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Resistance induction in the pathosystem tomato – *Alternaria solani*

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1 INTRODUCTION

Tomatoes (*Solanum lycopersicum L.*, syn. *Lycopersicon esculentum* Mill.) belong to the important fruit vegetables for human nutrition and are cultivated across all continents in fields or in protected culture. Early blight of tomato, caused by the necrotrophic fungus *Alternaria solani* (Ellis & Martin) Jones & Grout, is one of the most common foliar diseases of tomatoes. The disease can occur over a wide range of climatic conditions, but is most prominent in areas with heavy dew, rainfall and high relative humidity. On tomato it causes damping-off of seedlings, later collar rot, leaf spots, stem lesions and fruit rot. Infection of the plants can result in a complete loss of the crop as yields are reduced by destruction of foliage and the fruits are damaged directly by the pathogen and by sun blotch on defoliated plants (Rotem, 1994).

No major gene resistance towards early blight is known, genetic sources for partial resistance have been identified within wild species of tomato. The resulting lines from crosses of tomato with these wild species have still no satisfying crop qualities. Therefore, disease control of early blight is mainly conducted with chemical protective agents. However, these agents do not always prevent the infestation of the fruits and severe losses can still occur. Additionally, the used agents are often strongly fish poisonous, resulting in an increased risk for the environment in case of inappropriate application or storage.

An alternative to usual chemical plant protection methods could be to employ systemic acquired resistance (SAR) effects. These are processes whereby the

plant's own defence mechanisms are activated by biological or chemical resistance inducers. With SAR, the disease susceptibility of treated plants can be reduced for several weeks and the growing parts will also be protected. As SAR has only protective and no curing effects, it could be necessary to combine the resistance inducer with low-dosage of conventional fungicide application in order to stop pathogen development during the build-up of the induced resistance.

A third approach of plant protection is investigated in this study. The basic idea is to strengthen the plant by a symbiotic relation with arbuscular mycorrhizal fungi (AMF). Mycorrhiza is the symbiosis between land plants and AMF of the order *Glomales*. The AMF colonise the root cortex of a variety of host plants, among them tomato, and enhance the uptake of relatively immobile nutrients like phosphate or zinc. Other advantages for the colonised plant are (i) the supply with water is optimised, (ii) the tolerance to abiotic stresses is increased, and (iii) resistance to several root pathogens is improved. In return, the plant shares assimilated carbon with the fungal partner which depends on these sugars to complete its life cycle.

This work is part of a cooperative project with scientists of the National Centre for Agricultural and Livestock Health (CENSA) in La Habana, Cuba. In Cuba, *Alternaria solani* has far greater importance than in Central Europe because of the climatic conditions in the Caribbean region. Due to the political and economical situation of this country, modern fungicides and fertilisers are scarce and much too expensive for the majority of the farmers. Therefore, biological plant protection strategies are more commonly used and research for alternatives to chemical plant protection is very important.

The objective of this work was to develop a plant protection strategy which effectively combines systemic induced resistance, plant restoratives, symbiotic organisms like AMF, and – if necessary – minimum quantities of chemical fungicides to control the early blight disease on a long-term basis in an ecologically friendly manner.

2 LITERATURE SURVEY

This chapter provides an overview of the current state of research concerning (i) the examined pathogen of interest, *Alternaria solani*, (ii) its host plant tomato, (iii) the methods and achievements of chemical plant protection, (iv) induced resistance, and (v) improvement of plant health by symbiotic root colonising fungi.

2.1 The pathosystem tomato – *Alternaria solani*

The main focus of this section lies on the attributes of *Alternaria solani* (in the following *Alternaria*) and how they influence the infection process.

Tomatoes (*Solanum lycopersicum* L., syn. *Lycopersicon esculentum* Mill.) are a major contributor to the fruit vegetable diet of humans. They are cultivated in essentially all countries either in fields or in protected culture. In 2004, an area of 4,4 million ha was used for tomato production worldwide. The yield per ha differs widely: e. g. in Cuba an average yield of 13,4 t ha^{-1} is achieved, in Germany about 145 t ha^{-1} and in the Netherlands in high-input horticulture 454 t ha^{-1} (FAOSTAT data, 2004).

Early blight of tomato, caused by *Alternaria solani* (Ellis & Martin) Jones & Grout, is economically the most important disease of tomatoes in the USA, Australia, Israel, the UK, and India, where significant reductions in yield (35 up to 78 %) have

been observed (Datar & Mayee, 1972; Basu, 1974; Jones *et al.*, 1993). On tomato it causes damping-off of seedlings in the juvenile plant stage, on older plants collar rot, leaf spots, stem lesions and fruit rot. Typical symptoms for early blight disease are dark spots with concentric rings of spores surrounded by a halo of chlorotic leaf area (see Figure 2.1).

Figure 2.1: Tomato leaf with typical *Alternaria solani* symptoms. The dark brown necrotic spots have concentric rings in which new spores are produced and are surrounded by a chlorotic halo caused by the secreted toxins.

Infection of the plants can result in a complete loss of the crop as yields are reduced by the destruction of foliage and the fruits are damaged directly by the pathogen and by sun blotch on defoliated plants (Rotem, 1994). The disease progressively weakens the plant and increases its susceptibility to infection by reducing the photosynthetic leaf area and increasing the imbalance between nutrient demand in the fruits and nutrient supply from the leaves (Rowell, 1953). *Alternaria* has the ability to grow over a wide range of temperatures from 4 to 36 °C (Pound, 1951) and requires only a short wet period of at least four hours for successful infection (Vloutoglou & Kalogerakis, 2000). The disease is less frequent and less damaging on pepper, eggplants, and some other species of *Solanaceae* and other families (Rands, 1917; Neergaard, 1945). Typically, weakened plant tissues, either due to stress, senescence, or wounding, are more susceptible to *Alternaria* infection than healthy tissues (Thomma, 2003). *Alternaria* is a necrotrophic pathogen, i.e. that the invading fungus kills plant cells in order to feed

on the cell contents, instead of developing haustoria and keeping the plant tissue alive as biotrophic pathogens do.

Alternaria spp. has no known sexual stage or overwintering spores, but the fungus can survive as mycelium or spores on decaying plant debris for a considerable period of time, or as a latent infection in seeds (Rotem, 1994). The two major features of *Alternaria* species are the production of melanin, especially in the spores, and the production of non-specific as well as host-specific toxins in the case of pathogenic species (Thomma, 2003). One of the earliest identified non-specific toxins is alternaric acid, identified by Brian *et al.* (1952). This toxin, isolated from lesions or from culture filtrate, caused chlorosis and necrosis when introduced in tomato plants and also damaged nonhosts of *Alternaria* like cabbage, radish, spinach, pea, bean, and others, thus pointing to its nonspecificity (Pound & Stahmann, 1951). Germination fluids of *Alternaria* contain alternaric acid as well as a nontoxic substance that acts as susceptibility-inducing factor (Langsdorf *et al.*, 1990). It was concluded that alternaric acid alters the morphological and physiological characteristics of plasma membranes near plasmodesmata and thereby causes a permeability change which leads to a leakage of electrolytes (Langsdorf *et al.*, 1991).

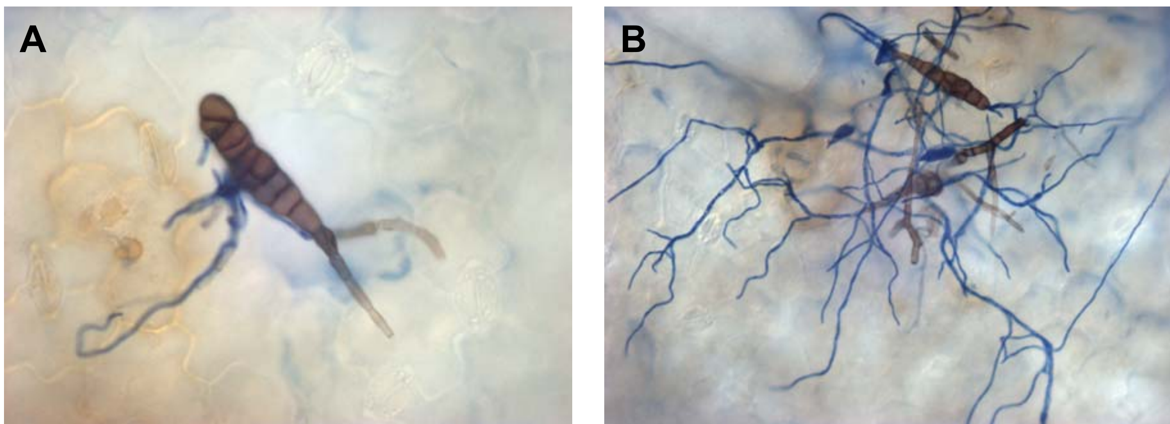


Figure 2.2 A and B: Germinated *Alternaria solani* spores on a tomato leaf surface. **A**: A single germinated spore and to the left dead, brownish epidermal cells that have been killed by toxins prior to penetration. **B**: Two spores and several mycel pieces with lots of new formed mycel (blue) ramifying on the surface of the leaf. Thick blue mycel dots could be appressoria for possible penetration but intracellular mycel is not visible. Note that toxins have already killed by some epidermal cells in the background.

Under favourable conditions, *Alternaria* spores germinate within hours and can produce more than one germ tube per spore as the spores consist of several cells.

The ability to penetrate the cuticule, stomata, and wounds has been described for most *Alternaria* species (Rotem, 1994). In case of less virulent strains or young and therefore resistant leaves, the germinating hyphae of *A. cassiae* and *A. alternata* tend to spread over the intact leaf surface and the only sites of infection are dead cells, suggesting that penetration is preconditioned by the secretion of toxins (von Ramm, 1962; van Dyke & Trigiano, 1987). This observation is consistent with our own microscopic examinations of *Alternaria solani* (see Figure 2.2).

Improving the resistance of tomato cultivars seems to be a promising option for control of early blight. But the lack of single-gene resistance and the complex patterns of inheritance have resulted in the availability of no commercial tomato cultivar that possesses adequate levels of resistance to *Alternaria* (Nash & Gardener, 1988). No genetic source of early blight resistance is known within the cultivated species of tomato (Martin & Hepperly, 1987; Foolad *et al.*, 2000). However, resistant accessions have been identified within related wild species of tomato, in particular the green-fruited species *Lycopersicon hirsutum* (Barksdale & Stoner, 1977; Maiero *et al.*, 1989) and the red-fruited species *Lycopersicon pimpinellifolium* (Martin & Hepperly, 1987; Kalloo & Banerjee, 1993). Resistant lines and cultivars provide a moderate resistance that enables the plants to tolerate an extended fungicide spray interval. This may contribute to a reduction in chemical inputs for early blight control in tomato (Gardner & Shoemaker, 1999). However, many of the more resistant lines are late-maturing or low-yielding, and/or the level of resistance is insufficient under field conditions (Foolad *et al.*, 2000).

2.2 Chemical plant protection

Currently, the following three early blight control measures are the most common in tomato production: (i) sanitation, (ii) long crop rotation to reduce the spore concentration on decaying plant material, and (iii) routine application of fungicides (Zhang *et al.*, 2003). The chemical control is conducted mainly with protective agents like MANEB® (in the following referred to as Maneb) or PROPINEB® and with inorganic fungicides (e.g. copper-hydroxide). Maneb is a dithiocarbamate which inhibits spore germination and penetration of the leaves. Since this agent

has only a preventive and no curative effect, leaf accession is not protected and the infestation of the fruits cannot be prevented so that crucial losses still occur. As Maneb is also easily washed off by rainfall, optimal plant protection requires repeated applications. A waiting period of three weeks between spraying and harvest has to be followed in order to ensure the degradation of the chemical remainders. Additionally, this plant protectant is strongly fish poisonous, which results in an increased risk for the environment in case of inappropriate application or storage.

In practice, different fungicides are often combined in mixtures. This approach is preferable mainly for three reasons: (i) to widen the spectrum of antifungal activity to control several diseases occurring simultaneously in a crop; (ii) to exploit additive and synergistic interactions between fungicides, by which the overall activity is increased and the concentrations of the compounds can be reduced without loss of activity; and (iii) to delay the selection process of resistant individuals in a pathogen population to one component of the mixture (Gisi, 1996).

In combination with the resistance inducer ASM, the fungicide Maneb could be added in very small dosages that could be sufficient to protect the plant until the build-up of induced resistance is completed. The interactions can be synergistic, if the combination has a greater activity than the activity of both agents applied alone. Also additive or, in the unfavourable case, antagonistic interactions are possible. All interactions can be demonstrated by special experimental designs and statistical analysis. The appropriate statistical approach has to be chosen depending on whether these agents function with the same or with different modes of action. Different action occurs if each pesticide affects a different physiological activity or vital system in the pest (Wadley, 1945), in this case, the Abbott procedure is proposed to determine synergism. As Maneb affects *Alternaria solani* and ASM activates the plant without direct activity against the pathogen, the modes of action are clearly different and the Abbott procedure (Abbott, 1925) the appropriate statistical method.

2.3 Induced resistance

An alternative to usual chemical plant protection agents could be the use of induced resistance. Two kinds of induced resistance are distinguished: (i) the systemically acquired resistance or SAR, and (ii) the rhizobacteria-mediated induced resistance, or ISR for short.

SAR is activated after infection by a necrotising pathogen or other biotic and abiotic stresses, rendering distant, uninfected plant parts resistant towards a broad spectrum of pathogens (Kuć, 1982). Generally, the SAR mechanism is effective against many types of pathogens including viruses, is associated with the production of pathogenesis related (PR) proteins and is mediated via a salicylic acid dependent process (Hammerschmidt, 1999). After an acquisition period that ranges from few hours to several days, this effect lasts for weeks and also protects the growing plant section in dicotyle plants.

The systemic signal prepares the tissue to react more rapidly and more efficiently to an infection challenge by a virulent pathogen. This phenomenon is often referred to as "conditioning" (Métraux *et al.*, 2002) or also "sensitising" (Sticher *et al.*, 1997). Since SAR protects the plants against a variety of diseases, it is assumed that the use of general chemical plant protection agents could be substantially reduced if substituted by SAR. It is likely that a combination of the resistance inducer with a fungicide in low dosage would give best results. This fungicide addition would stop the further development of attacking pathogens during the resistance build-up when the plants are still susceptible.

SAR can also be induced by a variety of chemicals which do not act systemically but cause lesion-like tissue damage at the points of application, suggesting that these chemicals mimic the biological SAR induction by necrotising pathogens. Examples for these chemicals are various salts, unsaturated fatty acids, harpin proteins, elicitin peptides, and sublethal concentrations of certain herbicides, as reviewed by Oostendorp *et al.* (2001). All these compounds share that they do not have direct antimicrobial effects on the pathogens.

Exogenous application of salicylic acid or structural analogues of SA such as benzo(1,2,3)thiadiazole-carbothioic-acid-S-methylester (BTH) and 2,6-dichloroiso-nicotinic acid (INA) that appear to act similarly to salicylic acid can induce SAR (Hammerschmidt, 1999). Spletzer & Enyedi (1999) could show that root feeding of salicylic acid to tomato plants significantly elevated foliar SA levels, induced PR-1B gene expression, and activated SAR that was effective against *Alternaria solani*.

Benzo(1,2,3,)thiadiazole derivates with S-methyl-benzo(1,2,3)thiadiazole-7-carbo-thiate, known as Acibenzolar-S-metyl (ASM), was the first commercial product marketed under the trade names BION®, ACTIGARD™ and BOOST®. These compounds do not show any antimicrobial activity *in vitro* and activate resistance against the same spectra of pathogens as the biological inducers of SAR (Kunz *et al.*, 1997). Resistance activation by ASM takes place without the accumulation of SA in wild type plants and it is still effective on *NahG* tobacco and *Arabidopsis thaliana* plants which lack SA and do not respond to biological induction of SAR (Oostendorp *et al.*, 2001), showing that the synthetic inductor itself functions as transmitter of the signal and is transported in the phloem to all plant parts. Like all other resistance inducers, both biological and chemical, ASM has to be applied well before the challenging pathogen attack as it has no curative effect and the build-up of resistance can take several days. ASM has shown activation of broad-spectrum disease resistance under field conditions on tomatoes, some other vegetable and fruit crops but this ASM-activated resistance is highly crop specific, for example on tomatoes, resistance is activated against late blight (*Phytophthora infestans*) while on potato no reliable activation against the same pathogen is observed (Tally *et al.*, 1999).

The second type of induced resistance develops systemically in response to colonisation of plant roots by certain plant growth promoting rhizobacteria (PGPR). This type of resistance, known as rhizobacteria-mediated induced systemic resistance (ISR), is transferred by a jasmonate/ethylene sensitive pathway and does not involve expression of PR proteins (van Loon *et al.*, 1998). Rhizosphere bacteria are present in large numbers on plant root surfaces, where root exudates and lyases provide nutrients (Lynch & Whipps, 1991). Generally, PGPRs are able

to control plant pathogens by antibiotic effects, site occupancy or competition for iron through siderophores (Métraux *et al.*, 2002). Certain strains of PGPRs promote plant growth and some are able to induce systemic resistance which extends to the above-ground plant parts and is phenotypically similar to SAR (Pieterse *et al.*, 2001).

Different rhizobacteria utilise different mechanisms for triggering systemic resistance: some trigger the SA-dependent pathway, others a jasmonate/ethylene-dependent one, and additional pathways are likely to be discovered in the future (Pieterse *et al.*, 2001). Silva *et al.* (2004) reported that PGPR isolate B101R reduced disease intensity in terms of average number of leaf lesions of *Alternaria solani*, *Stemphilium solani* (leaf spot) and *Oidium neolycopersici* (powdery tomato mildew) on tomato. ISR can be induced not only by the rhizobacteria themselves, but also by bacteria-synthesised macromolecules (Romeiro *et al.*, 2005). Based on work with gene-knockout mutants in *Arabidopsis*, SAR and ISR are proposed to confer resistance to pathogens according to their lifestyles, so that SAR primarily functions against biotrophic pathogens, and ISR against necrotrophic pathogens (Thomma *et al.*, 1998; 2001). Bacterial strains differ in their ability to induce resistance in different plant species, and plants show variation in the expression of ISR upon induction by specific bacterial strains (van Loon *et al.*, 1998). Simultaneous activation of both the jasmonic acid/ethylene-dependent ISR pathway and the SA-dependent SAR pathway resulted in an enhanced level of protection as investigated by van Wees et al. (van Wees *et al.*, 2000).

2.4 *Spirulina platensis* as plant restorative

Plant restorative agents are defined as agents which either (i) enhance the resistance of plants against pathogens without having a direct influence on the pathogen itself, (ii) protect plants against abiotic stresses, or (iii) prolong the life of cut flowers (Gesetz zum Schutz der Kulturpflanzen, 1998). In Germany, these plant restoratives have to be registered at the Biologische Bundesanstalt in Braunschweig and listed at the Bundesamt für Verbraucherschutz und Lebensmittelsicherheit. The acceptance to the list requires that the agents do not

have damaging effects on the health of humans or animals, or on the environment. Whereas a verification of the effectiveness is not necessary.

The cyanobacteria *Spirulina platensis* is one of the most widely sold microalgae on the health food market and contains highly effective scavenger complexes, minerals and trace elements, polyunsaturated fatty acids, a very high protein content of about 60 to 70% and high-molecular polysaccharides (Pulz *et al.*, 2001). Due to the exopolysaccharides of the microalgae, an effect on the plants pathogen defence system was expected and it is currently tested as potential plant restorative or resistance inducer.

2.5 Root symbionts

The following section focuses on fungi that colonise the root rhizosphere and live symbiotic on and in the roots of their host plants. This coexistence can have dramatic effects on the plant's status, also in respect to defence reactions towards attacking pathogens.

2.5.1 Plant growth promoting fungus *Piriformospora indica*

Piriformospora indica (in the following *Piriformospora*) Verma, Varma, Kost, Rexer & Franken is a newly discovered fungus which was isolated from an arbuscular mycorrhizal fungal spore from a desert soil in Rajasthan, India. The name refers to the pear-shaped chlamydospores that are formed asexually. The endophytic fungus belongs to the *Sebacinaceae* family, colonises the roots of a wide variety of plant species and promotes their growth in a manner similar to arbuscular mycorrhizal fungi (Peskan-Berghofer *et al.*, 2004). *Piriformospora* has a wide and diverse host spectrum that is very similar to that of AM fungi, but includes the non-mycorrhizal host *Arabidopsis thaliana* (Peskan-Berghofer *et al.*, 2004) and some terrestrial orchids (Blechert *et al.*, 1999).

Piriformospora enters the root cortex and forms inter- and intracellular hyphae, often forming dense hyphal coils, branched structures or round bodies within the

cortical cells. Like AM fungi, the hyphae multiply within the host cortical tissue and never traverse through the endodermis, also they do not invade the aerial portion of the plant (Singh *et al.*, 2000). Another attribute of *Piriformospora* is similar to AM fungi: the mobilization of insoluble phosphates and their translocation to the photosymbiont. Studies of Varma *et al.* (2001) have shown fungal-mediated uptake of radio-labelled phosphorus from the medium and its translocation to the host in an energy-dependent process, evident by a sharp increase in its content in the shoot.

Piriformospora shows growth-promoting effects on a broad range of host plants, as do the AM fungi, but has the added trait of being able to be grown in axenic cultures (Varma *et al.*, 1999). This cultivability of *Piriformospora* on economically viable synthetic media makes it suitable for mass scale inoculum production for application in agro-forestry and horticulture (Singh *et al.*, 2000). *Piriformospora* can also be used as biological hardening agent of mircopropagated plants, as it renders more than ninety per cent survival rate for laboratory to field transferred plantlets (Singh *et al.*, 2000). The positive growth responses of plants inoculated by *Piriformospora* have already been proved in field trials, e. g. on the medicinal plants *Spilanthes calva* and *Withania somnifera* by Rai *et al*. (2001).

Peskan-Berghofer *et al.* (2004) detected a promotion of root growth of *Arabidopsis thaliana* plants even before noticeable root colonisation. The growth promotional effects of *Piriformospora* on a compatible photosymbiont do not exclusively demand the physical contact of the mycelium, but could also be realised with the treatment of the host with small quantities of the culture filtrate (Singh *et al.*, 2000). But, the stimulating factor is not yet known. Varma *et al.* (1999) tested the possibilities of *Piriformospora* as biological control agent of soil-borne diseases. The pathogens *Gaeumanomyces graminis* and *Aspergillus sydowii* were placed in the centre of agar plates with four equidistantly surrounding mycel discs of *Pirirformospora*. Both pathogens were significantly suppressed in these agar plates, indicating the potential of *Piriformospora* to act as direct biocontrol agent.

2.5.2 Arbuscular mycorrhizal fungi

Vesicular-arbuscular mycorrhiza is the symbiosis between land plants and arbuscular mycorrhizal fungi (AMF) of the order *Glomales*. About 95 % of present-day plant species belong to families that are characteristically mycorrhizal (Smith & Read, 1997). Both fossil (Remy *et al.*, 1994) and molecular phylogenic (Simon *et al.*, 1993) evidence supports the hypothesis that terrestrial plants evolved with the aid of existing arbuscular mycorrhizal relationships. AMF are probably the most abundant fungi in agricultural soils, accounting for somewhere between 5 and 50% of the biomass of soil microbes (Olsson *et al.*, 1999). Arbuscular mycorrhizae are the most important microbial symbioses for the majority of plants and, under conditions of phosphate-limitation, influence plant community development, nutrient uptake, water relations, and above-ground productivity (Jeffries *et al.*, 2003). VA mycorrhizal fungi have been recognised as ecologically obligate symbionts of a very wide range of plant species. The symbiosis is biotrophic and normally mutualistic, the long-term compatible interaction being based on bidirectional nutrient transfer between plant and fungus (Smith & Read, 1997).

