium levels always decreased the fungal growth of a pathogen (Chardonnet *et al.*, 2000).

It has been suggested that high N fertilization increases host susceptibility to biotrophic fungi and decreases that of necrotrophic fungi. To explain this phenomenon one explanation could be that biotrophic pathogens rely mostly on the nutrient content of the apoplast, or on nutrient transfer from the host cells by a haustorium, while necrotrophic fungi that kill cells have a better access to the host nutrients (Divon and Fluhr, 2007; Solomon et al., 2003). In our studies we have also shown that *in vitro* conditions the strains responded positively, and similarly, to the N content of the medium used, at least up to a concentration of $2g.L^{-1}$ NH_4NO_3. In this study total N content increased with increasing N in the nutrient solution, so it could be hypothesized that any repression of fungal growth at the higher host N content was the result of a better ability of the plant to defend itself against the pathogen. As earlier studies shows that N play a vital role in the development of plant diseases, because in

all nutrient elements N is required by the plant in the greatest quantity also it is involved in metabolic processes, and is a component of proteins and metabolic compounds in disease defence. It is also consistent with reports that a high N nutrition can lead to production by the host of higher levels of some constitutive and induced defence molecules (Dietrich et al., 2004; Stout et al., 1998). The different responses of our strains to the host N status could be explained by the fact that infection strategies of *B. cinerea* (types and levels of secreted fungal toxins, cell wall degrading enzymes or oxalic acid) are strain-dependant (Choquer et al., 2007; Siewers et al., 2005). So here we thus conclude that balanced N fertilization can provide opportunities to fertilize the crops not only to maximise the over all yield and quality of the produce but also N could be use as a cultural control of many plant diseases in an indirect way. In case of tomato-Botrytis pathosystem high N fertilization reduced the over all disease severity for all isolates.

In second part of this study we have studies the effect of five different levels of N fertilization of the host plant influences production and aggressiveness of *B. cinerea* secondary inoculum. We already know that N fertilization can influence the susceptibility of plants to *B. cinerea* but there is lack of studies on the possible effects on sporulation of the pathogen and on the aggressiveness of resulting secondary inoculum. Sporulation is important for plant pathologists since spores are the major vehicle for the dissemination of fungal diseases (Dahlberg K R 1982). Abundance of fungal sporulation plays a key role in the development of epidemics, because long-distance dispersal in the air is an important survival strategy for many fungi, enabling them to colonize new territory rapidly or to migrate between summer and winter habitats. It is especially relevant for fungi pathogenic on crop plants, because wind dispersal of their spores for hundreds or thousands of kilometres has caused the spread of several important diseases on a continental or global scale and allows the regular reestablishment of diseases in regions where the climate is seasonally unfavourable.

Keeping in view the importance of the sporulation of *B. cinerea*, this study showed that level of N fertilization of tomato plants had a highly significant impact on the amount of spores produced by *B. cinerea* on diseased tissue as well as on the pathogenicity of this secondary inoculum. Sporulation was also affected by the type of plant tissue used as substrate. Overall sporulation by two strains of *B. cinerea* decreased significantly with increasing nitrate up to 15-30 mM. Aggressiveness of the spores was significantly influenced by the nutritional status of their production substrate. Disease severity was highest with spores produced on plants with very low or very high N fertilization (0.5 or 30 mM nitrate). It was lowest for inoculum from plants with moderate levels of N fertilization.

So our results are in confirmation with the earlier studies which showed that composition of substrate can affect the sporulation of B. cinerea and other fungi (Gao and Liu, 2010; Nicot et al., 1996), few studies have addressed the effect of plant fertilization on the production of secondary inoculum on diseased tissue for example, the disease severity and spore production of *Blumeria* (syn. *Erysiphe*) *graminis* (powdery mildew of barley) were increased with increase with increasing N fertilization (Bainbridge, 1974; Jensen and Munk, 1997), similarly in other study spore production by *Oidium neolycopersici* on tomato leaves (Hoffland et al., 2000b) and uredospore production per leaf lesion by *Puccinia triticina* on wheat was also increased with increasing N fertilization (Robert et al., 2004). In contrast, our study of *B. cinerea* on tomato shows that spore production was generally lowest with high N fertilization. Overall, this supports the hypothesis that biotrophs (such as the rusts and powdery mildews) and necrotophs (such as *B. cinerea*) may be differentially affected by N fertilization (Hoffland et al., 2000b; Solomon et al., 2003). However, the plant species might also influence this since increasing N from 1.8 to 28.8 mM in the irrigation solution of basil plants increased the incidence of sporulation by *B. cinerea* on stem segments (from nearly zero to over 75%) three days after inoculation (Yermiyahu et al., 2006).