Phosphate (P) is an important plant macronutrient, being a component of key molecules such as nucleic acids, phospholipids, and ATP. Like zinc, P is relatively immobile in the soil. It moves only by diffusion and its concentration in the soil solution is very low. The rapid uptake of both nutrients by plants results in depletion zones around the roots (Nye & Tinker, 1977). AMF are known to enhance uptake of relatively immobile nutrients as phosphate and zinc in their host-plants (Thompson, 1987) and to stimulate the growth of symbiotic plants. The positive effect of AM fungi on phosphate uptake has been attributed to: (i) an exploration of a larger soil volume by the extraradical mycelium; (ii) the small hyphal diameter leading to an increased phosphate absorbing area and, compared to non-mycorrhizal roots, higher phosphate influx rates per surface unit; (iii) the formation of polyphosphates by mycorrhizal fungi and thus lower internal inorganic phosphate concentrations; and (iv) the production of organic acids and phosphatases that catalyse the release of phosphate from organic complexes (Marschner & Dell, 1994).

The fungus provides a link between the soil and the plant and therefore has different parts of its thallus living in two quite different environments (Smith *et al.*, 2001). Extraradical hyphae can have a total surface area of several orders of magnitude greater than that of roots alone, which increases the potential for nutrient uptake, and possibly also water uptake (Rhodes & Gerdemann, 1975; Augé, 2001). In the root cortex, the AM fungi spread by forming intercellular hyphae and form arbuscules or hyphael coils and in some cases also vesicles, which are assumed to be storage organs. Arbuscules and other fungal structures do not penetrate host cell membranes, but invaginate them (Bonfante-Fasolo, 1984). Arbuscules consist of hyphae that branch dichotomously and profusely within root cortical cells (see Figure 2.3). They are assumed to be responsible for nutrient exchange between the host and the symbiont, transporting carbohydrates from the plant to the fungus and mineral nutrients, especially phosphate, and water from the fungus to the plant (Strack *et al.*, 2003). The phosphate obtained by the fungus is translocated through the hyphae and effluxed into the interfacial apoplast between root cortical cell and arbuscule before uptake by plant cells across the plasma membrane (Smith & Read, 1997).

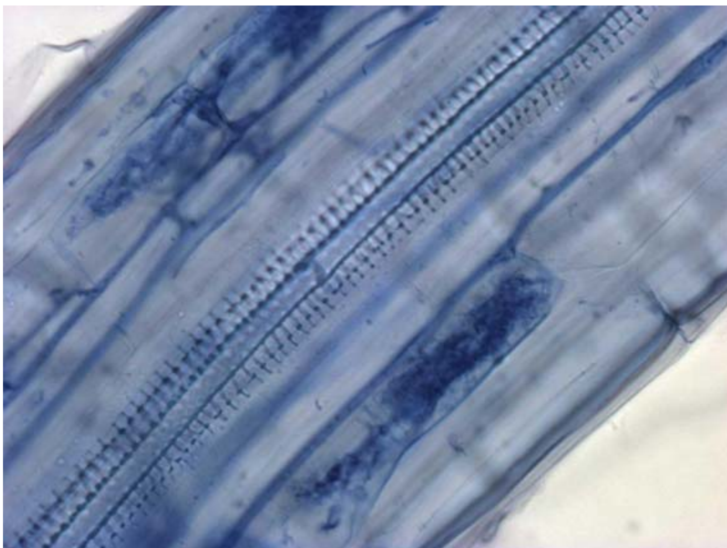


Figure 2.3: Part of a mycorrhizal tomato root. In the middle, from below left to top right, is the central cylinder of the root with the vascular system. The long straight blue lines are intercellular mycel, with which the fungus spreads in the root. The arbuscules are the spots of dense mycel that nearly fill the cells.

The formation of mycorrhizal symbiosis is closely related with the supply of phosphate: the colonisation of the roots decreases with increased phosphate available in the soil is for the host plant, but it may also be reduced at very low

phosphorus availabilities (Abbott *et al.*, 1984; Amijee *et al.*, 1989; Koide & Li, 1990). AMF are obligate biotrophs which completely depend on their hosts for C supply (Jennings, 1995) and require for up to 20 % of the host's assimilated carbon for establishment and maintenance (Jakobsen & Rosendahl, 1990; Douds *et al.*, 2000; Graham, 2000; Johnson *et al.*, 2002). The benefits of the host plants do not always outweigh the carbon costs and under conditions of sufficient and supraoptimal phosphate supply, a mycorrhizal infection may neither have positive effect on the phosphate absorption (Amijee *et al.*, 1993) nor on plant growth.

AMF also act as bioprotectants against pathogens and toxic stresses (Jeffries *et al.*, 2003). It is commonly accepted that mycorrhizal formation reduces infection with various soil-borne pathogens and nematodes (Azcón-Aguilar & Barea, 1996; Smith & Read, 1997). Regarding viral diseases and leaf pathogens on the other hand, plants in symbiosis with AMF are generally assumed to be more susceptible as summarised by Dehne (1982). Gernns *et al.* (2001) observed that mycorrhizal barley plants were more infected by *Blumeria graminis* f. sp. *hordei* but suffered less in terms of reduction of grain number, ear yield and thousand-grain weight.

In sustainable, low-input cropping systems the natural roles of microorganisms in maintaining soil fertility and biocontrol of plant pathogens may be more important than in conventional agriculture where their significance has been marginalised by high inputs of agrochemicals (Johansson *et al.*, 2004). Hyphae of AMF have been shown to play an important role in soil stabilization through formation of soil aggregates (Tisdall & Oades, 1979).

The strong influence of AMF on the plant's supply with P can make it difficult to distinguish between direct effects of the AMF on the plant and more indirect effects due to changed nutritional status. To ensure that effects were based on compensation and not on classical mycorrhizal growth promotion, a plant/AMF system has to be used that shows no difference in growth between mycorrhizal and non-mycorrhizal plants after sufficient fertilization with phosphate (Gernns *et al.*, 2001).

3 MATERIALS AND METHODS

The following chapter describes the materials and methods used in our studies of the pathosystem tomato – *Alternaria solani*: (i) the cultivation of plant material in section 3.1, (ii) all methods concerning the pathogen in 3.2, and (iii) the experimental designs and statistical analysis in 3.3. All used material and the manufacturers are listed in section 3.4.

3.1 Plant material

In this section, the growth and handling of the tomato plants is described. The cultivation of the plants had to be adjusted whether root symbionts were used in the experimental sets. When the plants shall be colonised by fungi, the growing substrate has to be free of microorganisms and shall be easily removable of the plant roots. In 3.1.1, the cultivation of tomato plants for all studies without root symbionts is described. Section 3.1.2 focuses on the plant growth for studies with *Piriformospora indica*. The growth of mycorrhizal plants is described in 3.1.3.

3.1.1 Tomato plants for general studies

In the following, we describe the growth conditions applied to those tomato plants that were not destined for any root symbiosis. These tomatoes supplied the plant

material for the leaf disc experiments and tests with plant restoratives and resistance inducers.

The following three tomato (*Solanum lycopersicum* L., syn. *Lycopersicon esculentum* Mill.) varieties were included in our studies: (i) Rheinlands Ruhm, (ii) Hellfrucht, and (iii) Campbell 28, the latter a Cuban cultivar developed at CENSA , San José de las Lajas, La Habana, Cuba. While cv. Rheinlands Ruhm was used in all experiments, the others were only used in studies in which different reactions of the cultivars were expected.

To sterilise the surface of the seeds, they were rinsed in 95 % ethanol, washed with *aqua dest.*, then soaked in 4 % NaOCl (v : v) for 10 min and washed again. The treated seeds were spread on filter paper for fast drying and stored in an air-tight and dark sterile container.

The seeds were sown in trays with P-soil (150 mg N l^{-1}; 150 mg P_2O_5 l^{-1}; 250 mg K_2O l^{-1}; pH 5.9) and transplanted to small pots or multipot-trays (5 cm diameter per pot) after full germination. After the development of two true leaves, the plantlets were transferred in circular pots of 12 cm diameter with T-soil (300 mg N l^{-1}; 280 mg P_2O_5 l^{-1}; 400 mg K_2O l^{-1}; pH 5.9). If no other age ist stated for the individual experiment, the inoculation with *Alternaria* was performed when the plants were about 5 weeks old and had developed 5 fully unfolded leaves.

The plants were grown at the University of Gießen either in a glasshouse at a 16 : 8 hours light : dark cycle, with 24 - 26 °C : 16 - 18 °C and about 65% relative humidity, or in climate chambers at constantly 20 $\pm$ 1 °C and about 65% relative humidity. For the inoculation with *Alternaria*, the relative humidity was increased to 95% for 24 to 48 hours to ensure germination and survival of the spores.

3.1.1.1 Plant protective agents and plant restoratives

The plant protective agent Maneb, resistance inducer ASM and plant restorative *Spirulina platensis* were applied as spray with a pressure sprayer. All agents were used in a variety of concentrations depending on the hypotheses of the studies.

The used concentrations are always given for the active ingredient for the single experiments in chapter 4. The plants were sprayed until run-off occurred. Both resistance inducers ASM and INA as well as *Spirulina* were also applied as soil drench. Plants were watered with a certain amount of the solution, afterwards some additional water was applied to ensure that the chemicals successfully infiltrate the soil. If more than one agent was tested, they were combined in the appropriate concentrations in one single spraying or watering solution. In all cases, control plants were treated first by replacing the spray or soil drench by tap water.

3.1.1.2 Leaf disc experiments

Leaf disc experiments were conducted (i) if the influence of the leaf age on infection was of interest, (ii) if a high number of replications was needed to detect differences, or (iii) if inoculation success was insecure during wintertime. Tomato plants for these studies were grown as described before until five to six weeks after germination. Leaves of defined leaf levels were picked and circular leaf discs with a diameter of 19 mm were cut. The leaf discs were placed in petri dishes with water agar (0.4% agar agar and an addition of 40 ml of 10% benzimidazol solution (w : v) per litre) and inoculated with droplets of *Alternaria* suspension (10^4 spores ml^{-1}). The spore suspension was thickened with 40 g l^{-1} Sofort Gelatine to make moving of the petri dishes possible without letting the droplets run off the leaf surface. The petri dishes were kept at 18 °C in a 16 : 8 hour day/night regime and the disease severity on the leaf discs was assessed after two to four days.

3.1.2 Plants with root colonising fungus *Piriformospora indica*

This section describes the cultivation of tomato plants used as host plants for the root colonising fungus *Piriformospora* and the culture of the fungus itself.

3.1.2.1 Plant material with *Piriformospora indica*

For experiments with *Piriformospora*, a 1 : 1 mixture (v : v) of Oil Dri and Seramis® was used. Per 400 g pot, 15 g of minced fungal mycel or the equivalent amount of water as control were used and mixed thoroughly in the soil. The pots were either

lined with plastic bags or laid out with filter paper at the bottom to prevent mycel-loss by run-off water. To optimise colonisation of the roots, the sterilised tomato seeds were sown directly into the pots. The plants were grown in a glasshouse under the same conditions as described in 3.1.1.

3.1.2.2 Culture of *Piriformospora indica*

The root colonising fungus *Piriformospora* was kindly provided Helmut Baltruschat, Institute for Applied Phytopathology and Zoology, Justus-Liebig-Universität Gießen, Germany.

When mycel material was required for experiments, *Piriformospora* was cultivated in liquid complete medium (C-medium) in bottles on a shaking machine at room temperature. The fungal growth was relatively slow, the production of sufficient mycel for one experiment took about two month. To harvest the mycel, the medium was filtered, the obtained mycel particles were then washed with sterile *aqua dest.* to remove nutrient remains of the medium, and minced with a blender. The fungus can be kept on petri dishes with C-medium plus agar agar for periods of several months.

Composition of medium used for *Piriformospora* cultivation

C-medium

50 ml salt solution (see below),
20 g glucose,
2 g peptone,
1 g yeast extract,
1 g casamino acids
and 1 ml trace element solution (see below),
filled up to 1 litre with *aqua dest.* and autoclaved. For use in petri dishes add 15 g l^{-1} agar agar.

Annotation: It is not necessary to add the vitamin solution as proposed by Pontecorvo *et al.* (1953).

Salt solution

120 g $NaNO_3$
10.4 g KCl
10.4 g $MgSO_4 \cdot 7H_2O$
and 30.4 g KH_2PO_4
dissolved in 1 litre *aqua dest.*

Trace element solution

For 100 ml solution:
2.2 g $ZnSO_4 \cdot 7H_2O$
1.1 g H_3BO_4
0.5 g $MnCl_2 \cdot 4H_2O$
0,5 g $FeSO_4 \cdot 7H_2O$
0.18 g $CoCl_2 \cdot 6H_2O$
0.18 g $CuSO_4 \cdot 5H_2O$
0.11 g $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$
and 5 g Na_2EDTA
are dissolved one ingredient after the other in about 80 ml *aqua dest*. Boil up the solution, then let to cool down to about 60 °C and set pH to 6,3 - 6,8 with KOH cookies or at least 10M KOH solution. After cooling down to room temperature solution is filled up to 100 ml and stored in dark bottle.

3.1.3 Plants with arbuscular mycorrhizal fungus *G. intraradices*

This section describes (i) the growth of mycorrhizal tomato plants, (ii) the harvest of the colonised roots, and (iii) the estimation of the degree of their colonisation. Further, the propagation of *Glomus intraradices* and the Cuban mycorrhizal inoculum EcoMic® are presented.

3.1.3.1 Plant material with *Glomus intraradices*

At the Risø National Laboratory, Denmark, a 1 : 1 (v : v) mixture of sand and irradiated soil (10 kGy, 10 MeV electron beam) was used for AMF-experiments. This growth medium, hereafter referred to as soil, had a bicarbonate-extractable P content of 9.8 $\mu g\ g^{-1}$ (Olsen *et al.*, 1954) and was enriched by adding nutrient solutions (Viereck *et al.*, 2004) as described in Table 3.1. The KH_2PO_4 solution was used to control P levels of 0, 3, 6, 9, 12, and 15 mg P kg^{-1} soil, or 0, 15 and 45 mg P kg^{-1} soil in AMF experiment 1 and 2, respectively. These concentrations do not include the above mentioned bicarbonate-extractable P. After the addition of the nutrient solutions, the soil was left to dry for three days and was then mixed thoroughly. Pots with 8 cm side length were lined with plastic bags and filled with 400 g soil mixture as control. Mycorrhizal plants were grown in a mixture of 368 g soil and 32 g *Glomus intraradices* inoculum (BEG 87 (28a); kindly provided by the Plant Nutrition Group of Risø).

Table 3.1: Nutrient solutions for mycorrhizal experiments.

Solution	Compound	Concentration in solution [mg ml^{-1}]	mg kg^{-1} soil	Solution addition [ml kg^{-1} soil]
I	K_2SO_4	25	75	3
II	$CaCl_2 \cdot 2\ H_2O$	25	75	3
III	$CuSO_4 \cdot 5\ H_2O$	0.7	2.1	3
	$ZnSO_4 \cdot 7\ H_2O$	1.8	5.4	
	$MnSO_4 \cdot H_2O$	3.5	10.5	
	$CoSO_4 \cdot 7\ H_2O$	0.13	0.39	
	$MgSO_4 \cdot 7\ H_2O$	15.0	45.0	
	$Na_2MoO_4 \cdot 2\ H_2O$	0.06	0.18	
IV	NH_4NO_3	28.57 (10 mg N ml^{-1})	30.0	3
V	KH_2PO_4	43.93 (10 mg P ml^{-1})	variable	variable

The third column shows amounts of compounds to prepare these solutions, the fourth column gives the desired concentrations of nutrients in the resulting soil and the fifth column the amount of solution which has to be added to the soil mixture to achieve these concentrations.

The surface sterilised tomato seeds were pregerminated on wet filter paper. The seedlings were transferred to pots after three days and reduced to one plant per pot after full germination. The soil surface was covered with plastic beads to prevent algae growth and evaporation. Plants were grown in climate chambers in a 16 : 8 hours light : dark cycle at 24 - 26 °C : 18 - 20 °C and irrigated daily to 60% field capacity.

3.1.3.2 Root harvest and estimation of mycorrhizal colonisation

At harvest, plant roots of mycorrhizal tomatoes were washed to remove the soil particles. The root material was cut into pieces of about 1 cm length, mixed thoroughly and a sample of about 1 to 2 g was taken from each plant. Samples were then cleared with 10% KOH and stained with 0,05% trypan blue in lactoglycerol (Phillips & Hayman, 1970, modified) with the omission of phenol from the solutions and HCl from the rinse. Percentage of mycorrhizal colonisation was determined using the following gridline intersection method as described by Giovanetti and Mosse (1980): Stained root pieces were spread on a petri dish with gridlines at 1 cm intervals. Using a binocular, all roots crossing these lines were assessed being whether mycorrhizal or not, recording the total number of roots

and the number of mycorrhizal roots separately, in order to determine the proportion of colonised roots.

3.1.3.3 Propagation of *Glomus intraradices*

Glomus intraradices was propagated in living plant cultures. For each culture, a pot (for 3 kg soil) was lined with a plastic bag, filled with 1500 g soil, then 950 g soil plus 50 g of the original inoculum and topped with 500 g of soil. A control pot was filled with the same amount of soil without inoculum. Pots were watered to 60 % field capacity and left for 1 week to stimulate AMF spore germination. Then 10 pregerminated seeds of host plants (*Medicago sativa* L. or *Trifolium resupinatum* L.) were sown and plants were reduced to five per pot after full germination. Pots were watered every second day to weight, increasing the weight slowly as plants were growing. After one month, a soil sample was taken, embodied roots were stained and checked for mycorrhizal colonisation. After approximately three months, the watering was stopped and the soil was left to dry for about two weeks. Then sprouts and thick roots of the host plants were removed, remaining roots were cut into fine pieces and mixed thoroughly with the soil. A quality control with the inoculum was performed by inoculating tomatoes and assessing the degree of colonisation after three weeks. The so produced inoculum can be stored in tightly closed dark containers either at room temperature or at 4 °C.

3.1.3.4 AMF inoculum EcoMic®

The biofertiliser EcoMic® is produced in Cuba by the National Institute for Agricultural Sciences (Instituto Nacional de Ciencias Agrícolas, San José de las Lajas, La Habana, Cuba). It is based on several AM fungi of the genus *Glomus*, more detailed information about the contents are not available as the production process and contents are subjects of business secrecy. For the field trial in Cuba, one pellet of EcoMic® was used per pot in the multipot trays.

3.2 Pathogen *Alternaria solani*

In this section, the culture of pathogen *Alternaria solani*, the inoculation of the host plant tomato and the assessment of the resulting disease severity for whole plants as well as leaf discs are described.

3.2.1 Culture of *Alternaria solani*

Alternaria isolates were cultured at 25 °C on plates and for long-term storage in test tubes. Potato dextrose agar (PDA) was produced from fresh potatoes and used as general culture medium. To ensure sporulation in spite of cultivation *in vitro* and without stimulating UV-C light, the isolates were cultivated every fourth cycle on dextrose agar (DA) which is a reduced PDA without potato cooking liquid. The sporulation enhancing S-medium after Shahin & Shephard (1979) was also used to induce sporulation.

List of media used for culture of *Alternaria solani*

PDA 200 g peeled potato pieces were boiled in a pot with lid with about 800 ml *aqua dest*. for 1 hour. The remaining cooking liquid is filtered through a sieve, mixed with 15 g agar agar and 20 g dextrose, filled up to 1 litre and autoclaved (Rotem, 1994).

DA 15 g agar agar and 20 g dextrose are dissolved in 1 litre *aqua dest.* and autoclaved. *Alternaria* isolates sporulated profusely on PDA media if they were grown on nutrient-poor DA media before.

S-medium 20 g sucrose, 30 g $CaCO_3$ and 20 g agar agar are dissolved in 1 litre of *aqua dest.* and autoclaved. This media can enhance profuse sporulation if cubes of PDA medium with *Alternaria* mycel are spread on its surface together with some sterile water (after Shahin & Shepard, 1979).

3.2.2 Inoculation of host plants

Tomato plants were inoculated with a mixture of spores from the isolates Greece-1, USA-1 and Cuba-141. To harvest the spores, 10-day-old cultures were brushed gently to loosen the spores from the mycel surface and then rinsed with a 0.01 % Tween 20 solution. The resulting spore suspension was filtered through a

fine nylon mesh to remove bigger mycel parts, quantified using a haemocytometer and generally adjusted to 10^4 spores ml^{-1} (differing concentrations are given with the single experiments in chapter 4).

Plants were inoculated by spraying the spore suspension until run-off. To ensure good spore germination, the plants were placed in plastic containers for 24 - 48 hours to increase the relative humidity. Studies in Germany were performed in fully controlled climate chambers where the relative humidity was increased to 95% for at least 24 hours.

3.2.3 Assessment of disease severity

To define disease severity, percentage of leaf area with necrotic spots and proportion of chlorosis were assessed separately for all unfolded leaves (assessment grades from 1 to 12, see Table 3.2) and arithmetic means for single plants were calculated. Dead, shedded leaves were rated with assessment grade 12 and were collected separately for every plant to determine the loss of biomass due to early blight infection.

Table 3.2: Assessment scheme for necrotic or chlorotic leaf areas of single leafs after Horsfall and Barrett (1945) which is based on a semi-logarithm with 50 % infection as midpoint.

% leaf area	0	< 3	< 6	< 12	< 25	< 50	< 75	< 87	< 94	< 97	< 100	100
grade	1	2	3	4	5	6	7	8	9	10	11	12

Tomato leaf discs were assessed with a reduced assessment scheme with grades from 0 to 5 as shown in Figure 3.1. All assessment grades of leaf discs in one petri dish were combined in an arithmetic mean, representing one leaf of a certain leaf level of one plant.

At harvest, the shoots were cut from the plants directly above the soil surface and weighed to measure the fresh matter. As *Alternaria* defoliates the host plants and causes chlorosis at infected leaves resulting in reduced turgor, the fresh matter can be used as an alternative measure for disease severity. The dry weight was determined after a drying period of at least 72 hours at 70 °C. Dead, shedded

leaves were collected separately for each plant to determine the percental loss of biomass due to fungal infection.

Figure 3.1: Assessment scheme for leaf disc experiments. Shown are leaf discs with the grades **0** to **4** from left to right: **0** no infection; **1** only single and small necrotic spots, less than half of the droplet area is necrotic; 2 more than half of the droplet area is necrotic; **3** the droplet area is fully necrotic; **4** the disease has spread over the whole leaf disc. Grade **5** (not shown) was given for discs with a disease severity similar to grade 4, but with fully chlorotic leaf material.

3.3 Experimental designs and statistical analysis

The following section focuses on the experimental designs of the performed studies and the statistical analysis of the collected data material. The choice of appropriate statistical tests for the different data is described.