In summary, the apparent contradictory effects of N fertilization on the sporulation of different fungi we can hypothesized that the N fertilization affects each fungus differentially because every fungus has different resource requirements for growth and development. However effect of each plant species can not be ruled out as in some studies the same pathogen act differentially according to host plant attacked. We conclude that the effect of N fertilization on fungal sporulation is highly pathogen-host specific and is probably dependent on differences in resource requirements of the pathogen or the sensitivity of the pathogen to plant resistance reactions or on both these factors.

In the 3rd part of the present study we examined the effect of five levels of N nutrition on the efficacy of two biocontrol agents *(Trichoderma atroviride* and *Microdochium dimerum)* to protect pruning wounds of tomato against *B. cinerea*. Although N fertilization is known to affect plant susceptibility to certain pathogens including *B. cinerea* in tomato, little is known on its possible effect on the efficacy of biological control. Our results showed that tomato plant N fertilization strongly affected disease development for both strains (BC1 & BC21) of the *B. cinerea* and it influenced the efficacy of both biocontrol agents. In control plants disease severity decreased with increasing N fertilization up to 10 mmol of nitrate per liter of nutrient solution. In the presence of either BCA (*M. dimerum* and *T. atroviride*), the AUDPC also decreased significantly with increasing N fertilization (up to 5-20 mM). So our results are in confermation with the earlier esults showing that high N nutrition decreased the susceptibility of tomato plants to *B. cinerea* (Lecompte et al., 2010, Hoffland et al., 1999).

In addition high nitrogen fertilization also increased biocontrol efficacy by delaying symptom development on the stems and slowing down lesion expansion. The efficacy of biocontrol globally increased with increasing N fertilization; however variations depending on the strain, the concentration of the pathogen's inoculum and the species of BCA were observed. *M. dimerum* provided higher levels of protection than *T. atroviride* and the efficacy

of either BCA was higher against the mildly aggressive than against the highly aggressive strain of *B. cinerea*. The protection index of *M. dimerum* increased sharply with increasing N fertilization, to reach nearly 80% and 100%, respectively, against the highly and the mildly aggressive strains of *B. cinerea*, while that of *T. atroviride* increased only moderately.

Few studies have examined the effect of soil fertilization on biocontrol. (Khattabi et al., 2004) reported that soil amendment with four types of N fertilizers resulted in decreased survival of sclerotia of soilborne pathogen *Sclerotium rolfsii* and that the antagonistic activity of biocontrol fungus *T. harzianum* against those sclerotia increased with increasing N doses. N amendments in nutrient agar media have also been shown to increase *in vitro* biocontrol activity of certain microorganisms. Thus, even if the major effect of plant N nutrition on biocontrol efficacy mostly occurred at the early infection phase, marked by a delay in symptom development, a reduction in lesion expansion was able, in some cases, to contribute to increased biocontrol at high N level when stems became infected.

Highly significant correlations ($P<0.001$) were observed between disease severity (AUDPC) and the concentration in the stems (mg per gram dry matter) of some of the tissue components: negative correlations with total N, NO_3^-, and fructose, and positive with sucrose (Table 3). Ccorrelations between susceptibility or resistance to diseases and various indicators of nitrogen status of the host plant (amino acids, nucleic acids, phenols, total N, protein content, C / N, etc) have been reported before in other plant-pathogen systems (Huber and Thompson, 2007). Also, a relationship between plant sugar content and susceptibility has been suggested by Bolton, (2009). Interestingly, no correlation was observed between disease severity and total soluble sugars, contrarily to the results of Hoffland et al. (1999) with total soluble carbohydrates, suggesting that other compounds than those measured in our study (glucose, fructose, sucrose) may be implicated, or that only some of the soluble sugars, fructose and sucrose, are implicated in the response to infection. The recent description of a

mutant of *B. cinerea* incapable of using fructose as a sole carbon source (Aguayo et al., 2011) should provide a useful tool to investigate this subject further.