3.3.1 Experimental designs

All experiments were conducted in completely randomised designs as the conditions in the green house and climate chambers were even. All experiments were repeated once, but both replications are only demonstrated if the results were not consistent. Most studies investigated one or two treatment factors. All experiments were made with the number of replications indicated at the figures and tables in the results chapter.

3.3.2 Statistical analysis

Most data, like fresh and dry weight data as well as mycorrhizal colonisation data, were evaluated using GLM and Tukey’s Test with α = 0.05. Disease assessment data were also analysed using GLM and Tukey's Test as arithmetic means were calculated for each plant or each leaf. Parallel, an analysis using H-Test followed by Nemenyi's Test was performed for these data. If these analyses resulted in diverging significant differences, both results are given in the results chapter.

Necrosis and chlorosis data of mycorrhiza experiments, which were surveyed over several days, were analysed with a linear mixed model analysis for repeated measurements. Thereby, the autoregressive covariance structure was used as it fitted best with respect to the experimental structure and minimized values for Akaike’s Information Criterion (AIC; Akaike, 1973 after Burnham & Anderson, 1998) and other information criteria. This statistical approach compares the treatments during the whole period of data collection and not only at certain time points by estimation of new means which are representing the whole time course and are therefore slightly different to the measured values. Degrees of freedom were estimated according to Satterthwaite’s formula (Satterthwaite, 1946 after Hocking, 1996). Levels of P addition and inoculation with *G. intraradices* were set as fixed factors, whereas the intervals between the assessments were used as covariates. Treatments were compared using LSD Test with Bonferroni correction. All calculations were performed using SPSS (SPSS for Windows, Rel. 12.0.1, 2003. Chicago, SPSS Inc.).

3.4 Chemicals and materials

In Table 3.3 on the following page, we list all chemicals applied , and provide their chemical formula and manufacturers.

Table 3.3 : Applied chemicals and their manufacturers

Chemicals	Chemical formula	Manufacturer
α-D-glucose		Carl Roth GmbH & Co., Karlsruhe, Germany
agar agar		
ammonium molybdate 4-hydrate	$(NH_4)_6Mo_7O_{24} \cdot 4\ H_2O$	
boric acid	H_3BO_4	
calcium carbonate	$CaCO_3$	
casamino acids		
cobalt chloride 6-hydrate	$CoCl_2 \cdot 6\ H_2O$	
copper II sulphate 5-hydrate	$CuSO_4 \cdot 5\ H_2O$	
iron II sulphate 7-hydrate	$FeSO_4 \cdot 7\ H_2O$	
glycerin		
lactic acid		
manganese II chloride 4-hydrate	$MnCl_2 \cdot 4\ H_2O$	
magnesium sulphate 7-hydrate	$MgSO_4 \cdot 7\ H_2O$	
potassium chloride	KCl	
potassium hydroxide	KOH	
sodium hypochloride	NaOCl	
sodium nitrate	$NaNO_3$	
peptone		
Tween 20		
zinc sulphate 5-hydrate	$ZnSO_4 \cdot 5\ H_2O$	
sodium ethylenediamine-tetraacetic acid	Na_2EDTA	Fluka Chemie GmbH, Buchs, Switzerland
yeast extract		Gibco Ltd., Paisley, Scotland
benzimidazole		Merck KGaA, Darmstadt, Germany
potassium dihydrogen phosphate	$KH_2PO_4 \cdot 4H_2O$	
trypan blue		Omikron GmbH, Neckarwestheim, Germany
ammonium nitrate	NH_4NO_3	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
calcium chloride	$CaCl_2 \cdot 2\ H_2O$	
cobalt sulphate 7-hydrate	$CoSO_4 \cdot 7\ H_2O$	
2,6-dichloroisonicotinic acid	INA	
manganese sulphate hydrate	$MnSO_4 \cdot H_2O$	
N,N-dimethylformamid	DMF	
potassium sulphate	K_2SO_4	
sodium molybdate 2-hydrate	$Na_2MoO_4 \cdot 2\ H_2O$	
sucrose		

The following Table 3.4 contains the trade names and manufacturers of the materials used in our experiments.

Table 3.4: Applied materials and their manufacturers

Materials	**Manufacturer**
Maneb®	Celaflor GmbH, Ingelheim am Rhein, Germany
Lycopersicon esculentum seeds cv. Campbell 28	Centro Nacional de Sanidad Agropecuaria, San José de las Lajas, La Habana, Cuba
Frustorfer P-Erde Frustorfer T-Erde	Hawita Gruppe Vechta, Germany
Oil Dri (Typ III R)	Importer: Damolin Mettmann, Germany
EcoMic® Patent No. 22641	Instituto Nacional de Ciencias Agrícolas San José de las Lajas, La Habana, Cuba
Lycopersicon esculentum seeds cv. Frembgens Rheinlands Ruhm cv. Hellfrucht	Juliwa-Enza & Co. KG Heidelberg, Germany
Seramis®	Masterfoods GmbH Verden, Germany
Sofort Gelatine, gemahlen	RUF Lebensmittelwerk Quakenbrück, Germany
host plant seeds for AMF inoculum: *Medicago sativa* L. and *Trifolium resupinatum* L.	Samenhaus Hesemann GmbH Gießen, Germany

4 RESULTS

In this chapter, the results of the studies of the pathosystem tomato – *Alternaria solani* are presented with special emphasis on induced resistance. The first section focuses on general studies about the pathogen itself and its interaction with the host plant. In the following sections, the effects of different agents for plant protection and resistance induction are demonstrated. The last section attends to the combination of tomatoes with symbiotic root colonising fungi and the resulting influences on disease severity.

4.1 General studies of *Alternaria solani*

This section describes the results of our general studies of *Alternaria* and its host plant tomato. Prior to the experiments, we had to develop a technique that induced sporulation in the isolates cultivated on artificial medium. The resulting new method is described in 4.1.1. The main objectives of the two following general studies were (i) to test whether the tomato leaves differing in age also differ significantly in suscptibility to early blight, which might influence the experimental results; and (ii) whether the toxins contained in the spore suspension could cause disease symptoms by themselves.

4.1.1 Induction of sporulation

A variety of *Alternaria* isolates were available but they had lost their ability to produce spores during the cultivation *in vitro* on axenic media. When high amounts of spores are required for inoculation, sporulation inducing methods are used to ensure sufficient spore production. The cultivation of *Alternaria* on Sporulation-medium (S-medium) after Shahin & Shepard (1979) rendered in profuse sporulation of several isolates. But, this method is very time-intensive, as single pieces of fungal mycel on PDA have to be spread on a layer of the S-medium and covered with sterile water. The harvest of spores also takes more time than from a planar mycel disc, so this method was rarely practiced.

Stress factors like nutrient deficiency can also induce sporulation, e.g. when the host plant is dying. We tested the cultivation on dextrose agar (DA), a modified PDA with the omission of potato cooking liquid. The mycel growth on DA plates was very slow and resulted in a fine mesh of mycel, but in no spores. The following transfer back on PDA yielded in sufficient and long-lasting spore production. With this two-step-method, isolates Greece-1, USA-1 and Cuba-141 (kindly provided by Simon Pérez Martinez, CENSA, Cuba) could be stimulated to sporulate continuously. These three isolates were used in as mixture for inoculation in all following experiments.

4.1.2 Leaf age dependent susceptibility

Tomato leaves of various leaf levels differ in their susceptibility to *Alternaria.* A leaf disc experiment was conducted to investigate if these differences are relevant and subsequently to decide on the most appropriate leaf age for the following studies. The leaves 2 to 7 of ten plants were picked and from each leaf, 12 leaf discs were cut. For the youngest leaves of levels 6 and 7, the number of leaf discs had to be reduced as these leaves were relatively small. These discs were infested with droplets of *Alternaria* spore suspension and assessed after 48 hours.

An overall leaf level effect could be shown by ANOVA ($P < 0.001$), with a maximal disease severity for level 2, i.e. for the oldest leaves. The disease severity decreased until leaf level 5, with minimal degree of infection. For levels 6 and 7,

the disease severity increased again. Leaves of level 7 (youngest) and 3 were significantly more susceptible than the leaves of level 5.

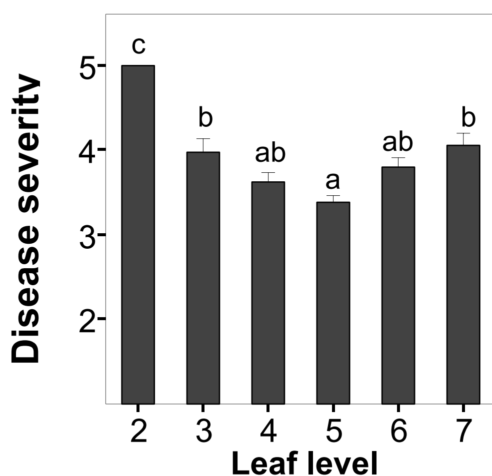


Figure 4.1: Disease severity of early blight disease susceptibility as a function of leaf age. Bars show arithmetic means and standard errors with *n* = 10, assessment scale from 0 to 5. Data with equal letters do not differ significantly.

4.1.3 *Alternaria* toxins

Alternaria produces several host-specific and non-specific toxins, some of which assist the fungus during the infection. Disease severity of normal spore solution and the toxins alone were compared in an experiment with the following three treatments: (i) control (tap water plus an addition of 0.01% of Tween 20); (ii) toxins (filtered suspension without spores); and (iii) spore suspension. Analysis of the severity data by ANOVA followed by Tukey's Test and H-Test followed by Nemenyi gave differing results, as listed in Table 4.1.

Table 4.1: *Alternaria* toxins and spore related disease severity.

Treatments	Disease severity
Control	0.03 ± 0.05 a A
Toxins	1.3 ± 0.13 b AB
Spores	4.2 ± 0.13 c B

Given are arithmetic means and standard errors with *n* = 4, assessment scale from 0 to 5. Data with equal letters do not differ significantly; lower case letters for Tukey after ANOVA, capital letters Nemenyi after H-test.

Alternaria toxins alone resulted in a slightly increased degree of infection, which was significant by Tukey's method but not by Nemenyi. But this effect was small compared to the damage caused by inoculation with spores, which resulted in a significant increase even by means of the conservative Nemenyi-method. These results show that we could retain to our method of inoculating the plants with spore suspension.

4.1.4 Summary

- Long-lasting ability for profuse sporulation could be induced in some *Alternaria solani* isolates by a growth period on nutrient-poor dextrose agar (DA). This method could be easily included in the cultivation cycles of the fungus and was an improvement compared to the previously used labour-intensive method of Shahin & Shepard (1979).
- The leaf levels of single plants do differ significantly in their susceptibility to early blight infection. The oldest leaves showed the highest susceptibility. The leaves of the levels 4, 5 and 6 were least susceptible. The susceptibility increased again in the youngest leaves.
- *Alternaria solani* toxins in the absence of spores, as contained in filtered spore suspension, can cause light disease symptoms. However, this effect is small compared to the inoculation with spores, so there was no need to adapt another method of inoculation.

4.2 *Spirulina platensis* as plant restorative

In this section we describe the effects of cyanobacteria *Spirulina platensis* as a plant restorative. Plant restoratives can help to increase plant health without any direct activity on the pathogen. First, the different methods of application and their influence on the activity are compared. Then, the results of a combination with resistance inducer ASM are described.

4.2.1 Comparison of soil drench and spraying

In the first approach, it was intended to test whether spraying or watering with *Spirulina* suspension has any influence on *Alternaria* disease development on leaf discs. For this purpose, 8 plants served as control and were treated only with *aqua dest.*, a second set of 8 plants were watered with *aqua dest.* and sprayed with *Spirulina* suspension, finally a third set of plants was watered with *Spirulina* suspension and sprayed with *aqua dest.*. Leaves of level 4 were picked five days after the treatment as modifications in the plants, e. g. a build-up of induced resistance, could require some time. Per plant, 8 to 10 leaf discs were inoculated with droplets of *Alternaria* spore suspension in the two concentrations 10^2 and 10^4 spores $ml^{-1}$and disease severity was assessed about 48 hours afterwards.

Table 4.2: Sprayed and watered *Spirulina platensis* related disease severity*.*

Treatments	10^2 spores ml^{-1}	10^4 spores ml^{-1}
Control	1.08 ± 0.06 a AB	2.58 ± 0.09 a
*S*prayed	1.39 ± 0.15 b B	2.57 ± 0.15 a
Watered	0.89 ± 0.03 a A	2.58 ± 0.08 a

Given are arithmetic means and standard errors with *n* = 8, assessment scale from 0 to 5. Data with equal letters in columns are not significantly different; lower case letters for Tukey after ANOVA, capital letters for Nemenyi after H-test.

For the lower *Alternaria* spore concentration, no significant different disease severity could be detected between control and *Spirulina* watered plants (Table 4.2), but sprayed plants showed significantly higher assessment grades than the two other treatments (according to Tukey). Spraying with *Spirulina* seemed to increase early blight infection. In case of the higher spore concentration, infection pressure was clearly higher and no differences between treatments could be seen. *Spirulina* showed no activity to increase the plants health. The results indicate that *Spirulina platensis* seems not to be suitable as plant restorative for the pathosystem tomato - *Alternaria*.

4.2.2 Combination of *Spirulina platensis* and ASM

While *Spirulina* alone turned out to be non-restorative, the following two experiments tested the activity of combinations of Spirulina and ASM against early

blight. Each agent was applied in 3 concentrations (*Spirulina*: 0, 2.5; 10 g l^{-1}; ASM 0, 424, 848 mg l^{-1}), all together 9 combinations. 11 tomato plants per treatment were sprayed, inoculated after one week, and 5 days later when the *Alternaria* infection was fully developed, plant sprouts were harvested. Since the disease assessment for the whole plants seemed too inaccurate, fresh and dry matter of the sprouts were measured instead. Especially data of fresh matter can reveal spreading *Alternaria* infection as leaves typically become chlorotic, start to wilt and are discarded already at early stages of the disease, thus reducing the biomass of the plant notably.

Table 4.3: Two-way ANOVA of fresh weight data of tomato plants for combined treatment with *Spirulina* and ASM in experiment 1.

Source	***df***	***SQ***	***MQ***	***F***	***P***
ASM	2	1957.776	978.888	18.697	0.000
Spirulina	2	126.775	63.388	1.211	0.303
Interaction	4	802.264	200.566	3.831	0.006
Error	90	4715.029	52.356		
Total	98	7598.844			

Using a one-way ANOVA for the analysis, the treatments with combinations of *Spirulina* and ASM differed significantly ($P < 0.001$) in their activity against early blight. A two-way ANOVA (Table 4.3) showed that only ASM had a significant influence ($P < 0.001$), whereas *Spirulina* treatments did not have any effect on the plants weight ($P = 0.303$). However, the interactions between both factors were significant with $P = 0.006$. The interactions are easily to detect as the mixtures of the two agents differed clearly in their effectiveness depending on the ASM concentration. Multiple comarisions did also not show any activity of *Spirulina*.

Plants treated only with *Spirulina* showed significantly lowest fresh matter values irrespective of the concentration (Figure 4.2). Fresh matter of the control plants (treatment A) and plants treated with ASM alone (B and C) were higher but not significantly higher than in *Spirulina* treatments. The combination of ASM in low dosage plus *Spirulina* in whatever concentration (F and G) resulted in highest fresh matter production. All other treatments were indifferent.

Concerning the dry weight, the results were slightly different since the number of indifferent treatments increased. Plants treated with the mixtures of ASM in low dosage with *Spirulina* had highest dry weights (F and G). However, we can conclude that none of the treatments could increase the fresh or dry weight of the plants significantly compared to the control. This means that no treatment in this study proved effective against early blight.

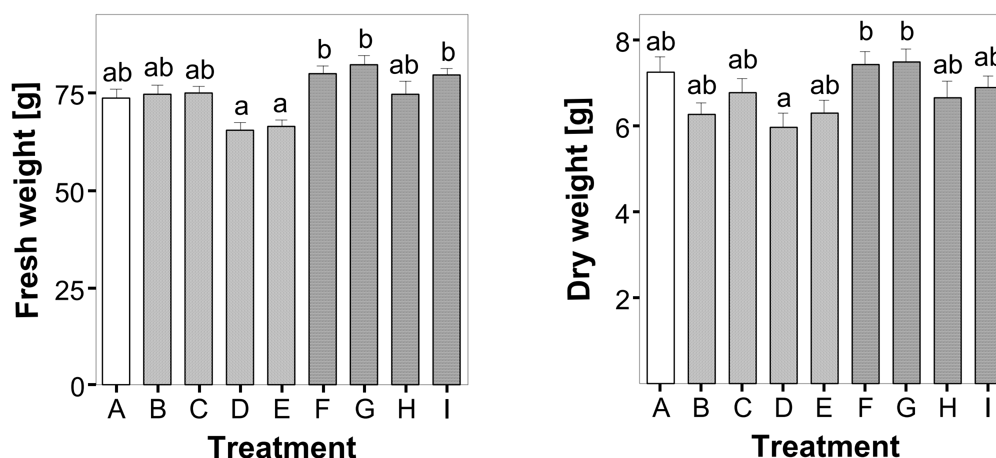


Figure 4.2: Fresh and dry weight data of combined treatment of tomato plants with *Spirulina* and ASM in experiment 1. Plants were sprayed with following solutions: **A** control, **B** 424 mg l^{-1} ASM, **C** 848 mg l^{-1} ASM, **D** 2.5 g l^{-1} *Spirulina*, **E** 10 g l^{-1} *Spirulina*, **F** 424 mg l^{-1} ASM + 2.5 g l^{-1} *Spirulina*, **G** 424 mg l^{-1} ASM + 10 g l^{-1} *Spirulina*, **H** 848 mg l^{-1} ASM + 2.5 g l^{-1} *Spirulina*, and **I** 848 mg l^{-1} ASM + 10 g l^{-1} *Spirulina*. Given are arithmetic means and standard errors with $n = 11$. Data with equal letters are not significantly different.

The experiment was repeated, but only for fresh matter. Experiment 2 gave some slightly differing results (Figure 4.3) and a two-way ANOVA analysis revealed again a significant influence of the factor ASM ($P = 0.006$) but none for the factor *Spirulina* ($P = 0.659$, compare Table 4.4). The interactions between both factors were again significant with $P = 0.006$.

Table 4.4: Two-way ANOVA of fresh weight data of tomato plants for combined treatment with *Spirulina* and ASM in experiment 2.

Source	***df***	***SQ***	***MQ***	***F***	***P***
ASM	2	701.221	350.611	5.390	0.006
Spirulina	2	54.408	27.204	0.418	0.659
Interaction	4	1012.317	253.079	3.891	0.006
Error	90	5854.111	65.046		
Total	98	7622.058			

The application of the higher concentration of ASM (C, Figure 4.3) led to significantly higher fresh weight compared to the control. Also, the combination of ASM in low concentration and *Spirulina* were significantly less infected with early blight than the control. These results differ from experiment 1, where none of the treatments showed any improvement of plant health. On the other hand, the *Spirulina* treatments had not such a negative impact on the plant weight as in experiment 1.

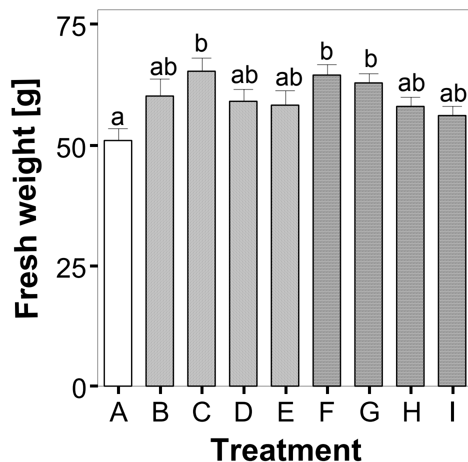


Figure 4.3: Fresh weight data of combined treatment of tomato plants with *Spirulina* and ASM in experiment 2. Plants were sprayed with following solutions: **A** control, **B** 424 mg l^{-1} ASM, **C** 848 mg l^{-1} ASM, **D** 2.5 g l^{-1} *Spirulina*, **E** 10 g l^{-1} *Spirulina*, **F** 424 mg l^{-1} ASM + 2.5 g l^{-1} *Spirulina*, **G** 424 mg l^{-1} ASM + 10 g l^{-1} *Spirulina*, **H** 848 mg l^{-1} ASM + 2.5 g l^{-1} *Spirulina*, and **I** 848 mg l^{-1} ASM + 10 g l^{-1} *Spirulina*. Given are arithmetic means and standard errors with n = 11. Data with equal letters are not significantly different.

4.2.3 Summary

- Spraying of *Spirulina* solution did rather increase the susceptibility of tomato plants to *Alternaria solani* instead of protecting the plants.
- When only low infection pressure occurs, watering with *Spirulina* solution can improve the plant health slightly. However, this effect was not significantly different to the control according to the conservative Nemenyi method.
- In the studies comparing treatments with ASM and *Spirulina* both alone and in mixtures, only the application of ASM had a significant influence on the plants fresh and dry weight. Since the interactions between both factors were also significant, *Spirulina* seemed to have some activity in the agent combinations.

- Spraying the plants with a combination of ASM in low concentration plus *Spirulina* resulted in higher plant fresh weights after early blight infection than application of *Spirulina* alone.
- Results for the treatments with ASM in higher concentration were slightly inconsistent in the two experiments.
- In experiment 1, no significant difference could be detected if the treatments with higher plant weight were compared to the control. None of the treatments could effectively control or at least reduce the infection with early blight.
- In experiment 2 however, the application of ASM alone in high concentration and of mixtures of ASM in low concentration plus *Spirulina* resulted in significantly higher plant fresh weights relative to the control.
- *Spirulina* had no significant influence in both experiments, but showed significant interactions with the ASM treatment. The application of *Spirulina* alone resulted in the lowest plant weights in experiment 1.
- All these findings together hint that spraying with *Spirulina* increases *Alternaria* infection on tomato.

4.3 Chemical plant protective agent Maneb®

If a resistance inducer is used, it can be necessary to mix the spraying solution with a curative chemical fungicide to eliminate already existing pathogens during the resistance build-up. As there is actually no curative agent against early blight available, the protective fungicide Maneb could be used in a mixture with a resistance inducer. By the addition of Maneb, the plants would be protected from the time of spraying and not as late as the resistance induction is completed.

This section focuses on the activity of Maneb if applied alone. Further experiments with Maneb in combination with ASM are described in later sections, see 4.4.3 and 4.4.5.

4.3.1 Maneb in recommended concentration

In a preliminary experiment, the fungicide Maneb was tested alone in the concentration recommended for tomato crops (2.4 g l^{-1}). Since it had been shown before (see 4.1.2) that leaves of varying levels differ in their susceptibility to *Alternaria*, it was of interest whether the activity of Maneb is also influenced by the leaf age. Plants were either sprayed with Maneb solution or with tap water for the control. After one week, leaves of different levels were picked and leaf discs were infected by droplets of *Alternaria* suspension.

Table 4.5: Maneb related disease severity.

Leaf level	Control	Maneb [2.4 g l^{-1}]
3	3.3 ± 0.06 b	1.1 ± 0.07 a
5	3.0 ± 0.07 a	1.8 ± 0.10 b
6	2.9 ± 0.06 a	2.0 ± 0.10 b

Given are arithmetic means and standard errors with n = 8, assessment scale from 0 to 5. Data with equal letters in columns are not significantly different. Data in lines are all significantly different.

The disease severity differed significantly between treatments as well as between leaf levels, and the interactions between the two factors were also significant (all $P \leq 0.005$, ANOVA not shown). On water control plants (Table 4.5), older leaves were significantly higher infected than younger leaves. This effect is consistent with results presented in section 4.1.2. Maneb treated plants were significantly less infected than plants of the control group. But, in Maneb treated plants, the younger leaves (levels 5 and 6) showed significantly more disease symptoms than the older leaves (level 3).

4.3.2 Summary

- The activity of Maneb against early blight on tomatoes was significant compared to the water control.
- However, this protection interacted significantly with the age of the examined leaves. The grade of protection was significantly lower for the younger leaves, which had not been fully grown or unfolded at the time of application.

- Maneb is only active on the treated parts of the plant and has no systemic effect, therefore it cannot protect growing plant tissue.
- It could be demonstrated again that the older tomato leaves are significantly more susceptible to *Alternaria* than the younger ones.

4.4 Resistance inducer ASM

Acibenzolar-S-methyl, or short ASM, is the active agent of the commercially used resistance inducer BION®. This resistance inducer can be applied either as spray or as soil drench and should not have any direct inhibitory effect on pathogens. This section focuses first on the activity of ASM depending on the way of application, and later on its activity in combination with the fungicide Maneb. Finally, the direct activity of ASM against *Alternaria* is demonstrated.

4.4.1 ASM as soil drench or as spray

To test ASM activity, two leaf disc experiments were performed separately, one with sprayed and one with soil drench application of ASM.

The first experiment was performed by soil drenching ASM to 10 plants per treatment. ASM solutions in 5 concentrations (0, 5, 25, 50, and 100 mg l^{-1}) were used and leaf discs were prepared after one week after application, using leaves of levels 4 and 7.

Table 4.6: Two-way ANOVA for disease severity after ASM soil drench.

Source	***df***	***SQ***	***MQ***	***F***	***P***
Concentration	4	3.700	0.925	7.001	0.000
Leaf level	1	39.005	39.005	295.244	0.000
Interaction	4	7.387	1.847	13.980	0.000
Error	90	11.890	0.132		
Total	99	61.982			

Table 4.7: Disease severity after ASM soil drench.

Concentration	Leaf level 4	Leaf level 7
Control	3.67 ± 0.20 a	3.47 ± 0.08 a
5 ASM	4.61 ± 0.17 b	3.28 ± 0.08 a
25 ASM	4.81 ± 0.11 b	3.43 ± 0.11 a
50 ASM	4.92 ± 0.04 b	3.20 ± 0.10 a
100 ASM	4.76 ± 0.11 b	3.16 ± 0.05 a

All concentrations are given in mg l^{-1} of the active ingredient. Given are arithmetic means and standard errors with $n = 10$, assessment scale from 0 to 5. Data with equal letters in columns are not significantly different.

A 2-way ANOVA of the data showed significant differences for both the factor leaf level and the ASM concentrations, and also significant interactions (all $P < 0.001$, compare Table 4.6). For level 4, differences in disease severity between control and ASM-treatment were significant (Table 4.7). But, *Alternaria* infection was clearly higher for the ASM-treated plants, independent of the concentration used. The application of ASM as soil drench increased the tomato susceptibility to early blight. Ratings of level 7 showed no significant differences between treatments. The disease severity was generally lower relative to the leaves of level 4.

The second experiment with sprayed ASM was performed with level 4 leaves alone, since the preceding experiment indicated no effects at level 7. The number of tested concentrations were also reduced to 0, 5, 50 and 100 mg l^{-1} ASM.

Table 4.8: Disease severity after sprayed ASM.

Concentration	Leaf 4
Control	3.2 ± 0.08 a
5 ASM	4.6 ± 0.14 b
50 ASM	4.5 ± 0.47 b
100 ASM	4.6 ± 0.12 b

All concentrations are given in mg l^{-1} of the active ingredient. Given are arithmetic means and standard errors with $n = 10$, assessment scale from 0 to 5. Data with equal letters in columns are not significantly different.

The disease severity differed significantly depending on the ASM concentration. Leaf discs of control plants had the lowest ratings (Table 4.8) again. All ASM treatments showed higher disease severity that did not differ depending on the

concentration used. These results show that ASM seemed to enhance early blight on tomato plants, independent of the mode of application.

4.4.2 Comparing ASM spray and soil drench in one experiment

This study combines the two separate experiments described in 4.41. Five week old tomato plants were either sprayed or watered with ASM solution containing 100 mg l^{-1} active ingredient. Control plants were watered and sprayed with tap water. In this experiment, we included the leaf levels as factor again.

Table 4.9: Two-way ANOVA of comparison of ASM soil drench and spray.

Source	*df*	*SQ*	*MQ*	*F*	*P*
Application	2	11.743	5.872	22.845	0.000
Leaf level	3	4.251	1.417	5.513	0.002
Interaction	6	5.361	0.894	3.476	0.005
Error	60	15.421	0.257		
Total	71	36.776			

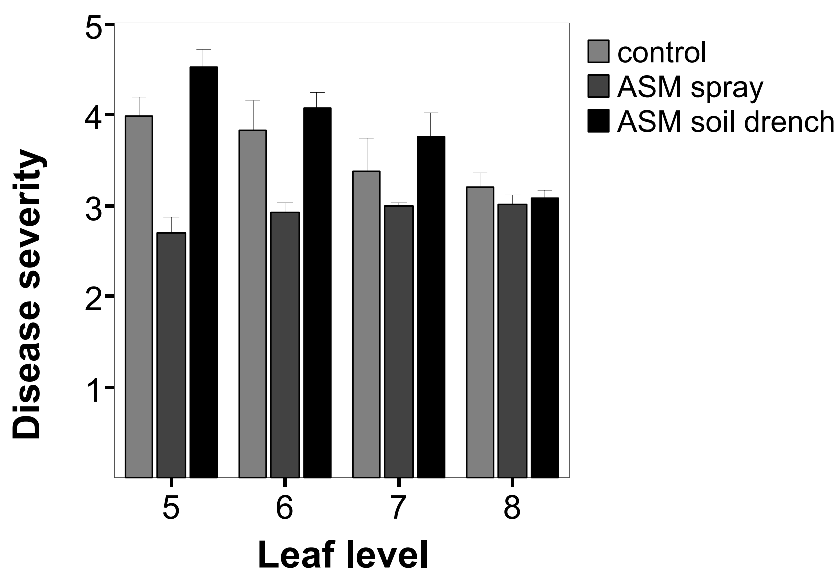


Figure 4.4: Disease severity on tomato leaf discs of 4 different leaf levels. The plants were either treated with water, sprayed with ASM solution or watered with ASM solution. Given are arithmetic means and standard errors with *n* = 6, assessment scale from 0 to 5.

Both factors, application mode and leaf level, were significant with *P*-values $P = 0.000$ and $P = 0.002$, respectively. The interactions between both factors were also significant with $P = 0.005$ (Table 4.9). These interactions are obvious as the effect of the ASM-application was reduced for younger leaves (compare Figure 4.4). The application of ASM as spray significantly reduced the disease for the levels 5 and 6, for the younger leaves (levels 7 and 8) the difference was no longer significant. The application of ASM as soil drench increased the infection with *Alternaria* relative to the control, but this difference was not significant. These findings were inconsistent with the results of the previous experiments in 4.4.1.

4.4.3 Combination of Maneb and ASM

The following study investigated the activity of ASM in combination with the fungicide Maneb, which was used in reduced concentration. As the foregoing experiment had shown only a positive effect of ASM if it was sprayed, and as Maneb cannot be applied as soil drench, the four combinations (400 mg l^{-1} Maneb, 100 mg l^{-1} ASM plus 400 mg l^{-1} Maneb, 50 mg l^{-1} ASM plus 80 mg l^{-1} Maneb, and 100 mg l^{-1} ASM) as well as the control were applied as spray. The leaf discs were cut of leaf level 5 one week after the application.

Results of a one-way ANOVA showed significant differences between the treatments (Figure 4.5). Only the higher concentration of Maneb was effective against *Alternaria solani,* with no distinction if ASM was added or not. This reduction of disease severity was significant, but the improvement was very small, showing that the concentration of 400 mg l^{-1} Maneb was only slightly effective. No difference could be seen between all other treatments. Inconsistent to the results in section 4.4.2, ASM sprayed in the concentration of 100 mg l^{-1} showed no activity here. Thus, it can be concluded that application of Maneb in lower concentration (20% of the recommended dose) with or without ASM has only minor effects against early blight. The reduction of the Maneb concentration to only 4% of the recommended dose plus an addition of ASM had no influence at all. Different experimental results with regard to ASM seem to be inconsistent.

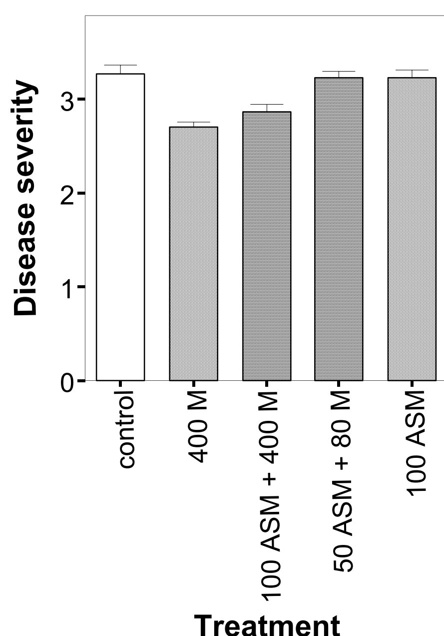


Figure 4.5: Disease severity in the leaf disc experiment with mixtures of Maneb (M) and ASM. All concentrations are given for the active ingredient in mg l^{-1}. Bars represent arithmetic means, error bars the standard errors with n = 8, assessment scale from 0 to 5. Data with equal letters in columns are not significantly different.

Re-analysing the current data with GLM, the factors were used as covariates. According to this analysis, ASM application did not have any significant influence, whereas Maneb had significant activity (Table 4.10). The interactions between both factors were not significant. The estimated parameters of the regression revealed a significant negative slope for Maneb. Thus, a higher concentration of Maneb would result in less disease severity.

Table 4.10: GLM of the leaf disc experiment with mixtures of Maneb and ASM, factors were used as covariates.

Source	*df*	*SQ*	*MQ*	*F*	*P*
ASM	1	0.010	0.010	0.213	0.647
Maneb	1	1.443	1.443	30.696	0.000
Interaction	1	0.090	0.090	1.922	0.174
Error	36	1.692	0.047		
Total	39	3.818			

4.4.4 Direct effects of ASM on *Alternaria solani*

In an *in vitro* experiment, it was tested whether ASM has a direct inhibitory effect on the growth of *Alternaria* isolates. Isolates Greece-1 and USA-1 were chosen for this study since their mycel discs tend to regular growth so that mycel radii could be measured accurately. Both fungi were grown in petri dishes on PDA media with ASM addition in the following concentrations: 0, 2.6 mg l^{-1}, 10.6 mg l^{-1} and 42.4 mg l^{-1}.

Table 4.11: Mycel radii of 2 *Alternaria* isolates grown on culture media containing ASM.

ASM [mg l^{-1}]	Greece-1	USA-1
Control	37.7 ± 0.3 a	29.0 ± 1.0 a
2.6 mg l^{-1}	38.3 ± 0.9 a	26.3 ± 0.3 a
10.6 mg l^{-1}	31.7 ± 0.3 b	23.0 ± 0.6 b
42.4 mg l^{-1}	27.7 ± 0.7 c	21.0 ± 0.6 b

Mycel radii are given in mm. Given are arithmetic means and standard errors with *n* = 3. Data with equal letters in columns are not significantly different.

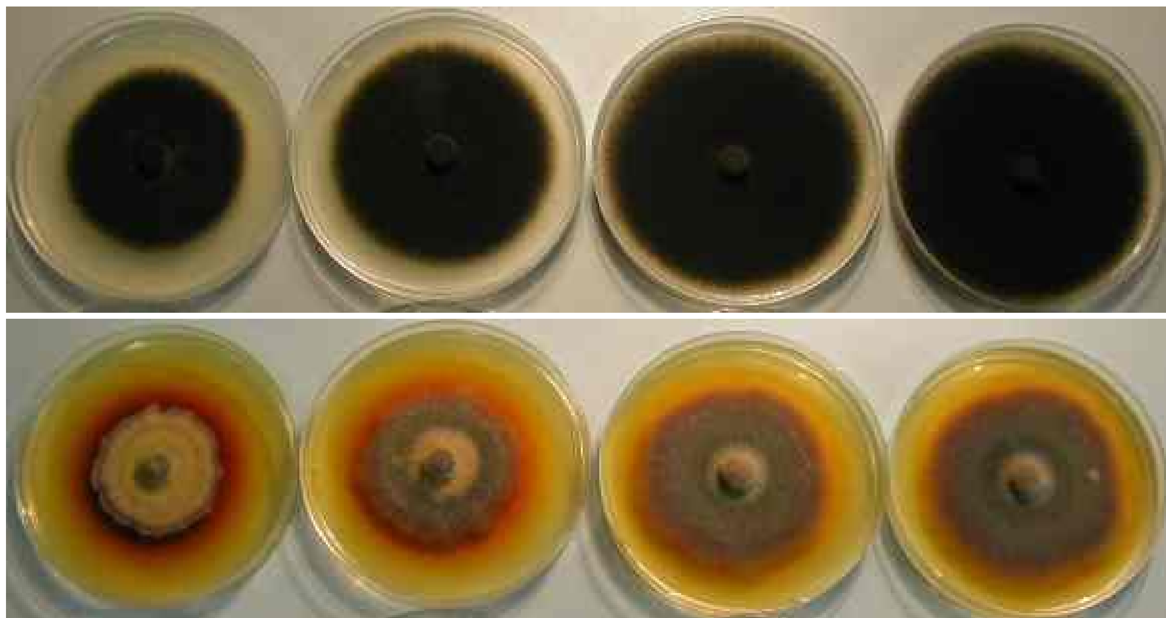


Figure 4.6: Direct fungitoxic effect of resistance inducer ASM on *Alternaria solani* isolates Greece-1 (above) and USA-1 (below) *in vitro*. From left to right the concentrations of ASM in mg l^{-1}: 42.4; 10.6; 2.6 and control. Note the change in colour of isolate USA-1 with increasing ASM concentration.

A direct fungitoxic influence of ASM on *Alternaria* could be proved as shown in Table 4.11 and Figure 4.6. Isolate Greece-1 responded to increasing ASM concentration with significantly reduced mycelia growth. The isolate from USA showed a very apparent change of colour from grey to brownish-white in addition

to the significant size reduction. A radius reduction of 27% as observed for isolate Greece-1 results in a reduction of the covered area to only 53% of the possible size. With these results, we can conclude that ASM obviously directly inhibits the growth of *Alternaria*.

4.4.5 Interactions between Maneb and ASM

The experiment described in section 4.4.3 had not revealed any interactions between ASM and Maneb. But since direct effects of ASM on *Alternaria* were discovered, an *in vitro* study was performed. By measuring the growth reduction, precise calculated efficiencies (expressed in percent control) could be determined. These were needed to estimate the extent of interaction. Mycel discs of three *Alternaria* isolates were placed in petri dishes with PDA media to which Maneb and ASM were added in various concentrations. One week later, the diameters of the mycel were measured and transformed to percent control by standardisation with control dish values. As Maneb and ASM have very different modes of action, the Abbott formula (Abbott, 1925) was used to calculate the expected efficacy of the different mixtures.

Abbott formula (Abbott, 1925):

$\% \, C_{exp} = A + B - (AB / 100)$

with $\% \, C_{exp}$ as expected efficacy of fungicide mixture and A and B as % control levels given by the single fungicides.

If the ratio between the experimentally observed efficacy of the mixture (C_{obs}) and the expected efficacy C_{exp} is greater than 1, synergistic interactions are present in the mixture. If the ratio is less than 1, the combination of fungicides has antagonistic effects. A ratio of 1 indicates an additive action of the mixture.

The addition of Maneb to the growth media had very drastic effects on *Alternaria*, only the lowest concentration (100 mg l^{-1}) allowed at least some growth. In petri dishes with higher Maneb concentration, the fungi were killed. Synergistic

interactions decrease rapidly with increasing control levels of single components (Cohen, 1986; Gisi, 1996; Samoucha & Cohen, 1988; Scardavi, 1966), so the maximum efficiencies should not exceed 70% (Gisi, 1996). Only in variants with the lowest Maneb concentrations synergism could be verified and data of treatments with higher concentration are not presented.

Table 4.12: Ratios between C_{obs} and C_{exp} for certain Maneb and ASM mixtures.

ASM [mg l^{-1}]	Maneb [mg l^{-1}]	*Alternaria* isolate	Ratio	Interaction
42.4	100	Cuba-141	1.22	synergistic
		Greece-1	3.41	synergistic
		USA-1	0.79	antagonistic
10.6	100	Cuba-141	1.24	synergistic
		Greece-1	2.61	synergistic
		USA-1	0.97	additive
2.65	100	Greece-1	1.39	synergistic
		USA-1	1.03	additive

One data set for isolate Cuba-141 is missing as this isolate tends to mutations which altered the growth and inhibited correct measurements. Ratio values are calculated as described above, *A* (for ASM alone) and *B* (for Maneb alone) as well as C_{obs} were computed with $n = 5$. Ratio values close to 1 were assessed as additive interaction.

As seen in Table 4.12, the possible interactions of Maneb and ASM did depend on the specific *Alternaria* isolate. For the Cuban and especially the Greek isolate, synergistic effects of the combination of ASM and Maneb *in vitro* could be shown. These results hint that ASM has a direct fungitoxic effect on *Alternaria* isolates *in vitro*.

However, for the isolate from the USA only additive and even antagonistic interactions could be demonstrated. Possibly, ASM or Maneb has a lower inhibitory effect on this isolate. This could be due to increased resistance of this isolate towards one or both agents. Unfortunately, Cuban isolate 141 mutated too often in some of the tested variants so that no round mycel area was developed and radii could not be measured. These data were discarded.

4.4.6 Summary

- Using ASM as soil drench significantly increased the susceptibility of old tomato leaves against *Alternaria*. For younger and therefore relatively resistant leaves, no influence could be demonstrated.
- Also spraying with ASM solution increased the disease severity on treated tomato plants.
- These results indicate that ASM enhances disease severity of *Alternaria* on tomato plants independent of the way of application.
- A study comparing the applications of ASM as spray and as soil drench gave inconsistent results. Here, ASM showed a significant reductive activity when it was applied as spray. This effect was also not significant for the youngest leaves.
- Combinations of ASM with the fungicide Maneb in very low concentration did not have any plant protecting effect and resulted in the same disease severity as for the water control. A concentration of 20% of the recommended Maneb dose could reduce early blight to a minor extent. In this experiment, the addition or omission of ASM had no effect on the disease.
- No interactions between ASM and Maneb were found when tested *in vivo* on living inoculated plants.
- A strong, direct inhibitory effect of ASM *in vitro* on *Alternaria* isolates was documented. Mycel discs were reduced to only 53% of the size of the control.
- When fungicide Maneb and resistance inducer ASM were examined together *in vitro* in petri dishes, synergistic interactions between the two agents could be demonstrated. These interactions seemed to depend on the *Alternaria* isolate, hinting that the individual isolates differ in their resistance towards Maneb and/or ASM.

4.5 Resistance inducer INA

As treatments with ASM did not result in consistent results concerning *Alternaria* infection, a set of experiments with 2,6-dichloroisonicotinic acid (INA) was plan-ned. This resistance inducer is also a derivate of salicylic acid, but is not commercially used like ASM.

4.5.1 Phytotoxic side-effects of INA

In a first study, the formulation with the wetting agent Tween 20 and solution additive DMF (N,N-Dimethylformamid) as well as feasible concentrations of INA were tested. Plants were treated with soil drench of either 30 ml tap water (as control), 20 ml formulation plus 10 ml tap water, or 20 ml formulation with 30 μg respectively 60 μg INA plus 10 ml tap water. The plants were watered with these solutions 3 weeks after germination and harvested one week later.

Table 4.13: Tomato fresh and dry weight after treatment with resistance inducer INA.

Treatments	Fresh weight [g]		Dry weight [g]	
Control	10.3 ± 1.14	bc	0.9 ± 0.17	b
Formulation	11.2 ± 1.89	c	1.0 ± 0.27	b
30 μg INA	8.5 ± 1.08	b	0.7 ± 0.12	a
60 μg INA	6.4 ± 1.75	a	0.5 ± 0.15	a

Given are arithmetic means and standard errors with *n* = 10. Data with equal letters in columns are not significantly different.

Figure 4.7: Tomato plants treated with resistance inducer INA. From left to right the water control, the formulation control and the treatments with 30 and 60 μg INA, respectively.

Results in Table 4.13 show that the formulation with DMI plus Tween 20 alone was not phytotoxic for tomato plants as neither fresh nor dry weights of the plants differ significantly from the control. However, the treatment with INA had very dramatic effects reducing fresh as well as dry matter of these plants significantly (see Figure 4.7). In case of fresh matter production, a significant difference between the two concentrations could be seen: the higher the concentration of INA, the smaller the plant. These findings show that the use of INA as plant protective agent for

tomatoes is not practicable due to the strong phytotoxicity. Further experiments with this resistance inducer were not conducted as the reduction of biomass could mimic a strong infection with early blight.

4.5.2 Summary

- Resistance inducer INA showed strong phytotoxic side effects which made a use as second resistance inducer impossible.
- As the aim of this work was to find an optimal combination of different plant health increasing agents for horticulture, further studies with INA were stopped.

4.6 Growth promoting fungus *Piriformospora indica*

The recently isolated fungus *Piriformospora* is known to stimulate plant growth as it colonises the roots of its host plants. Since susceptibility to *Alternaria* is high for weak, stressed or senescent plants, the symbiosis with *Piriformospora* could possibly strengthen the plants and increase the resistance.

4.6.1 *P. indica* growth promotion in three tomato cultivars

These two experiments investigated the growth-promoting effect of *Piriformopsora* on the three tomato cultivars Campbell 28 (C), Hellfrucht (H) and Rheinlands Ruhm (R). The growth substrate of the tomatoes was enriched with either (i) autoclaved *Piriformospora* mycel as a control for the included nutrients, (ii) fresh *Piriformospora* mycel, or (iii) as control with wet sand. Two approaches were taken: experiment 1 focused on the test whether a growth induction is achieved, and experiment 2 studied the growth promotion over a longer time period.

In the first experiment, 10 tomato plants were grown per pot to keep the necessary amount of *Piriformospora* mycel as low as possible. Plants were harvested 3 weeks after germination.

Both factors tomato cultivar and *Piriformospora* treatment were significant with P-values of $P = 0.002$ and $P = 0.000$, respectively (ANOVA not shown). Also significant interactions between both factors could be detected. These are clearly visible in cultivar C that differed from the two other cultivars in its reaction to the addition of living *Piriformospora* mycel (Figure 4.8). Instead of a more pronounced growth induction, the C plants showed a significant growth depression. For the other cultivars H and R, the autoclaved material induced a significant growth promotion and an even more pronounced effect by the living fungus.