Competition for nutrients is a common mode of action of biocontrol agents on plant surfaces. Although no information is available for the two BCAs included in our study, competition for fructose and nitrate has been identified as a factor in the mode of action of certain BCAs (Jacobsen, 2006). This indicated that the effects of these components were not different for plants inoculated with *B. cinerea* alone or with *B. cinerea* plus a BCA, and conflicted with the hypothesis of a direct effect of the plant components on biocontrol. Rather, the results suggest that the tissue content affected the capacity of the pathogen to infect the plant, and that the outcome of the biocontrol was in turn affected by this modified interaction between the host and the pathogen. This hypothesis is in accordance with the observation that at each N regime, the efficacy of biocontrol was directly related to the pressure exerted by the pathogen, in terms of inoculum concentration and strain aggressiveness. Increasing the plant N content would either enhance plant defenses or reduce the ability of the fungus to colonize the tissue, and by this direct reduction of fungal growth, indirectly facilitate the action of the BCAs and hence their efficacy.

Conclusions and Perspectives

1: In conclusion, we have shown here that the the tomato plants grown with different regimes of N fertilization (0.5 to 20 mM), overall disease severity was lower for all isolates on plants with higher nitrogen inputs, but the effect of host N on *Botrytis* development depends on the strain and the concentration of inoculum leading to primary infection. One explanation for this could be that the fungal development depend both on trophic and defence processes, and that the availability of nutrients for the pathogen ultimately depends of its ability to explore new sources of unexplored plant tissues. Upon N scarcity, low substrate availability would impair growth of moderately aggressive isolates, those against which plant defences are more efficient, while resources available to aggressive strains would not be limiting, whatever the nutrition status of the host. More research is needed in order to find how N fertilization increase or decrese disease tolerance or resistance to different host-pathogen systems, what the changes are in plant metabolism and how this can be used to control plant disease. In addition, further research is required to use different isolates of the pathogen on different patho systems.

2: The nutritional status of the host plant had a significant impact on the abundance and pathogenicity of secondary inoculum produced by plant pathogenic *B. cinerea*. This information could lead to exciting new studies to elucidate cellular mechanisms involved and suggests that fertilization can influence the development of epidemics on a field scale. Conversely, it opens the possibility of manipulating crop fertilization not only to reduce plant susceptibility but also to limit the impact of secondary inoculum and minimize the need for pesticide use.

3: The results of this study clearly indicated that the level of N fertilization provided to the plant can influence not only the susceptibility of tomato stems to *B. cinerea* but also the

efficacy of biocontrol. High N fertilization enhanced the efficacy of both biocontrol agents tested against *B. cinerea*. To our knowledge this is the first report of an effect of N fertilization on the efficacy of biocontrol against an airborne disease. It suggests that the manipulation of N fertilization could provide a new useful tool for integrated protection of greenhouse tomato. To devise specific recommendations for growers, further work is needed to validate the present results in conditions of commercial production and to evaluate possible side effects on other diseases or pests in the tomato greenhouse. Thus, the novel results presented here on the effect of N fertilization on the efficacy of two biocontrol agents against *B. cinerea* should stimulate further work to better understand which mechanisms are involved at the cellular and molecular level.

4: This study showed that at cyto-histological levels high N fertilization greatly influences *B. cinerea* mycelial development in tomato tissues. It is also enhanced the efficacy of fungal biological control agents. Thus the manipulation of N fertilization makes it possible not only to reduce the disease severity but also improve the efficacy of biological control against *B. cinerea* in green house tomato. To our knowledge, this is the first *in planta* observation of the interaction between N fertilization and biological control agents with *B. cinerea*.