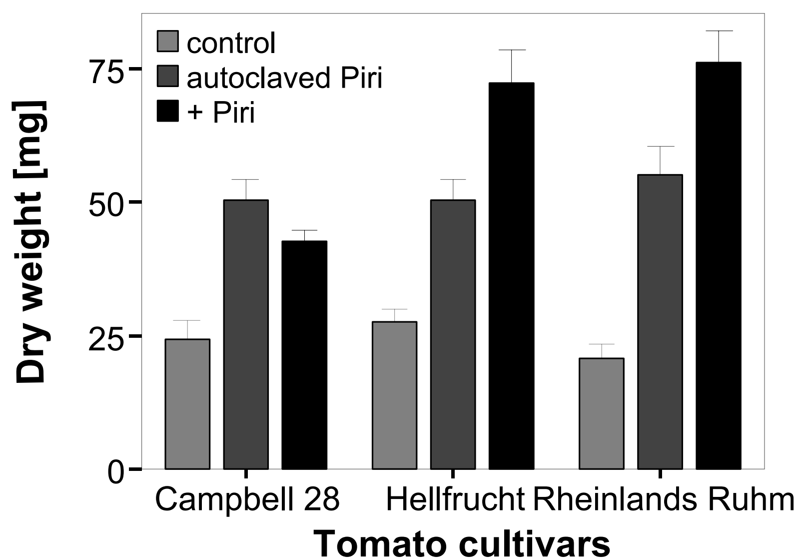


Figure 4.8: Dry weight of tomato sprouts of 3 cultivars grown in the presence or absence of *Piriformospora* in the soil. Control plants were grown without *Piriformospora* mycel, some plants were grown in soil added with autoclaved mycel, and the other plants were grown in soil enriched with living mycel. Bars represent arithmetic means, error bars the standard errors with $n = 10$.

In the second experiment, single plants were grown in the pots, but replicates were limited to three due to the little amount of mycel material. Plant sprouts were harvested at three time points: 15, 30 and 50 days after germination. The dry weight was measured to analyse if the differences in growth are stable over a longer time period.

Table 4.14: Two-way ANOVA of the *Piriformospora* time-course experiment.

Source	*df*	*SQ*	*MQ*	*F*	*P*
P. indica	2	0.340	0.170	0.765	0.470
Cultivar	2	3.759	1.880	8.460	0.001
Harvest	2	187.410	93.705	421.745	0.000
P. indica * Cultivar	4	1.172	0.293	1.319	0.275
P. indica * Harvest	4	2.149	0.537	2.418	0.060
Cultivar * Harvest	4	4.715	1.179	5.306	0.001
P. indica * Cultivar * Harvest	8	2.152	0.269	1.211	0.311
Error	54	11.998	0.222		
Total	80	213.696			

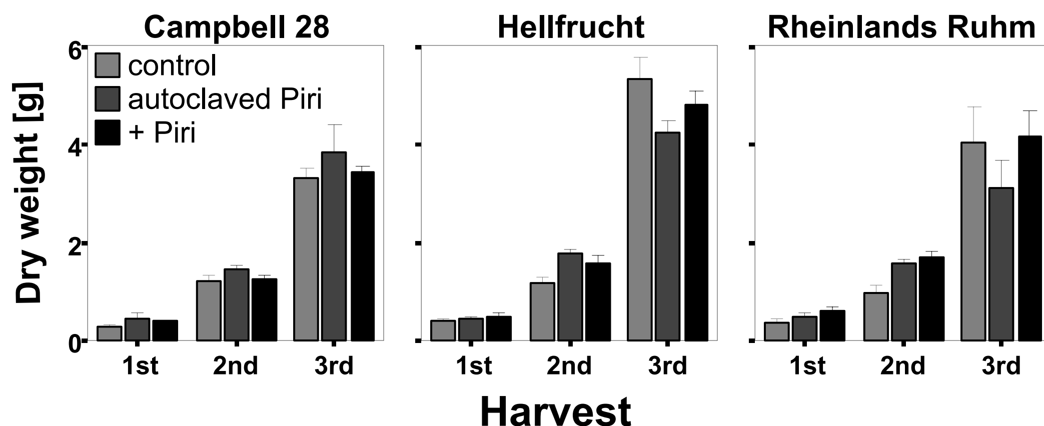


Figure 4.9: Dry weight of tomato sprouts in the *Piriformospora* time-course experiment. Control plants were grown without *Piriformospora* mycel, some plants were grown in soil added with autoclaved mycel, and the other plants were grown in soil enriched with living mycel. Bars represent arithmetic means, error bars the standard errors with *n* = 3.

When the data were analysed using a three-way ANOVA, only the time points of the harvests, the tomato cultivars and the interaction between these two factors were significantly influencing the tomato weight (Table 4.14). As the main focus of the experiment laid on the possible growth-inducing effect of *Piriformospora*, the data were split and analysed separately for the three time points and for the three cultivars. These evaluations resulted in the significant differences closely described below.

The results of the first harvest are similar to the results of the previous study, but due to the small number of replications and therefore higher variability, the

differences between the *Piriformospora* treatments are not significant. For cultivar C, no significant influence of the treatment with *Piriformospora* on the dry weight was found. The addition of *Piriformospora* had a significant growth-inducing effect only for the cultivars H and R with $P = 0.029$ and $P = 0.012$, respectively (see Figure 4.9, 2nd harvest). At the 3rd harvest, both H and R showed slight growth depressions in the treatment with autoclaved *Piriformospora* mycel, but this was also not significant. It is likely that the decaying fungal material had negative effects on soil and roots of these plants. No growth-induction due to colonisation with *Piriformospora* could be documented at the time of the 3rd harvest.

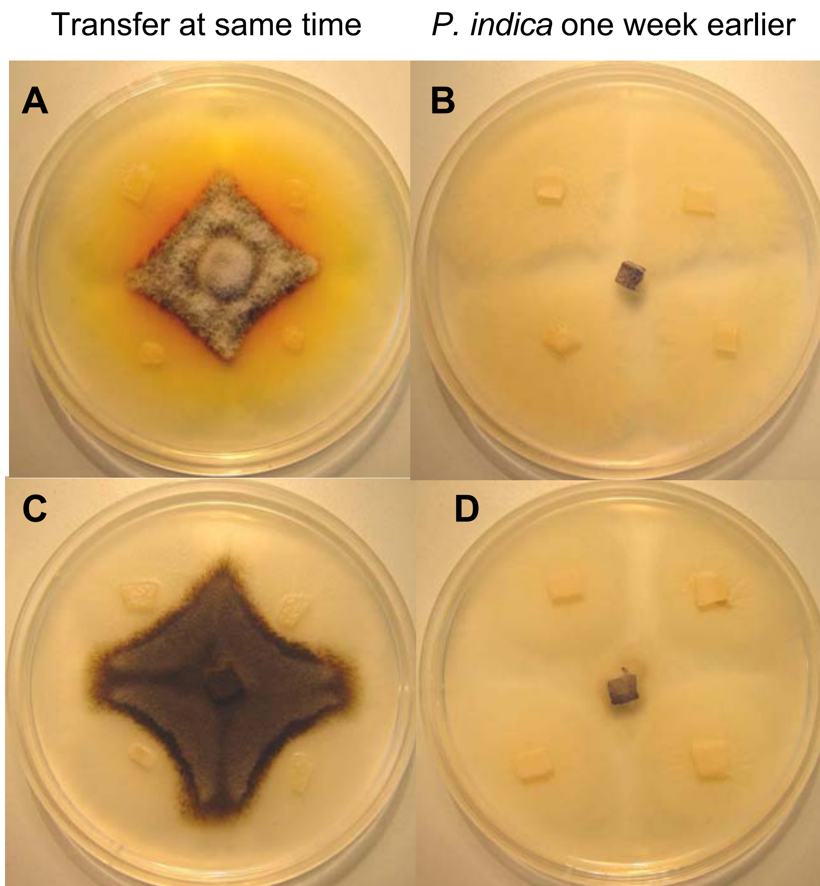
4.6.2 Influence of *P. indica* on *Alternaria in vitro*

Varma *et al.* (2001) tested *in vitro*, whether *Piriformospora* could be used as biological control agent against soil-borne diseases. *Alternaria*, that can outlive on decaying plant material or seeds, can infest germinating tomato plants in the seedbed. Here, an addition of *Piriformospora* could help to prevent damping-off of the plants and increase the survival rate when the seedlings are transplanted.

The *Alternaria* isolates and *Piriformospora* were arranged together in different patterns in petri dishes, similar to the study described by Varma *et al.* (2001). One mycel disc of *Alternaria* was growing either alone (as control), or in the middle of four *Piriformospora* mycel discs. Mycel growth was measured after one week. To compensate the slow growth of *Piriformospora*, it was placed in petri dishes one week before *Alternaria* in one treatment. It was also examined if the culture media C-medium and PDA influence the growth.

Evaluation showed that no chemical mechanisms between the fungi exist to repress growth of one another. When both fungi were placed in the dishes at the same time, the mycel grew in usual speed until hyphae nearly touched (Figure 4.10 A and C) and then stopped. This showed that there were no direct interactions between the fungi. Growth of both fungi was clearly better on PDA medium, even if the brim of the *Piriformospora* mycel was less sharp than on C medium (compare Figure 4.10 B and D). But all *Alternaria* isolates were repressed drastically if they were placed in the middle of already growing *Piriformospora*

mycel discs (Figure 4.10 B and D). Statistical analysis showed that culture media and fungal growth as well as *Alternaria* isolate and *Piriformospora* treatment had significant interactions (ANOVA not shown).



Figures 4.10: A-D: Interaction of *Piriformospora* and *Alternaria in vitro*. **A** and **B**: isolate Cuba-141 in the middle, both on PDA medium; **C** and **D**: isolate Gre-1 in the middle, both on C-medium. For the plates on the left, both fungi were placed at the same time in the petri dish. For the plates on the right, the 4 mycel pieces of *Piriformospora* were placed in the petri dish one week before the addition of *Alternaria*. Note the production of yellow-red pigment by isolate Cuba-141 in treatment A, which is stopped in treatment B. Also note the slightly different look of the *Piriformospora* mycel in the plates B and D.

4.6.3 Influence of *P. indica* on *Alternaria* infection *in vivo*

A clear growth-inducing effect of *Piriformospora* could be seen in the previous experiments in section 4.6.1, even though the tested tomato varieties did differ in their reaction on root colonisation. In the next study we investigated the reaction of *Piriformospora* colonised tomato plants on *Alternaria* inoculation. With only small amount of *Piriformospora* mycel available, all plants of one treatment were grown together in a single pot. This resulted in an early harvest and relatively small dry weights.

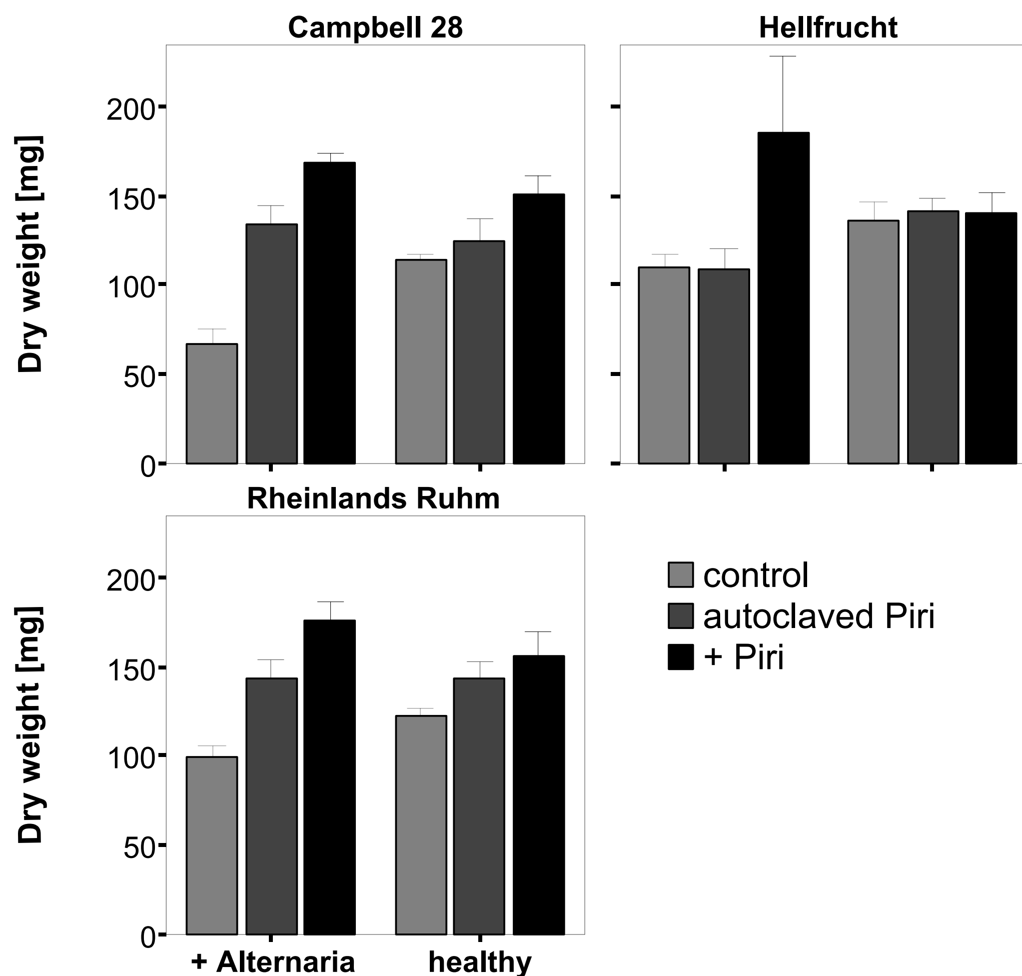


Figure 4.11: Dry weight of tomato sprouts of 3 cultivars, inoculated with *Alternaria* (left) or healthy (right). Control plants were grown without *Piriformospora* mycel, some plants were grown in soil added with autoclaved mycel, and the other plants were grown in soil enriched with living mycel. Bars represent arithmetic means, error bars standard errors with $n = 10$.

The data for healthy tomato plants were slightly inconsistent to the previous described experiments as growth induction of tomato cultivar C was this time higher for the living mycel material. And, for cultivars H and R, no significant growth induction could be demonstrated (Figure 4.11). The dry weight of C plants was significantly higher than the control when living *Piriformospora* mycel was added. In case of plants inoculated with *Alternaria*, the dry weight increased with the addition of autoclaved *Piriformospora* mycel and even increased more, if living mycel material was used. But, this effect was only significant for cultivar C due to the relatively high variances of the data. For all three cultivars, healthy plants of the control treatment were bigger than *Alternaria*-infected plants.

4.6.4 Summary

- *Piriformospora* mycel showed a clear growth inducing effect on tomato plants. A smaller growth promotion was achieved if autoclaved *Piriformospora* material was added to the soil.
- The growth promotion was not long-lasting, as plants in symbiosis with *Piriformospora* were not bigger than control plants 60 days after germination, even if the growth induction was obvious in the weeks before.
- *In vitro*, *Piriformospora* had an inhibitory effect on the growth of different *Alternaria* isolates, when it was placed in the dishes one week prior to the addition of *Alternaria*.
- In a later experiment, the reactions of the single tomato cultivars to the colonisation with *Piriformospora* were slightly inconsistent to the studies before. Tomatoes in symbiosis with *Piriformospora* suffered less after inoculation with *Alternaria* than control plants. The addition of autoclaved *Piriformospora* mycel also significantly increased the dry weight of infected tomato cultivars C and R, but not to the same extend as living mycel.

4.7 Influence of nutrient supply on *Alternaria* infection

Experiments described in the following sections 4.7.1 and 4.7.2 were performed during a stay at the Risø National Laboratory, Roskilde, Denmark. The studies presented here were performed additionally to the experiments presented in chapter 6.

Studies examining the influences of different nutrient supplies on the development and severity of early blight are presented in this section. We focused on the investigation of nitrogen (N), to determine optimal provision with this nutrient, and on phosphorus (P), which is known to interact with AM fungi. All plants were grown in soil enriched with the nutrients by addition of nutrient solutions as described in chapter 3.

4.7.1 Influence of phosphorus on early blight

This study was performed using the three tomato cultivars Campbell 28 (C), Hellfrucht (H) and Rheinlands Ruhm (R), in order to choose a suitable cultivar for the planned experiments with AMF and with different P supply levels. 6 plants per treatment were grown in soil with the following amounts of P: 0, 15 mg P kg^{-1} soil, and 45 mg P kg^{-1} soil. Half of the plants were harvested 4 weeks after germination to measure the dry weight of the sprouts.

Regarding the dry weight, the supply with P resulted in significantly better plant growth compared to the plants without additional P. For the cultivars H and R, the treatments with additional P differed significantly to the 0 P treatment. Increasing the P supply from 15 to 45 mg kg^{-1} soil did not result in even higher dry weights. Low-input cultivar Campbell 28 seemed to react inconsistently to increased P supply (see Figure 4.12). Here, the treatments 0 P and 15 P differed significantly, whereas the 45 P treatment was intermediate.

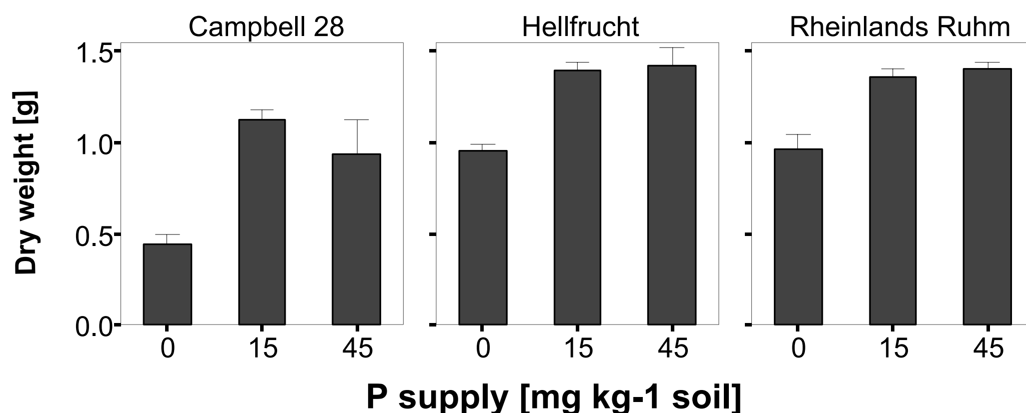


Figure 4.12: Influence of three P supply levels (0, 15 and 45 mg kg^{-1} soil) on the growth of three tomato cultivars. Bars show arithmetic means, error bars the standard errors, both with *n* = 3.

The remaining plants were inoculated with *Alternaria* spores and the disease development was assessed during 2 to 7 days after inoculation (dai). There were no significant differences in the severity of early blight infection (compare Figures 4.13 and 4.14) concerning necrosis and chlorosis, with all *P*-values well above 10% in the mixed model analysis.

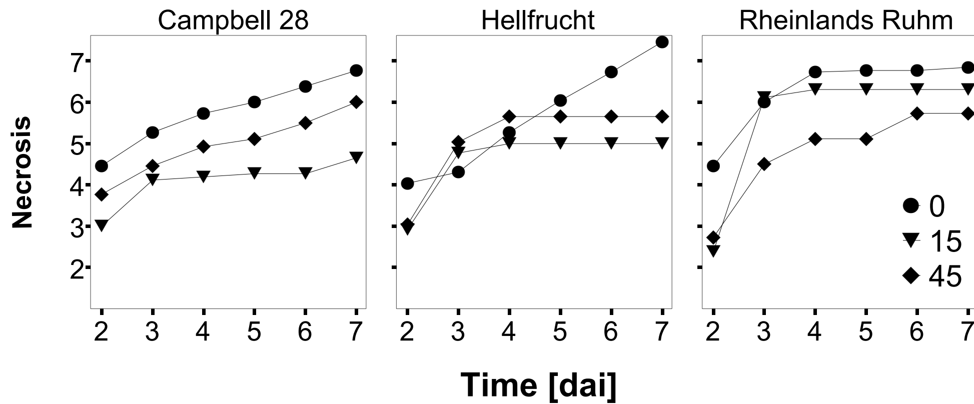


Figure 4.13: Influence of three different P supply levels (0, 15 and 45 mg kg^{-1} soil) on early blight necrosis development during 2 to 7 days after inoculation (dai) on three tomato cultivars. Dots represent arithmetic means with n = 3, assessment scale from 1 to 12.

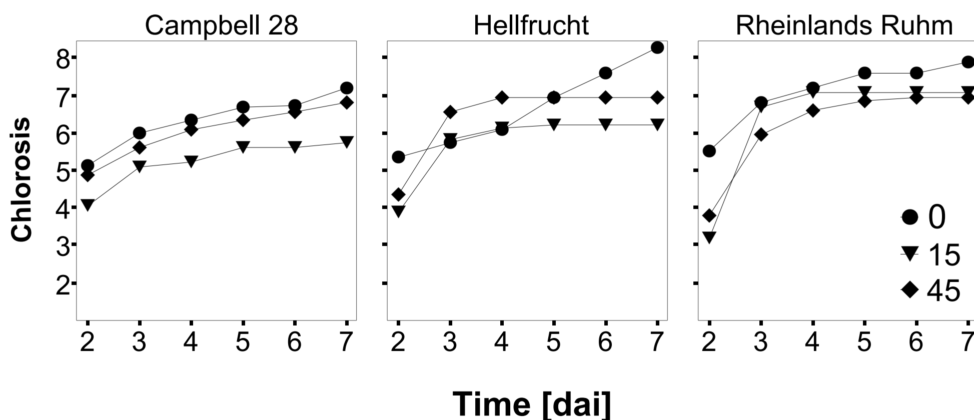


Figure 4.14: Influence of three different P supply levels (0, 15 and 45 mg kg^{-1} soil) on early blight chlorosis development during 2 to 7 days after inoculation (dai) on three tomato cultivars. Dots represent arithmetic means with n = 3, assessment scale from 1 to 12.

Table 4.15 gives the estimated means of necrosis and chlorosis for all cultivars. The means were not significantly different, but the disease severity was in nearly all cases higher for plants grown without additional P. Therefore, tomato plants supplied an increased levels of P tended to a reduced disease severity. For cultivar H, the necrotic symptoms increased dramatically at the end of the assessment period in the treatment 0 P. Cultivar C had slightly lower disease symptoms, but this difference was also not significant.

Table 4.15: Necrosis and chlorosis of three tomato cultivars grown in soil with different P supply levels and inoculated with *Alternaria solani*.

	Necrosis			Chlorosis		
P supply	Mean	*SE*	*df*	Mean	*SE*	*df*
Campbell 28						
0 P	5.691	0.527	5.608	6.233	0.495	5.375
15 P	3.935	0.527	5.608	5.016	0.495	5.375
45 P	4.931	0.527	5.608	5.908	0.495	5.375
Hellfrucht						
0 P	5.727	0.797	5.196	6.738	0.723	5.031
15 P	4.203	0.797	5.196	5.333	0.723	5.031
45 P	4.643	0.797	5.196	5.975	0.723	5.031
R. Ruhm						
0 P	6.033	0.667	6.096	6.929	0.670	5.562
15 P	5.132	0.667	6.096	5.832	0.670	5.562
45 P	4.593	0.667	6.096	5.830	0.670	5.562

Estimated means were calculated in a mixed model analysis with repeated measures data over the whole time period. Necrosis and chlorosis were assessed using an assessment scale from 1 to 12.

4.7.2 Influence of nitrogen on early blight

As the study concerning P supply did not show any objections against the commonly used tomato cultivar Rheinlands Ruhm, the experiment concerning possible influences of the nitrogen (N) supply was conducted with this cultivar only. The hypothesis of the experiment was to test whether a high supply with N could reduce *Alternaria* infection of the plants. Four different N supply levels (0, 25, 50 and 75 mg kg^{-1} soil) were examined with 4 plants each. Inoculation with *Alternaria* occurred 4 weeks after germination. The disease development was assessed during 2 to 8 days after inoculation (dai).

Disease severity data from the days 2 to 8 after inoculation were analysed using a linear mixed model approach. Table 4.16 gives the estimated means calculated over the whole time period. There were no significant differences between the four N treatments for both necrosis (P = 0.302) and chlorosis (P = 0.231). Analogue to the previous study concerning the P supply, the plants of increased nutrient levels tended to show the lowest early blight disease symptoms.

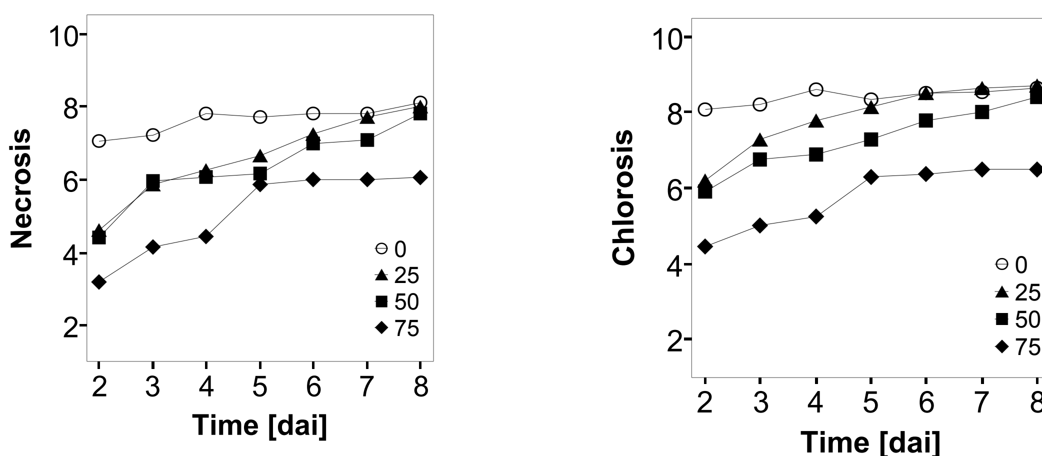


Figure 4.15: Influence of four different N supply levels (0, 25, 50 and 75 mg kg^{-1} soil) on early blight disease development during 2 to 8 days after inoculation (dai) on tomato cultivar Rheinlands Ruhm. Dots represent arithmetic means with n = 4, assessment scale from 1 to 12.

Table 4.16: Necrosis and chlorosis of tomato plants grown in soil with different N supply levels and inoculated with *Alternaria solani*.

	Necrosis	95% Confidence Interval		**Chlorosis**	95% Confidence Interval	
N supply	Mean	Lower Bound	Upper Bound	Mean	Lower Bound	Upper Bound
0 N	7.598	5.417	9.779	8.377	6.345	10.409
25 N	6.383	4.202	8.564	7.523	5.491	9.555
50 N	6.166	3.985	8.347	7.173	5.141	9.205
75 N	4.751	2.570	6.932	5.522	3.490	7.554

Estimated means were calculated in a mixed model analysis with repeated measures data over the whole time period, n = 4. The standard error of necrosis means is SE = 1.00 and the degrees of freedom are df = 12.14 for all treatments, whereas the standard error and df for chlorosis means are SE = 0.93 and df = 12.08. Necrosis and chlorosis were assessed using an assessment scale from 1 to 12.

4.7.3 Summary

- Concerning the nutrient experiments, we can summarise that neither N nor P have a significant influence on early blight development, at least in the concentrations used in the studies.
- Phosphate addition did increase the dry weight of the treated plants significantly without differing between 15 and 45 mg P kg^{-1} soil.
- Early blight disease was not influenced significantly by the supply with P, but tomatoes without any additional P showed the worst disease symptoms.

- In the case of N, a high supply with this nutrient seemed to reduce early blight symptoms, but this effect was not significant. Plants without N supply showed the highest disease severity for both necrosis and chlorosis, but this was also not significant.
- As the tendency of N to reduce early blight was not significant, subsequent studies with normal N supply could be performed.
- All tested tomato cultivars reacted in the same way to the different nutrient supply. Therefore, we decided to use cultivar Rheinlands Ruhm also for the following studies. Since we used it in the foregoing experiments, the results of all studies together would be complementary.

4.8 Arbuscular mycorrhizal fungi

Experiments described in section 4.8 were performed during a stay at the Risø National Laboratory, Roskilde, Denmark. The here presented studies were performed additionally to the experiments presented in chapter 6.

In this section, two experiments are described, which are testing the possible influence of colonisation of tomato plants with AM fungi on the infection with *Alternaria*. As phosphate nutrition and rate of colonisation are closely interacting, all studies include different levels of P supply.

4.8.1 AMF and low P supply influence on *Alternaria* infection

The first experiment focused on a reduced range of concentrations from 0 to 15 mg P kg^{-1} soil. *Glomus intraradices* (AMF) was also added to the treatment with 3 mg P to test if a presumably lower colonisation of roots, as described by Mosse (1973), still has a positive influence on plant health. This resulted in an unbalanced experimental design and the two additional treatments 0 P + AMF and 3 P + AMF.

Estimations of the degree of colonised roots revealed that there was no significant difference between treatments 0 P + AMF and 3 P + AMF in formation of

mycorrhiza (about 80% root length, data not shown), the low amount of offered phosphate did not have any influence on formation of the symbiosis.

Table 4.17: Necrosis and chlorosis of mycorrhizal (+ AMF) and non-mycorrhizal tomato plants after infection with *Alternaria* in experiment 1.

	Necrosis	95% Confidence Interval		**Chlorosis**	95% Confidence Interval	
Treatment	Mean	Lower Bound	Upper Bound	Mean	Lower Bound	Upper Bound
0 P	5.38 bc	4.60	6.17	5.86 b	5.05	6.68
0 P + AMF	2.92 a	2.13	3.71	3.39 a	2.58	4.21
3 P	6.25 c	5.46	7.07	6.86 b	6.05	7.68
3 P + AMF	4.21 ab	3.42	5.00	5.01 ab	4.19	5.82
6 P	5.80 bc	5.01	6.59	6.61 b	5.79	7.42
9 P	6.12 c	5.33	6.91	7.12 c	6.31	7.94
12 P	5.98 bc	5.19	6.77	6.90 b	6.09	7.72
15 P	6.13 c	5.35	6.92	7.30 c	6.49	8.12

Estimated means were calculated in a mixed model analysis with repeated measures data over the whole time period, $n = 4$. The standard error of necrosis means is $SE = 0.38$ and the degrees of freedom are $df = 22.20$ for all treatments, whereas the standard error and df for chlorosis means are $SE = 0.39$ and $df = 20.56$. Necrosis and chlorosis were assessed using an assessment scale from 1 to 12. Means in columns followed by equal letters are not significantly different.

Concerning the infection with *Alternaria*, the disease severity was significantly least on tomatoes of the treatment 0 P + AMF (see Fig. 4.16 and Table 4.17). Plants of treatment 3 P + AMF showed a more severe infection, however, it did not reach damaging levels as with non-mycorrhizal tomatoes. A linear mixed model analysis revealed significant differences depending on the addition or omission of AMF. The phosphate treatments as well as the interactions were both not significant with $P = 0.160$ and $P = 0.587$, respectively.

The colonisation of tomato plants by *Glomus intraradices* reduced the infection with early blight significantly. But it seemed that P nutrition could reduce the beneficial susceptibility-reducing effect of mycorrhiza even if the degree of colonisation is not influenced. The *Alternaria* infection in terms of necrosis and chlorosis increased significantly during the experiments in all treatments.

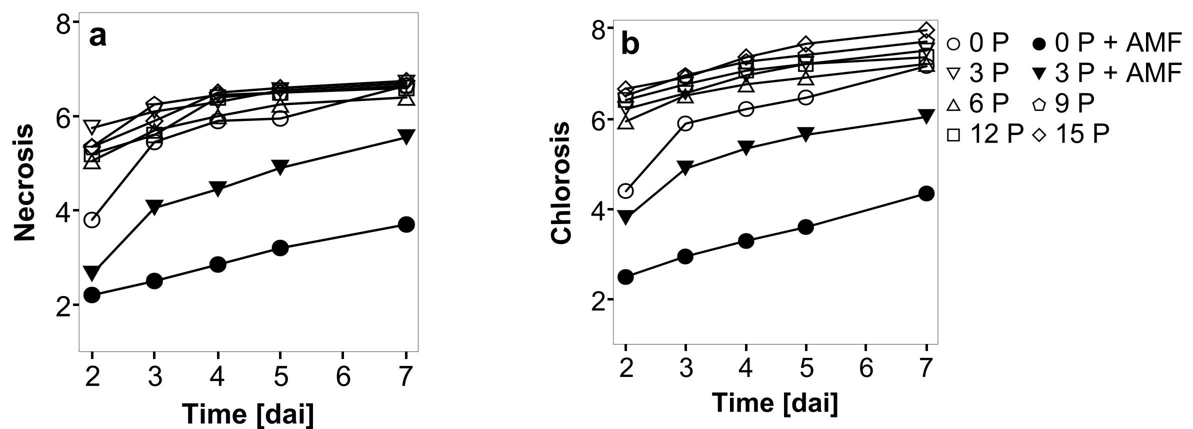


Figure 4.16: Necrosis (a) and chlorosis (b) caused by *Alternaria* infection in experiment 1 monitored over days 2 to 7 after inoculation (dai) of tomato leaves. 0 to 15 P represent non-mycorrhizal plants with different P supply (in mg kg^{-1} soil) and 0P + AMF and 3 P + AMF represent mycorrhizal plants. Data points show arithmetic means with $n = 4$, assessment scale from 1 to 12.

During this study it was observed that tomatoes of 0 P + AMF were significantly smaller in terms of sprout length compared to plants of treatment 0 P (Figure 4.17). This could probably be attributed to the strong competition for carbon between the two sinks plant and fungus.

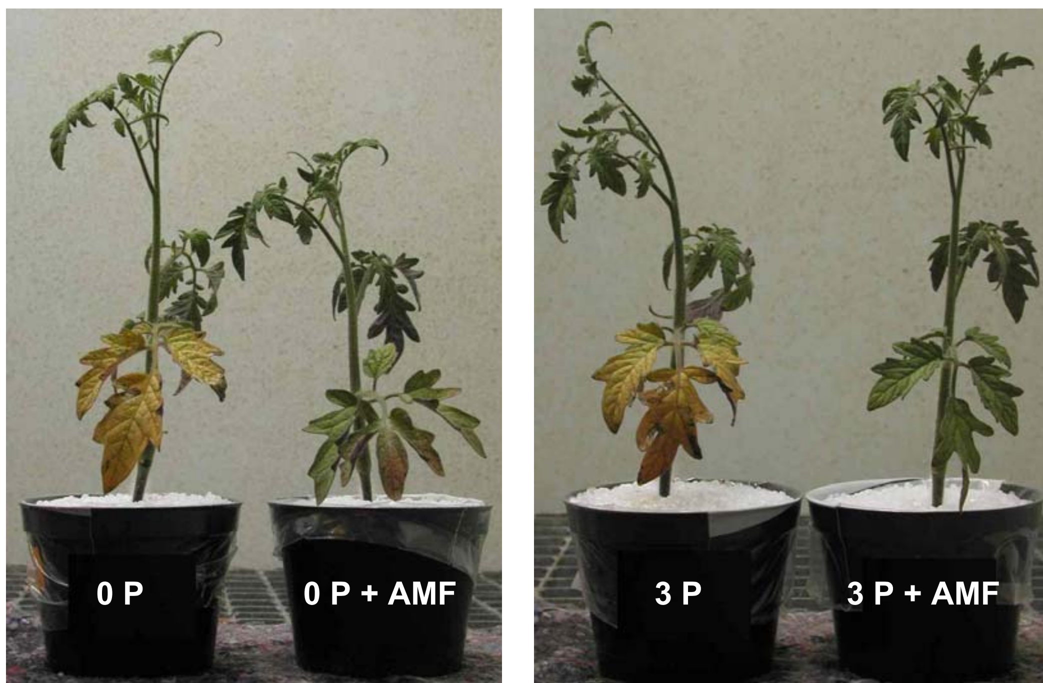


Figure 4.17: Tomato plants of the first experiment combining different P supply and AMF. From left to right the treatments 0 P, 0 P + AMF, 3 P and 3 P + AMF. Note the different height of 0 P plants and the varying amounts of chlorotic leaf area of plants with and without AMF.

Plants of treatment 0 P + AMF showed red colouring of some lower leaves, hinting a P deficiency. The difference in disease severity between the treatments with and without addition of AMF is clearly visible in amount of chlorotic leaf area. Plants of the treatments 3 P and 3 P + AMF did not show a great difference in height but the same distinction in early blight infection.

4.8.2 AMF and high P supply influence on *Alternaria* infection

A balanced design was used in the second study, meaning that all P supply levels were combined with and without the addition of AMF. This resulted in the six treatments: 0 P, 0 P + AMF, 15 P, 15 P + AMF, 45 P and 45 P + AMF. This study is a replication of the second experiment presented in the article in chapter 6, but inoculation success with *Alternaria* was notably smaller than in other experiments.

Mycorrhizal colonisation of plants with no added P was significantly higher ($P = 0.000$) than the colonisation of plants with 15 and 45 mg P kg^{-1} soil: 65% for 0 P + AMF and 46% and 36% for 15 P + AMF and 45 P + AMF, respectively. The sprout dry weight was significantly reduced in both treatments without added P and was not influenced by the presence or absence of *Glomus intraradices*.

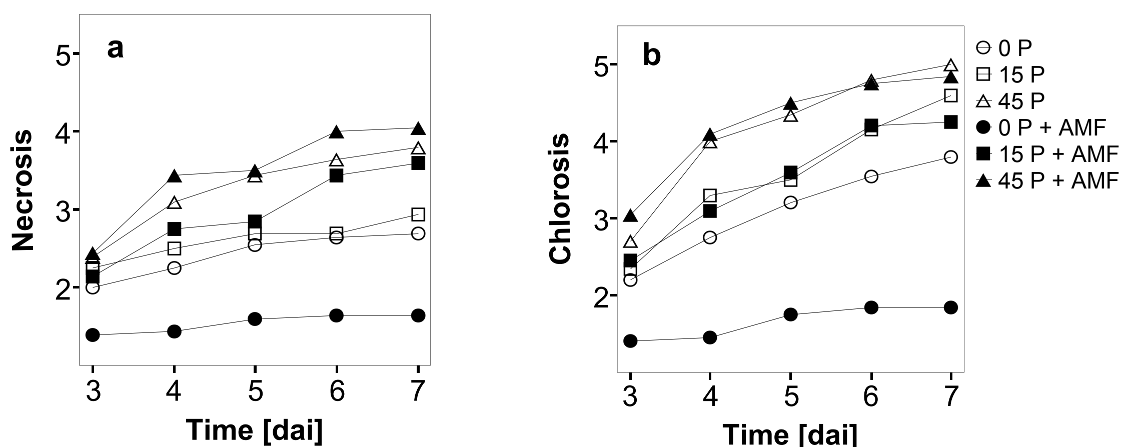


Figure 4.18: Necrosis (a) and chlorosis (b) caused by *Alternaria* infection in experiment 2, monitored during 3 to 7 days after inoculation (dai) of tomato leaves. 0, 15 and 45 P represent non-mycorrhizal plants with different P supply (in mg kg^{-1} soil). 0P + AMF, 15 P + AMF and 45 P + AMF represent mycorrhizal plants. Data points show arithmetic means with $n = 4$, assessment scale from 1 to 12.

Concerning early blight disease, a linear mixed model analysis of the time course assessment could only support a significant influence of the P supply (Table 4.18). As visible in Figure 4.18, the inoculation success with *Alternaria* was very low in this experiment. Mean assessment grades reached only about 4 for necrosis, corresponding to 6 to 12% of the leaf area, which is very low compared when to all other experiments. Though not significant, the treatment 0 P + AMF showed for both necrosis and chlorosis the lowest disease severity which did increase only very slightly over time. The higher infection levels of treatments 15 P + AMF and 45 P + AMF could be a result of the reduced mycorrhizal colonisation.

Table 4.18: Necrosis and chlorosis of mycorrhizal (+ AMF) and non-mycorrhizal tomato plants after infection with *Alternaria* in experiment 2.

	Necrosis	95% Confidence Interval		**Chlorosis**	95% Confidence Interval	
Treatment	Mean	Lower Bound	Upper Bound	Mean	Lower Bound	Upper Bound
0 P	2.376	1.432	3.320	3.029	1.824	4.234
0 P + AMF	1.533	0.589	2.477	1.635	0.430	2.840
15 P	2.606	1.663	3.550	3.505	2.300	4.711
15 P + AMF	2.902	1.959	3.846	3.399	2.194	4.604
45 P	3.158	2.215	4.102	3.943	2.738	5.148
45 P + AMF	3.328	2.384	4.271	4.037	2.832	5.242

Estimated means were calculated in a mixed model analysis with repeated measures data over the whole time period, $n = 4$. The standard error of necrosis means is $SE = 0.45$ and the degrees of freedom are $df = 19.82$ for all treatments, whereas the standard error and df for chlorosis means are $SE = 0.57$ and $df = 18.12$. Necrosis and chlorosis were assessed using an assessment scale from 1 to 12.

4.8.3 Summary

- Colonisation of tomato plants with *Glomus intraradices* significantly reduced early blight disease symptoms in terms of necrosis and chlorosis.
- The amount of mycorrhizal colonisation is reduced significantly if amounts like 15 and 45 mg P kg^{-1} soil are added.
- A smaller amount of added P, as described in the first experiment, did not reduce the mycorrhiza formation, but decreased the resistance as well. This showed that the AMF-induced resistance is strongly influenced by the P supply.
- In the first experiment, plants of the treatment 0 P + AMF were notably smaller than plants of the treatment 0 P, which hinted a strong competition for carbon

between the two sinks plant and fungal symbiont. For plants with an addition of 3 mg P, no such growth difference was observed.

4.9 Field trial with ASM, *Spirulina* and AMF

The following section describes a field trial that was performed at the CENSA, La Habana, Cuba. The induction of resistance by (i) AM fungi, (ii) chemical resistance inducer ASM, and (iii) plant restorative *Spirulina* was tested alone and in combinations under field conditions. It was planned to analyse SAR-related enzymatic systems like PR proteins (pathogenesis related proteins), peroxidase, polyphenoloxidase, chitinase, and lypoxigenase both after treatment of the plants and after inoculation with *Alternaria solani*. Leaf samples were to be taken at 0, 12, 24 hours, and the following 6 days. Assessment of the disease severity was to take place during flowering (60 days after germination) and before the ripening of the fruits (90 days after germination). The following treatments were planned: AMF, ASM, *Spirulina*, AMF + ASM, AMF + *Spirulina*, ASM + *Spirulina*, and the untreated control.

The seedlings were first grown in multipot-trays in a glasshouse. Mycorrhizal plants were inoculated using pellets of the Cuban AMF inoculum EcoMic®. Regrettably, the used soil for the multipots was of poor quality which resulted in bad seedling growth and especially bad root development. And, the tomato plants were not sown in time, so that at the time of transplanting to the field, they had developed only one leaf besides the cotyledons. The seedlings were transplanted in a randomised complete block design with three replications to compensate slight soil differences in the grounds. Every plot contained 5 rows with about 20 plants. It was not tested, whether the plants of the "+ AMF" treatments show mycorrhizal colonisation. It can be assumed that all plants normally become mycorrhizal during the growth in fields. This test could have proved mycorrhization at the time of transplanting for the "+ AMF" tomatoes and could have helped to explain possible differences in the later results.

High damage by leaf cutter ants occurred and in some plots were only very few plants left. In Cuba, chemical plant protectants are scarce and the only strategy to minimise plant loss was offering of cabbage to the ants to detract them from the tomato plantlets. After two weeks, the ant population had enormously increased and more tomato plants than before were lost. In some plots, not enough plant material was left, so that these plots had to be excluded from sampling. The analysis of the enzymatic systems is still in work due to shortage of staff at CENSA.

Figure 4.19: Leaf cutter ants transporting tomato plant pieces to their nest.

5 DISCUSSION

It is well-known that the use of induced resistance in plant protection can be problematic, especially under practical conditions. In this work, we investigated the pathosystem tomato – *Alternaria solani* with special emphasis to resistance induction to understand more about mechanisms and factors influencing the success or failure of plant protection. A multitude of experiments was accomplished to analyse aspects such as application, interaction with other agents, and different inductors. Within the scope of these studies, SAR emerged to be rather unreliable in controlling *Alternaria*. However, it was found that focusing on arbuscular mycorrhizal fungi can be a promising option. In the discussion, we interpret and summarise our findings with regard to achieve a general view.

5.1 General studies of *Alternaria solani*

In the first studies with *Alternaria*, we could (i) develop a new and more efficient method to induce sporulation, and (ii) prove the age-dependent susceptibility of tomato leaves. We could show also that the toxins in the spore suspension can cause disease symptoms by themselves, but not to a degree that would falsify the inoculation of the plants and the later assessment of the disease severity.

Alternaria is readily isolated from infected plant tissue but it is difficult to maintain these isolates on agar media in forms that produce conidia. Often sporulation

capacity declines or is lost after a few serial transfers on these media and the colonies may become completely mycelial or produce only very few conidiophores and conidia. The ability to still produce spores when growing on artificial media differs widely from isolate to isolate, but mostly an induction of sporulation is required when high amounts of spores are needed for experiments.

Commonly used methods for sporulation induction include exposure to sun- or ultraviolet light (Charton, 1953; Rands, 1917), mycelial wounding (Douglas & Pavek, 1971), medium dehydration or the use of chemical additives (Charton, 1953). The normal method used in our laboratory, developed by Shahin & Shepard (1979), combined wounding of the mycel, reduction of nutrient supply and increased stress by the addition of sterile water. This method is very effective but also time-consuming and work-intensive, as single mycel cubes have to be spread on a second medium and the harvest of spores has to be done more carefully. Our new method focuses on nutrient stress for the induction of sporulation. The isolates were transferred first to DA medium without any contents of host plants and are transferred back to normal PDA medium 7 to 10 days before the spores are needed. This method is easily included in the normal maintenance of the cultures as only a change of media is required. The change in nutrient supply resulted in profuse sporulation and could establish continuous sporulation in isolate Greece-1. It can be concluded that the new method is an improvement compared to the previously used sporulation induction process.

In leaf disc experiments, the oldest leaves were the most susceptible to *Alternaria*. This was expected as the susceptibility of tomatoes to early blight increases with senescence (Rotem, 1994). Barna & Györgyi (1992) list several reasons for a generally increased susceptibility of senescent tissues to necrotrophic pathogens, among these reasons are higher sensitivity of senescent plant tissues to pathogen toxins, to cell wall degrading enzymes and autolysis of membrane lipids.