5: The main conclusion of our work is that, at least for one of the two necrotrophs in this study, the effect of N fertilization on susceptibility appeared statistically mediated by the metabolism of sugars. As sugar accumulation is specific in each plant species, and for a given species, in its different organs, it might explain why the variation of plant susceptibility to necrotrophs in response to N fertilization is not unequivocal. Genotypes with contrasted sugar accumulation should be useful to test a general relationship between specific sugars and resistance to necrotrophs in lettuce and other species. Clearly more work is needed in this area and it is, of course, likely those different fungi will respond in different ways to the application of N fertilization. It is important that future studies combine measurements of

fungal growth and symptom production with plant biomass and in plant nutrient levels. Further studies are thus required to understand the mechanisms involved at the level of gene expression. Trophic interactions related to primary metabolism are unlikely to be the only mechanisms implicated. Other compounds related to plant defense mechanisms were not examined in our study but would merit further attention as they could influence fungi indirectly through their effect on plant tissue colonization by the pathogen.

In the end we conclude that in most of the studies reported here clearly shown that optimum N fertilization of the host plant has decreased the susceptibility of tomato plants to *B. cinerea*, their sporulation and aggressiveness and also high N fertilization enhanced the efficacy of biocontrol agents against this pathogen for the protection of pruning wounds of tomato. This is probably because N nutrient is involved in the tolerance or resistance mechanism of the host plant.

In sustainable agriculture balanced nutrition is an essential component of any integrative crop protection programme because in most cases it is more cost-effective and also environmentally friendly to control plant disease with the adequate amount of nutrients with no pesticides. Also, balanced nutrient fertilization can reduce disease to an acceptable level, or at least to a level at which further control by other cultural practices or conventional biocides are more successful and less expensive.

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Abstract: Nitrogen (N) fertilization is known to influence the susceptibility of many plants to a variety of diseases. In the case of diseases caused by *Botrytis cinerea*, the role of N fertilization appears to be variable, with high levels either fostering or reducing severity depending on the studies. To test whether this variability could be due to possible differences in the host plants, inoculum pressure or in the behavior of different strains of the pathogen, studies were carried out to investigate the effect of different N fertilization regimes on the susceptibility of tomato and lettuce to six isolates of *B. cinerea*. Possible epidemiological effects of N fertilization through the sporulation of the pathogen and on the pathogenicity of resulting secondary inoculum were also investigated on tomato. Plants were grown in a soil-less drip-irrigation system. Differential N nutrition ranging from 0.5 to 30 mM NO_3^- was applied for the last four weeks prior to inoculation on the leaves (lettuce) or on leaf pruning wounds (tomato) and incubation of the plants in conditions conducive to disease development. On the tomato stems, disease onset was delayed and overall severity was lower for all isolates on plants with higher N inputs, regardless of inoculum concentration. However, the rate of stem lesion expansion was differentially affected depending on the strains, decreasing with increasing N fertilization levels for the more aggressive isolates, while increasing for the less aggressive isolates. In contrast with tomato, high N fertilization increased disease severity on lettuce for all isolates tested. On tomato plant tissue, sporulation of *B. cinerea* decreased significantly with increasing N fertilization up to 15-30 mM NO_3^- and the pathogenicity of the spores was significantly influenced by the nutritional status of their production substrate. It was highest for spores produced on plants with very low or very high N fertilization (0.5 or 30 mM NO_3^-) and lowest for those from plants with moderate levels of N fertilization. Plant fertilization also strongly affected the efficacy of two biocontrol agents *(Trichoderma atroviride* and *Microdochium dimerum)* to protect pruning wounds of tomato against *B. cinerea*. The highest levels of protection were obtained with high N fertilization and related to a delay in symptom development on the stems, sometimes associated with a slowdown in lesion expansion. Histological studies showed that the decrease in disease severity at high N fertilization was associated to structural alteration of *Botrytis* mycelial cells. In the presence of a biocontrol agent, the effect on the pathogen was further associated to vacuolisation, glycogen deposition and mycelial cell death. Hypotheses to explain these results are discussed in light of the possible physiological effects of nitrogen fertilization on nutrient availability for the pathogen in the host tissue and of possible production of defense metabolites by the plant. These results also open new possibilities for including the manipulation of N fertilization as a tool for the integrated protection of vegetable crops.