The leaves of levels 6 and 7 showed unexpectedly high susceptibility. These leaves were not completely developed at the time of picking. Leaf discs cut from these young leaves were not planar but slightly folded towards the leaf vein. When droplets of the spore solution were placed on these discs, they covered a bigger

area of the disc due to the water tension. Additionally, the discs of young leaves seemed to wilt faster than other leaf discs. These two points, bigger area for *Alternaria* attack and additional stress by reduced turgor, could have eased early blight infection. Furthermore, it was impossible to cut as many leaf discs from these small leaves as from leaves of the other levels, resulting in fewer measurement replications for the youngest leaves and providing less reliable estimates. This could have been avoided by reducing the repeated measurements to the smallest possible number, but this reduction of measurement repetitions would have reduced accuracy at the same time.

Rotem (1994) states that many hosts of *Alternaria* species are susceptible in the juvenile stage, with a second peak of highest susceptibility in the senescent stage. The best-known case is tomato, where *Alternaria* causes collar rot in seedlings. Past the susceptible juvenile stage, plants become relatively resistant, but later enter the susceptible senescent stage (Moore, 1942; Moore & Thomas, 1943). Our results suggest that not only tomato seedlings are highly susceptible, but also young leaves.

Tomato leaf discs inoculated with filtered, spore-free suspension, which is likely to contain some fungal toxins, showed significantly higher assessment grades than the control discs inoculated with pure water droplets. Not surprisingly, inoculation with unfiltered spore suspension showed by far highest disease severity indicating that toxins alone caused some disease symptoms but no severe damage. Germination fluids of *Alternaria* contain alternaric acid (Langsdorf *et al.*, 1990), so we can conclude that the filtered spore suspension contained at least this toxin. The marginal effect of the toxin itself showed that inoculation using a spore suspension will not create an artefact but represent the true inoculation success.

The involvement of a toxic compound produced by *Alternaria in vivo* was suggested very early in *Alternaria* research since the disease symptoms enlarge rapidly and coalesce from small necrotic spots (Hooker, 1981). Pound and Stahmann (1951) showed that alternaric acid, produced by *Alternaria solani*, plays an important role in the emerge of early blight symptoms and in defoliation, as it can be spread in the vascular tissue of an infected plant and enhance chlorosis

and shedding of the leaves. Alternaric acid alters the morphological and physiological characteristics of plasma membranes near plasmodesmata and thereby causes a permeability change, which leads to a leakage of electrolytes (Langsdorf *et al*., 1991). Various toxic metabolites produced by *Alternaria* isolates in culture were reported, e. g. alternaric acid (Brian *et al*., 1945), anthroquinones like altersolanol A and macrosporin (Stoessl, 1969), solanopyrones (Ichihara *et al*., 1983) and other chemically not yet fully identified compounds. The importance of these toxins for the infection process could be demonstrated by their susceptibility-inducing properties: when spores on non-pathogenic races of these pathogens which were unable to infect the host plants *per se* were supplemented with host-selective toxins, they were able to penetrate the host plants and initiate disease (Yoder & Scheffer, 1969; Yamamoto *et al*., 1984). However, no correlation was found between the virulence of specific strains of *Alternaria* and their ability to produce toxins (Stancheva, 1989).

5.2 Plant restorative *Spirulina platensis*

The studies concerning *Spirulina platensis* as plant protective showed that the activity depends strongly on the method of application. Application as spray increased the disease severity relative to the control or decreased the plants weight. However, if *Spirulina* was used as soil drench, it could reduce the susceptibility of tomato to *Alternaria* at low infection pressure. For normal infection pressure, no differences between the two application methods and the control were observed. In mixtures with resistance inducer ASM, *Spirulina* had no significant activity. Since the influence of ASM was significant in this experiment, these results are discussed in section 5.4.

The slight disease reduction after use of *Spirulina* as soil drench may be explained by (i) a fertilising effect or (ii) a kind of induced resistance. *Spirulina* contains a multitude of proteins and amino acids, vitamins, macro- as well as micronutrients, and pigments such as chlorophyll and carotenoide (Pulz *et al*., 2000). These substances, and especially the soluble exocellular polysaccharides secreted by *Spirulina* (Filali Mouhim *et al*., 1993; De Philippis & Vincenzini, 1998; Nicolaus *et*

al., 1999), could enhance the plants nutrition or interact with the plant and induce an alteration in the plants defence.

In contrast to soil drenching, *Spirulina* as spray increased the early blight disease symptoms significantly. The remains of the spraying solution could have had a positive effect on *Alternaria* spores, possibly by stimulating germination or providing nutrients prior to penetration into the host. It is also likely, that the exopolysaccharides of *Spirulina* interacted with the plant cells and increased the susceptibility. It also seems possible that spraying with the dark-green *Spirulina* solution covered the canopy with cell residues and reduced the light intensity. This could have resulted in decreased photosynthesis and reduced the plant growth on a long-term basis. However, this cannot explain the leaf-disc results, where no photosynthesis is at work but nevertheless an increased disease severity was measured.

5.3 Chemical plant protective agent Maneb

In a preliminary study preparing for the subsequent interaction experiments, Maneb was applied in the recommended concentration for tomato crops. The observed disease severity after inoculation with early blight dependent strongly on leaf age. For older leaves, necrosis and chlorosis symptoms were significantly reduced compared to the control. For the younger leaves, the disease severity was still significantly reduced, but to a lesser extend.

At the time of spraying, the leaves of levels 5 and 6 were not fully developed and therefore the Maneb layer was thinner or more irregular than for the older leaves. *Alternaria* spores could germinate easier on the less protected younger leaves. Maneb, as a strictly protective fungicide without systemic action, can only protect treated plant parts and not growing tissue. Under practical conditions, the application with Maneb would have to be repeated in regular time intervals.

Additionally, this leaf disc experiment showed that untreated leaves of different ages had characteristically unequal levels of susceptibility, with the lowest susceptibility for the youngest leaves. This effect is already discussed in section 5.1.

5.4 Resistance inducer ASM

Results of the various ASM studies seem rather inconsistent. The application of ASM as soil drench significantly increased disease severity, whereas sprayed ASM increased in some cases, and decreased in others the outbreak of early blight.

The induction of systemic resistance in tomato, especially against *Phytophthora infestans*, is reported by several authors (Heller & Gessler, 1986; Kovats *et al.*, 1991; Enkerli *et al.*, 1993). Spletzer & Enyedi (1999) activated SAR in tomato plants by root feeding of SA. Also Senaratna *et al.* (2000) could induce multiple stress tolerance in tomato by growing tomatoes from seeds imbibed in solutions with SA and acetyl salicyl acid (ASA). It is possible that both SA and ASA are more readily absorbed via the roots than ASM. There are also reports of successful use of ASM against *Alternaria solani*, or of resistance induction by ASM in tomato against a variety of pathogens and pests. Spraying with ASM gave almost complete control of *Alternaria* in potato under glasshouse-conditions, and at least a reduction of disease severity in the field (Bokshi *et al.*, 2003). Treatment of tomato seedlings with ASM reduced the disease severity of bacterial canker as well as the growth of the disease causing bacteria *Clavibacter michiganensis in planta* to up to 75% (Soylu *et al.*, 2003). Audenaert *et al.* (2002) reported resistance induction of tomato to the necrotrophic pathogen *Botrytis cinera* after ASM soil application. ASM treatment (i) reduced the population growth of potato aphid on a susceptible tomato cultivar, and (ii) enhanced aphid control on a resistant cultivar, both by a direct negative effect on the phloem-feeding insect (Cooper *et al.*, 2004).

In contrast to the positive reports above, is the efficacy of resistance inducing chemicals rather unpredictable, and resistance strategies by chemical induction

are so far inapplicable for farmers (Kumar *et al.*, 2002). For example in the barley – mildew-pathosystem, one application of ASM under laboratory conditions is usually sufficient for protection during the whole growing season, whereas under field conditions, the action of ASM is uncertain and the reasons for this are still unclear (Wiese *et al.*, 2003).

The diverging results could be due to the relatively high phytotoxicity of ASM. The threshold between the concentration to achieve induced resistance and the concentration damaging the plant seems to be very narrow. Audenaert *et al.* (2002) reported that higher concentrations of ASM caused a decline in induced resistance and dramatic changes in the tomato plants morphology: leaves turned dark green and became shrunken, and spontaneous necrotic lesions developed. Already in the concentration of 10 mg ASM kg^{-1} soil, the treatment resulted in lengthening of internodes and shrinking of leaves. In our studies, plants treated with ASM as soil drench in high concentration (> 25 mg ASM l^{-1}) had a darker leaf colour and in some cases, the single leaflets were slightly smaller than in control plants. Tomato plants stressed by the phytotoxic ASM could be more susceptible to *Alternaria*.

Watering tomato plants with ASM solution resulted in significantly increased infection compared to the water control. Instead of increasing the resistance, this treatment led to a higher susceptibility to *Alternaria*. As the plants were significantly more infected, we cannot only emanate from missing effectivity but have to ask why the susceptibility was increased. Therefore, missing absorption into the plant cannot explain the worsening of the disease.

Induced resistance caused by ASM mainly protects the plants by formation of papillae (Kogel & Hückelhoven, 1999) and accelerating the hypersensitive response (HR) of the challenged cell. The current knowledge predicts that the effects of reactive oxygen intermediates in plant pathogenesis depend on many factors, of which the lifestyle of the pathogen (biotrophy or necrotrophy) is a major one (Hückelhoven & Kogel, 2003). In general, it seems that SA-dependent defence responses (SAR) are effective against biotrophic pathogens, while

jasmonic acid-dependent defence responses (ISR) are effective against necrotrophic pathogens (Thomma *et al.*, 1998; 2001a; Tierens *et al.*, 2002).

As described before, *Alternaria* is necrotrophic. Involvement of pathogenesis-related (PR) proteins has been implicated in *Alternaria* resistance in tomato through the observation that it correlates with high and rapid accumulation of PR-proteins. However, it is speculated that those PR-proteins, many of which possess hydrolytic activity, are not necessarily directly involved in arresting the pathogen but rather release elicitors from the pathogen cell wall, thus triggering a hypersensitive response (Lawrence *et al.*, 2000). The HR is considered to be a major element of plant disease resistance as it disrupts the pathogen from food supply and confines it to the initial infection site.

Necrotrophic pathogens, such as *Botrytis cinera*, *Sclerotinia sclerotiorum* and *Alternaria solani*, however, can utilise dead tissue and HR does not protect plants against infection (Gorvin & Levine, 2000). By contrast, *B. cinera* triggers HR, which facilitates its colonisation of plants.

So, when a pathogen is necrotrophic and requires dead cells for its survival, the HR is not a viable resistance mechanism. While necrotrophs may produce toxins to facilitate plant cell death for their survival, there also remains the possibility that they take advantage of disease resistance mechanisms such as HR (Gorvin & Levine, 2000), evolved by the plant to defend against biotrophs (Thomma *et al.*, 2001b). The observations that oxidative stress is higher in senescing leaves (Lin & Kao, 1998) and most necrotrophic fungi prefer to grow on senescent leaves (Agrios, 2005), would lend support to this theory.

Alternatively, necrotrophic pathogens may be directly responsible for reactive oxygen species (ROS) production as means of increasing lipid peroxidation (Gönner & Schlösser, 1993), to cause cell death (von Tiedemann, 1997). Recent reports suggest that a successful pathogenesis of some necrotrophic or hemi-biotrophic fungal pathogens relies on or is at least supported by a high concentration of hydrogen peroxide (von Tiedemann, 1997; Govrin & Levine, 2000; Kumar *et al.*, 2001).

Able (2003) observed two bursts of ROS production during the early stages of challenge in barley by the fungal necrotroph *Rhynchosporium secalis*, with the first burst occurring in both resistant and susceptible responses and the second burst being specific to the susceptible response. A similar situation has been observed during the response of susceptible pear cells to challenge with the necrotrophic bacteria *Erwinia amylovora* (Venisse *et al*., 2001), suggesting a role in signalling for the first oxidative burst.

Since *Alternaria* has a necrotrophic lifestyle, it could penetrate and spread more easily in a host with enhanced ability to perform hypersensitive response. In contrast to this, all less susceptible tomato lines have higher constitutive levels of the PR-proteins chitinase and β-1,3-glucanase, and constitutive enzymes can release elicitors of plant cell death from the cell walls of *Alternaria solani* (Agrios, 2005).

ASM was also tested in combination with the plant protective agent Maneb. Maneb concentrations were reduced to 20%, respectively only 4% of the recommended dosage, to check if this fungicide in such low concentrations could protect the plant during the build-up of SAR. ASM again showed no activity against early blight. Only treatments with the higher Maneb concentration significantly reduced *Alternaria* infection compared to the water control and the other variants. Still, these leaf discs were not fully protected and an infection of plants could only have been delayed and not held off. The combinations of ASM and Maneb did not show results that would hint synergistic interactions. Though, the experimental design was not sufficient to test for interactions as the agents were not tested in a sufficient amount of different concentrations.

However, in a study combining ASM with the cyanobacteria *Spirulina*, ASM alone in high concentration and ASM in low concentration plus an addition of *Spirulina* could increase the plant shoot weight significantly compared to the control.

Using *in vitro* studies, we demonstrated a significant direct fungitoxic activity of ASM on *Alternaria* isolates. In a second *in vitro* experiment, ASM and Maneb were tested in different concentrations and combinations. To enable correct

measurements of percentage control data, it was necessary to conduct this test *in vitro* with mycel discs of *Alternaria* growing on PDA mixed with the plant protectives. This means that the normal mode of action of ASM, which would induce mechanisms of SAR in a plant and have only indirect influence on the pathogen, could not take place. Still, treated plants are often covered in dry remainders of the spraying solutions and spores on the leaf surface would have direct contact with the components, which have not diffused into the plant. Here, ASM could unfold its fungitoxic activity and directly control the pathogen. For the protective fungicide Maneb, this contact is necessary to inhibit spore germination. This test proved interactions between the two agents that seem to depend highly on the individual isolate. For the isolates Cuba-141 and Greece-1, we could document clear synergistic effects. For isolate USA-1 however, the interactions between ASM and Maneb were only additive and for the combination of Maneb and ASM with the highest concentrations even antagonistic. These findings hint different levels of resistance of the *Alternaria* isolates against ASM and/or Maneb. Since isolate USA-1 showed significant growth depressions in the experiment with ASM alone, we assume that this isolate is relatively resistant against Maneb.

Kunz *et al*. (1997) state, that BTH derivates, with ASM as commercial product, do not show any antimicrobial activity *in vitro*. In the case of *Alternaria*, we could clearly demonstrate that this general assumption is not true. Since the activity of ASM depends on the individual isolates of *Alternaria*, it is likely that there are also isolates that would not be suppressed by an addition of ASM in the growth media.

It would be very interesting to rerun this study essay with leaf discs and later with plants to check for interactions with the normal modes of action of both plant protectives. To achieve general conclusions, the experimental design should include the following qualities: (i) correct measuring of the infected leaf areas for later percentage control calculation, (ii) more than two or three different concentrations, (iii) a high number of combinations, (iv) an adequate number of replications, and finally (v) the test of either many individual isolates or a mixture of them.

5.5 Resistance inducer INA

In a preliminary study to test useful concentrations and the formulation of INA, the phytotoxic effects on tomato plants were dramatic. Further experiments were not performed, as this resistance inducer could not be an alternative to ASM. Oostendorp *et al*. (2001) explained that none of the INA derivates were commercialised, mainly due to insufficient crop tolerance.

5.6 Growth promoting fungus *Piriformospora indica*

The tested tomato varieties differed in their reactions to colonisation with *Piriformospora indica*. In contrast to the other varieties, the Cuban cultivar Campbell 28 produced less dry matter when living *Piriformospora* mycel was added than with the addition of autoclaved mycel. The growth improvement in combination with the autoclaved mycel, in relation to the control, seemed to rely on the load with nutrients. These results show that no general growth induction for all cultivars of the host plants of *Piriformospora* can be expected.

For both other tomato varieties, *Piriformospora* showed a clear growth-inducing effect. But when plants grew older, the difference between colonized and non-colonized plants was more and more reduced until the plant size did not differ significantly at the time of the third harvest. It is likely that the beneficial effects of the fungal colonisation are no longer sufficient for plants reaching maturity, or that fungal growth is limited in the pots and so is the stimulus for the plants.

An *in vitro* experiment showed that preinoculation of the agar plates with *Piriformospora* inhibited the growth of *Alternaria*. Either, *Piriformospora* released some substances that inhibit the growth of *Alternaria*, or it had already diminished the essential nutrients in the media. These fungi do not share the same habitats and so do not come in contact with each other, so this finding may not have any practical use. On the other hand, when both fungi were transferred to the petri dish at the same time, they would both grow at usual speed and then stop before the mycel discs touch each other. This shows that there should not be some toxic

substances involved in the suppression of *Alternaria*. Varma *et al*. (2001) reported equal findings for *Gaeumannomyces graminis* and several other established root pathogenic fungi.

In the last study with *Piriformospora*, tomato plants were inoculated with *Alternaria*. For both Campbell 28 and Rheinlands Ruhm, the plants dry shoot weight was highest in the treatment with living *Piriformospora* mycel, whether the plants were affected with *Alternaria* or not. Cultivar Hellfrucht did not show any growth induction in the healthy plants. This findings are analogue to Kumar *et al.* (2002), who reported that colonisation of wheat roots by *Piriformospora* considerably increased growth and yield relative to non-colonised control plants up to approximately 35% and the number and size of *Bipolaris sorokiniana* leaf lesions were significantly reduced.

5.7 Influence of nutrient supply on *Alternaria* infection

It is well established that nitrogen availability can influence the resistance of plants to pathogens (Engelhard, 1989; Marschner, 1995). These effects may depend, among others, on the form of N nutrition to the host (Huber & Watson, 1974) or the type of pathogen, i.e. if the pathogen is biotroph or necrotroph (Büschbell & Hoffmann, 1992). Biotrophic diseases are often increased by high rates of nitrogen fertilizer (Marschner, 1995). The opposite situation exists with the senescent type pests, i.e. pests that attack the more mature plant tissues, such as *Alternaria* (MacKenzie, 1981). Since *Alternaria* is known to be primarily a pathogen of senescent tissue, any factor that delays maturity will also reduce the severity of the disease. Conversely, plants suffering from a lack of nitrogen are weaker, slower growing, and faster aging. Such plants, therefore, are susceptible to pathogens that are best able to attack weak, slow-growing plants (Agrios, 2005).

It has been commonly observed that early blight disease appears to be worse (i) on plants located in the less fertile parts of a field, (ii) on earlier transplanted plants, and (iii) on earlier maturing varieties (Thomas, 1948). MacKenzie (1981) could prove that increased rates of nitrogen fertilizer application reduced the

apparent infection rate and also the final amount of early blight disease in potato. In carrots, disease severity of leaf blight, caused by *Alternaria dauci*, was significantly decreased by high nitrogen supply (Vintal *et al.*, 1999). By providing additional applications of nitrogen fertilizer, the grower may forestall the senescence of the crop and indirectly control the pathogen. Thomas (1940) found that tomato seedlings grown in sand with a high nitrogen supply were consistently less susceptible to the collar rot type of stem canker caused by *Alternaria* and had smaller lesions than those plants raised in medium- or low-nitrogen solutions. Horsfall & Heuberger (1942) explained reduced infection of plants fertilized with sodium nitrate by an overvegetative condition of the plants that was accompanied by a poor fruit set. High nitrogen levels are known to prolong plant vigor and delay maturity, especially when other factors are limiting (Blachinski *et al.*, 1996).

It is commonly accepted that high nitrogen levels, together with low phosphorus and medium to high potassium levels, decrease host susceptibility to *Alternaria* (Barclay *et al.*, 1973; Soltanpour & Harrison, 1974; MacKenzie, 1981; Kumar *et al.*, 1983). In contrast to this, Thomas (1948) reported that plants grown at high levels of phosphorus, irrespective of nitrogen and potassium levels, had smaller leaf spots and a significantly smaller percentage of dead leaves than plants grown with low P fertilization. P seems to increase resistance either by improving the balance of nutrients in the plant or by accelerating the maturity of the crop and allowing it to escape infection by pathogens that prefer young tissues (Agrios, 2005). The improvement of the nutrient balance, as P is often more limiting nutrient than N, could probably explain the lower disease severity in the studies of Thomas (1948). Whereas an acceleration of senescence caused by higher P supply would rather increase an early blight infection.

In both performed studies concerning possible interactions between early blight and the supply with nitrogen or alternatively phosphorus, we could not find significant effects of the nutrients. This might be due to the small number of replications, resulting in relatively high data variability and inhibiting the demonstration of significant differences. As visible in the according figures, there was a tendency to lower disease severity if the plants were provided with the highest amount of nitrogen or respectively phosphate. A second factor could be

the general amount of nutrients in the used soil. Even in the zero treatments, this soil provided some nitrogen or phosphorus. It is likely that this general nutrient supply was too high to result in nutrient deficiency or measurable differences during the short growing phase of the experiments. And, we did not test very high levels of nutrient supply. These problems could be avoided by planning further studies with a higher number of replications, more extreme nutrient concentrations and by using a substrate low in nutrients, e. g. pure sand.

5.8 Arbuscular mycorrhizal fungi as bioprotectants

In the first experiment, mycorrhizal colonisation of tomatoes led to significantly lower infection-levels of *Alternaria solani* than in non-mycorrhizal tomato plants. Additional to the protective effect of AMF against stress (Jeffries *et al.*, 2003), bioprotection of AM colonised plants against various pathogens like nematodes and root diseases has been described by several authors (Boedker *et al.*, 1998; Cordier *et al.*, 1996; Dugassa *et al.*, 1996; Elsen *et al.*, 2001; Slezak *et al.*, 2000; Vaast *et al.*, 1998). Newsham *et al.* (1995) found that AM inoculation did not affect P concentration of the annual grass *Vulpia ciliata*, but mycorrhizal plants were protected from the deleterious effects of *Fusarium oxysporum* infection on shoot and root growth. Apparently, the AM suppressed pathogen development in the roots. Niemira *et al.* (1996) were able to demonstrate, even in a high-input commercial greenhouse and very low AM colonization and no evidence of enhanced P nutrition, a suppression of tuber dry rot (*Fusarium sambucinum*) in minitubers of potato (*Solanum tuberosum*) treated with peat-based medium containing *Glomus intraradices*. And furthermore Caron (1989) observed a reduction in *Fusarium* populations in the soil surrounding mycorrhizal tomato roots, and suggested that there was a potential role for AM fungi in biocontrol of soil-borne diseases.

Some studies have shown mycorrhizal protection of tomato plants against the root pathogens *Erwinia carotovora* and *Pseudomonas syringae* (García-Garrido & Ocampo, 1988; García-Garrido & Ocampo, 1989) but nothing is known with

respect to *Alternaria solani* and mycorrhizal tomatoes. As Azcón-Aguilar *et al.* (2002) explain, competition for carbon compounds may be a cause of pathogens depression in mycorrhizal plants as the growth of both symbiotic and pathogenic organisms depends on host photosynthates.

For leaf pathogens however, increased activity on mycorrhizal plants is well documented (Schönbeck & Dehne, 1979; Linderman, 1994; Dugassa *et al.*, 1996). Dehne (1982) suggests that the systemic influence of AMF may be attributed to (i) enhanced nutrition, (ii) plant growth, and (iii) physiological activity of mycorrhizal plants. Therefore, with increased levels of assimilates, such plants can serve better as nutrient sources for plant parasitic organisms. Shaul *et al.* (1999) provide an alternative mechanism by explaining the increased disease severity by the suppression of the plant defence response by AMF shortly after the early events of root colonization.

Tomatoes with AMF did not show a positive growth effect due to mycorrhization. In fact, the mycorrhizal tomatoes of the 0 P level in the first experiment were notably smaller than corresponding non-mycorrhizal plants. This growth depression was expected as it was described previously in other studies (Burleigh *et al.*, 2002) and seems to occur mainly under conditions of increased competition for carbon, e.g. light deficiency. Also Burleigh *et al.* (2002) reported growth depressions in tomato and medic colonized by *Gigaspora rosea* for 5 week-old plants. AM fungi are an additional sink for the carbon resources of the host and mycorrhizal plants require improved production of assimilates (Drüge & Schönbeck, 1992; Gernns *et al.*, 2001; Smith & Read, 1997). As carbon costs can be as high as 20% (Douds *et al.*, 2000; Graham, 2000; Jakobsen & Rosendahl, 1990), the competition for carbon between host and AM fungus can be very strong. On the other hand, Azcón-Aguilar *et al.* (2002) state that there is no relationship between the ability of AMF to protect the plant, and their ability to promote growth.

Alternaria fungi are saprophytes and facultative parasites with necrotrophic nature, which explains why they require a weakened, stressed, or senescent host plant for infection (Rotem, 1994). Rotem *et al.* (1990) showed for *Alternaria macrospora* that the disease severity of cotton plants grown in cool regime, causing delayed aging, was lower than for those grown under hot conditions (causing accelerated

aging), demonstrating that physiological rather than chronological age governs the age-conditioned susceptibility. Due to the competition for carbon, mycorrhizal tomatoes could be physiologically younger than non-mycorrhizal ones and therefore harder to infect by *Alternaria solani*. A delaying effect of AM on senescence has been observed in barley (Gernns *et al.*, 2001; West, 1995) and in roots of herbaceous plants (Gavito *et al.*, 2001; Lingua *et al.*, 2002). Induction of juvenility inhibits tissue necrosis or cell death and affects only indirectly the development or multiplication of pathogens, which means that development of necrotrophs, like *Alternaria* and *Botrytis*, is reduced in such tissues (Barna *et al.*, 2003).

Alternaria itself destructs the foliage of the host plant by defoliation and blightening. In addition to this reduction of green leaf area, the photosynthetic activity is decreased and the respiration is increased in apparently healthy tissue (Rotem, 1994). These alterations might be explained by the action of a phytotoxic material that diffuses into the leaf area surrounding necrotic lesions (Ephrath *et al.*, 1989) that affects the photosynthesis and reduces the yield to a higher proportion than defoliation alone would do (Rotem, 1990). Gernns *et al.* (2001) found that mycorrhizal barley without mildew showed an increased net assimilation of 34%, demonstrating again that AM fungi have a stimulatory effect on photosynthesis of their host plants (Drüge & Schönbeck, 1992; Smith & Read, 1997). It is likely that this increased photosynthetic activity of mycorrhizal plants could compensate the negative influence of *Alternaria* on photosynthesis and could help to reduce the disease severity.

The involvement of preactivation of plant defence responses by AMF in bioprotection is an apparent paradox because at the early stages of root colonization only a weak and transient defence response is induced (Azcón-Aguilar & Barea, 1996; Gianinazzi-Pearson, 1996) and this response is suppressed later on (Kapulnik *et al.*, 1996). By using a split root experimental system it has been shown that a decrease in the development of *Phytophthora* in mycorrhizal and non-mycorrhizal roots of mycorrhizal tomato plants was associated with accumulation of phenolics and plant cell defence responses (Cordier *et al.*, 1998), this being the first evidence of the induction of systemic

resistance by mycorrhiza formation. AMF are able to induce systemic protection against pathogens as parallel aspects to these described for rhizobacteria-mediated ISR have been found for mycorrhiza-induced defence response in plants (Pozo *et al.*, 2002).

Certain non-pathogenic microorganisms, such as some plant growth promoting rhizobacteria (PGPR) have been shown to evoke in plants a resistance response phenotypically similar to SAR induced by pathogens, this phenomenon has been called induced systemic resistance (ISR) (van Loon *et al.*, 1998). In contrast to SAR, which is mediated via a salicylic acid dependent process and is associated with the production of pathogenesis related proteins (reviewed by Hammerschmidt, 1999); ISR is generally mediated by a jasmonate/ethylene sensitive pathway and does not involve expression of PR proteins (Pieterse *et al.*, 1998; van Loon *et al.*, 1998). But, different rhizobacteria utilise different mechanisms for triggering systemic resistance: some trigger the SA-dependent pathway, others a JA/ethylene-dependent one, and additional pathways are likely to be discovered in future (Pieterse *et al.*, 2001). Silvia *et al.* (2004) could show that rhizobacteria induced systemic disease resistance in terms of average number of lesions as compared to the treatment control, thus increasing protection of tomato plants against *Alternaria solani*, *Stemphilium solani* (leaf spot) and *Oidium neolycopersici* (powdery mildew). In general, it seems jasmonic acid-dependent defence responses (ISR) are mainly effective against necrotrophic pathogens (Thomma *et al.*, 1998; 2001a; Tierens *et al.*, 2002), so we can assume to have triggered an induced resistance similar to ISR that reduced the susceptibility of the mycorrhizal tomato plants.

In the second experiment, the disease severity of all tomatoes increased together with the P supply and resulting in significantly higher early blight infection for plants with 45 P than in plants with 0 P. Plants of the 15 P level were intermediate. In the two-way linear mixed model analysis, the mycorrhiza treatment had no significant influence. Still, the plants of treatment 0 P + AMF had definite less necrotic lesions and lower chlorotic areas. The inoculation success seemed reduced in this experiment as the disease severity was notably lower than in all other experiments of this series. This remote *Alternaria* infection could attribute to

the missing significances in this study. It seems to be more difficult to inoculate tomato plants with *Alternaria* during late autumn and winter months, even if conditions in the glasshouse or climate chamber are constant.

An early mycorrhizal inoculation, previous to pathogen attack, has been shown to be a successful practice to increase disease tolerance/resistance in economically important crop species mainly for those involved in horticultural and fruit production systems (Jaizme-Vega *et al.*, 1997; Pinochet *et al.*, 1998). In the second experiment, the mycorrhizal colonization of the tomato roots was significantly reduced for the treatments 15 P + AMF and 45 P + AMF. The addition of P has therefore an indirect influence on the disease severity of the mycorrhizal plants, as the reduced mycorrhizal colonisation could not induce adequate resistance. Cordier *et al*. (1996) proved for tomato plants infected with *Phytophthora parasitica*, that only a well-established mycorrhizal colonization could protect plants. And bioprotection by *Glomus mosseae* against *Aphanomyces euteiches* was shown to depend on a fully established symbiosis with presence of arbuscules (Slezak *et al*., 2000).

We can conclude that the reduction of susceptibility to *Alternaria* in mycorrhizal tomato plants is likely to be caused by a mechanism similar to ISR. This effect is indirectly influenced by the supply with phosphate as this nutrient interferes with the development of root colonisation, so that a reduced colonisation leads to reduced resistance. To exploit optimal protection by AMF, the phosphate provision via fertilizers should be reduced, which is possible as the mycorrhiza will ensure a sufficient supply with this nutrient.

6 MYCORRHIZA REDUCES SUSCEPTIBILITY

Arbuscular mycorrhiza reduces susceptibility of tomato to *Alternaria solani**

6.1 Summary

- Mycorrhiza frequently leads to the control of root pathogens, but appears to have the opposite effect on leaf pathogens. Here we studied mycorrhizal effects on the development of early blight in tomato (*Lycopersicon esculentum*) caused by the necrotrophic fungus *Alternaria solani*.
- *Alternaria*-induced necrosis and chlorosis of all leaves was studied in mycorrhizal and non-mycorrhizal plants over time course and at different soil P levels.
- Mycorrhizal tomato plants had significantly less *A. solani* symptoms than non-mycorrhizal plants, but neither plant growth nor phosphate uptake was enhanced by mycorrhizas. An increased P supply had no effect on disease severity in non-mycorrhizal plants, but led to a higher disease severity in mycorrhizal plants. This was parallel to a P supply-induced reduction in mycorrhiza formation.
- The protective effect of mycorrhizas towards development of *A. solani* has some parallels to induced systemic resistance, mediated by rhizobacteria:

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both biocontrol agents are root-associated organisms and both are effective against necrotrophic pathogens. The possible mechanisms involved are discussed.

6.2 Introduction

Early blight of tomato, caused by the fungus *Alternaria solani*, is one of the common foliar diseases of tomatoes. This disease can occur over a wide range of climatic conditions, but is most severe in areas with high relative humidity caused by heavy dew or rainfall combined with high averaged temperatures. Tomato crops are damaged by damping-off of seedlings, collar-rot, destruction of foliage as well as direct destruction of fruits by fruit rot and sun-scald of fruits on defoliated plants (Rotem, 1994). *Alternaria* spp. fungi produce many non-host-specific as well as host-specific toxins (Thomma, 2003), which kill the host cells prior to or at the time of invasion and the fungus subsequently lives as saprophyte on the decaying tissue. In contrast to such necrotrophic life style, biotrophic fungi feed on living host cells and are very host specific pathogens. The toxin alternaric acid, produced by *A. solani*, causes chlorosis and necrosis itself and therefore plays a major role in early blight symptoms and defoliation (Pound & Stahmann, 1951).

Strategies for the control of *A. solani* include the activation of various forms of induced resistance. Systemic acquired resistance (SAR) is activated after infection by a necrotising pathogen or other biotic and abiotic stresses, rendering distant, uninfected plant parts resistant towards a broad spectrum of pathogens (Kuć, 1982; Durrant & Dong, 2004). Certain strains of plant growth promoting rhizobacteria (PGPR) are able to induce systemic resistance, which extends to the above-ground plant parts and is phenotypically similar to SAR. This second type of induced disease resistance is commonly referred to as rhizobacteria-mediated induced systemic resistance (ISR), as reviewed by Van Loon *et al.* (1998). Based on work with gene-knockout mutants in *Arabidopsis*, SAR and ISR are proposed to confer resistance to pathogens according to their lifestyles, so that SAR primarily functions against biotrophic pathogens, and ISR against necrotrophic pathogens (Thomma *et al.*, 1998; 2001). Parallel with the induction of resistance, significant

changes in the plant occur: inoculation with root-promoting *Pseudomonas* results in a significant increase of phenylpropanoid content in sprouts (Leinhos & Bergmann, 1995) and root exudates (Azcón-Aguilar & Barea, 1995). ISR can be induced not only by the rhizobacteria themselves, but also by bacteria-synthesized macromolecules (Romeiro *et al.*, 2005).

Root colonisation by arbuscular mycorrhizal fungi (AMF) has been frequently reported to reduce root infection by various soil-borne pathogens (Azcón-Aguilar & Barea, 1996; Smith & Read, 1997). The mechanisms involved in this biocontrol are not clear, but the common increase in plant P status in response to mycorrhiza formation appears to be involved (Graham & Menge, 1982; Graham & Egel, 1988; Graham, 2001). In contrast, mycorrhiza is generally assumed to increase susceptibility to leaf pathogens such as fungi, viruses and aphids (Dehne, 1982; Gange & West, 1994; Lindermann, 1994; Dugassa *et al.*, 1996).

The objective of this work was to investigate effects of mycorrhiza on susceptibility to *A. solani* in tomato. Disease symptoms were studied over time in mycorrhizal and non-mycorrhizal plants and the possible role of mycorrhiza-induced changes in plant P status was investigated by supplying different amounts of P to both non-mycorrhizal and mycorrhizal plants.

6.3 Materials and methods

6.3.1 Experimental design

Two experiments were carried out: Experiment 1 had four treatments: 0 P, 25 P, 75 P, and 0 P + AMF (numbers refer to mg P added kg^{-1} soil). Experiment 2 had eight treatments resulting from combining four P levels with the presence or absence of mycorrhizas: 0 P, 0 P + AMF, 3 P, 3 P + AMF, 6 P, 6 P + AMF, 24 P, and 24 P + AMF. The experiments were conducted in a completely randomised design and repeated once. Generally, all treatments had 4 replicates as indicated by Figures and Tables. For experiment 2, seven plants per treatment were grown and three of these were harvested before inoculation with *Alternaria solani*,

reducing the replications to 3 for these data. Later data of this experiment were obtained from the 4 remaining plants.

6.3.2 Biological materials

Lycopersicon esculentum cv. Frembgens Rheinlands Ruhm was grown in both experiments. Seeds were surface sterilized (95% EtOH, soaking in 4% NaOCl (v : v) for 10 min and a final rinse in dist. H_2O) and pre-germinated on wet filter paper for 2 days. Mycorrhiza was established in both experiments by inoculation with *Glomus intraradices* (BEG 87), which had been propagated on *Trifolium subterraneum*. The dry inoculum consisted of soil, hyphae, spores and colonised root pieces. The *Alternaria solani* isolate Greece-1 was kindly provided by Simon Pérez Martinez, CENSA, Cuba, and was cultured on potato dextrose agar at 25 °C. To harvest the spores, 10-day-old cultures were brushed gently to loosen the spores and then rinsed with a 0,01% Tween 20 solution. The resulting spore suspension was filtered through a fine cloth, quantified using a haemocytometer and adjusted to 10^4 spores ml^{-1}.

6.3.3 Experimental setup

Plants were grown in square pots (8 cm side length) lined with plastic bags and filled with 400 g growth medium, which was a 1 : 1 (w : w) mixture of quarts sand and irradiated soil (10 kGy, 10 MeV electron beam). The growth medium, hereafter referred to as soil, had nutrients uniformly incorporated at the following concentrations (mg kg^{-1} dry soil): K_2SO_4 75.0; $CaCl_2 \cdot 2H_2O$ 75.0; $CuSO_4 \cdot 5H_2O$ 2.1; $ZnSO_4 \cdot 7H_2O$ 5.4; $MnSO_4 \cdot H_2O$ 10.5; $CoSO_4 \cdot 7H_2O$ 0.39; $MgSO_4 \cdot 7H_2O$ 45.0; $Na_2MoO_4 \cdot 2H_2O$ 0.18; NH_4NO_3 30.0 (Viereck *et al.*, 2004). The soil had a bicarbonate-extractable P content of 9.8 μg P g^{-1} (Olsen et al., 1954). The various P levels in the two experiments were established by thorough mixing of KH_2PO_4 into the soil. Mycorrhizal treatments (+ AMF) had 32 g of the soil replaced by *G. intraradices* inoculum. The soil was moistened to 60% of water holding capacity and pots were left for one week for incubation of AMF inoculum. Three pregerminated seeds were planted in each pot and were thinned to one plant per pot after establishment. The soil surface was covered with plastic beads. Plants were grown in a climate chamber with a 16 : 8 h light : dark cycle with 24-26 : 18-20 °C temperatures respectively, and watered daily to 60% water holding capacity.

Plants were inoculated 35 days after sowing by spraying with spore suspension until run-off occurred. To ensure high relative humidity that is needed during spore germination, the plants were placed in tight closing clear plastic containers for 24 to 48 h.

6.3.4 Monitoring of disease severity, harvest and analyses

Early blight disease severity was assessed daily or every second day in experiment 1 and experiment 2, respectively, by assigning a grade in the range 1-12 reflecting the percentage of leaf area with necrotic spots or respectively chlorosis of all unfolded leaves. Grade 12 was assigned to dead and shed leaves. For each time point, arithmetic means for individual plants were calculated. Tomato shoots were harvested 5 or 8 days after infection (dai) with *A. solani* in experiment 1 and 2 respectively, and weighed after drying at 70 ^{o}C for two days. Ground shoot material was digested in a 4 : 1 mixture (v : v) of nitric and perchloric acids. Total P content was measured by the molybdate blue method (Murphy & Riley, 1962) on a Technicon Autoanalyser II (Technicon Autoanalysers, Analytical Instruments Recycle, Inc., Golden, CO, USA). Root systems were washed and root samples of mycorrhizal tomatoes were cleared with 10% KOH and stained with 0.05% trypan blue in lactoglycerol (Phillips & Hayman, 1970) with the omission of phenol from the solutions and HCl from the rinse. Percentage of AMF colonisation was determined using a gridline intersection method (Giovanetti & Mosse, 1980).

6.3.5 Statistical analysis

Data for dry weight, phosphate content and mycorrhizal colonisation were analysed using GLM and Tukey's Test. Necrosis and chlorosis data which were surveyed over several days in all experiments, were analysed with a linear mixed model for repeated measurements, using the autoregressive covariance structure which fitted best with respect to the experimental structure and minimized values for Akaike's Information Criterion (AIC) and other information criteria. This statistical approach compares the treatments during the whole period of data collection and not only at certain time points by estimation of new means which are representing the whole time course and are therefore slightly different to the measured values. Degrees of freedom were estimated according to

Satterthwaite's formula (Satterthwaite, 1946 after Hocking, 1996). Levels of P addition and inoculation with *G. intraradices* were set as fixed factors, whereas the intervals between the assessments were used as covariates. Treatments were compared using LSD Test with Bonferroni correction. All calculations were performed using SPSS (SPSS for Windows, Rel. 12.0.1, 2003. Chicago, SPSS Inc.).

6.4 Results

In experiment 1, the *Alternaria solani* development was determined (i) on non-mycorrhizal tomatoes with increasing P supplies and (ii) on mycorrhizal and non-mycorrhizal tomatoes given no additional P. Disease severity in terms of necrosis ($P = 0.005$) and chlorosis ($P = 0.000$) was significantly reduced by mycorrhiza. Whereas the effect of increased P supply to non-mycorrhizal plants was not significant ($P = 0.208$ and $P = 0.089$ for necrosis and chlorosis, respectively) (Fig. 1; Table 1). Necrosis and chlorosis increased significantly (both $P = 0.000$) over time in all treatments. Interactions could not be tested due to the incomplete experimental design.

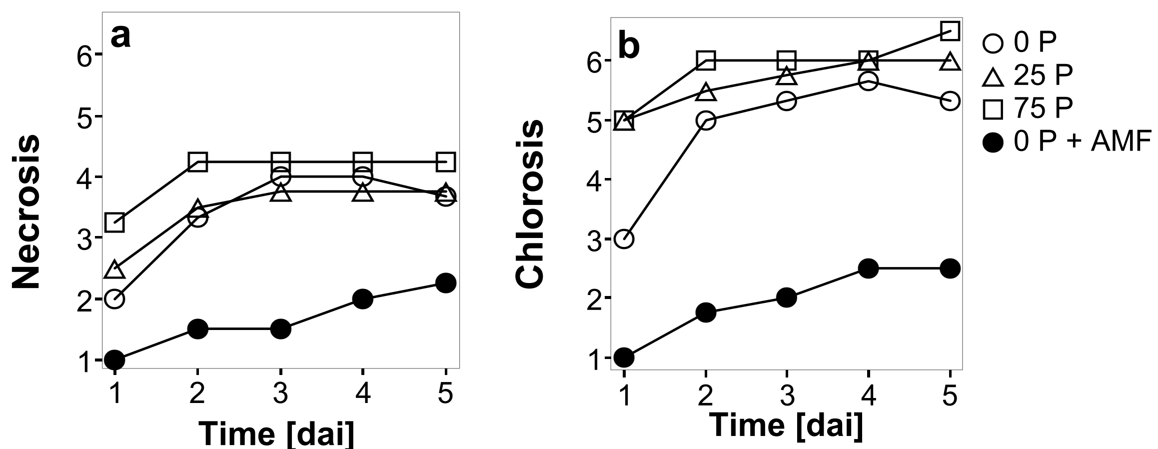

Figure 1: Necrosis (a) and chlorosis (b) caused by *Alternaria solani* infection monitored over 5 days after inoculation (dai) of tomato leaves. 0, 25 and 75 P represent non-mycorrhizal plants with different phosphate (P) supply (mg kg^{-1} soil) and 0P + AMF represent mycorrhizal plants. Data points show arithmetic means with $n = 4$. Experiment 1.

The increased degree of necrosis in response to P addition was not significant. For the range of concentrations used in this study, phosphate nutrition had no

influence on severity of early blight. The more pronounced effect on disease development arose from the addition of *Glomus intraradices* to the 0 P treatment (0 P + AMF) such that mycorrhizal tomato plants had significantly less *Alternaria* symptoms in terms of necrosis and chlorosis than non-mycorrhizal plants (Fig. 1, Table 1).

Table 1: Necrosis and chlorosis ranking of mycorrhizal (+ AMF) and non-mycorrhizal tomato plants estimated over a time period of five days after infection with *Alternaria solani*.

Treatment	Necrosis	Chlorosis
0 P	3.16 b	4.55 b
25 P	3.31 b	5.58 b
75 P	3.92 b	5.83 b
0 P + AMF	1.64 a	1.86 a

Estimated means were calculated in a mixed model with repeated measures data. The standard errors of necrosis means are $SE = 0.34$ for 0 P and $SE = 0.29$ for all other treatments. For chlorosis data, the standard errors are $SE = 0.41$ and $SE = 0.36$, respectively. Degrees of freedom are $df = 11.89$ for necrosis data and $df = 11.81$ for chlorosis. Means followed by equal letters are not significantly different. Experiment 1.

In order to test whether the reduced *Alternaria* symptoms in the mycorrhizal plants in the first experiment were caused by an improved phosphate-level, a second experiment was set up where mycorrhiza was combined with all phosphate levels in the range between 0 and 24 mg kg^{-1}, resulting in a complete factorial design. Phosphate concentrations in shoots were analysed to study whether disease severity was somehow correlated to any change in leaf P status caused by AMF inoculation or P supply.

A two-way statistical analysis showed that disease severity was significantly influenced by the addition or absence of AMF ($P = 0.000$ for both necrosis and chlorosis). Mycorrhizal plants had significantly less disease symptoms (Fig. 2a and Table 2). Necrotic symptoms did not differ at various levels of P supply ($P = 0.253$) but chlorosis of leaves was significantly influenced by the amount of additional P ($P = 0.014$).

Table 2: Necrosis and chlorosis ranking of mycorrhizal (+ AMF) and non-mycorrhizal tomato plants estimated over a time period of third to seventh day after infection with *Alternaria solani*.

Treatment	Necrosis	Chlorosis
0 P	4.42 d	4.50 b
0 P + AMF	1.35 ab	1.63 a
3 P	3.31 cd	3.75 b
3 P + AMF	1.15 a	1.50 a
6 P	3.48 cd	4.38 b
6 P + AMF	1.29 ab	2.00 a
24 P	3.08 bcd	3.88 b
24 P + AMF	2.50 abc	3.75 b

Estimated means were calculated in a mixed model with repeated measures data. The standard error of necrosis means is $SE = 0.37$ and the degrees of freedom are $df = 25.79$ for all treatments, whereas the values are $SE = 0.33$ and $df = 24.00$ for chlorosis data. Means followed by equal letters are not significantly different. Experiment 2.

Also, there were significant interactions between mycorrhiza and the level of P supply, with $P = 0.019$ and $P = 0.002$ for necrosis and chlorosis, respectively. These interactions were very obvious as an increase in P supply caused more early blight symptoms in mycorrhizal plants. At the 24 P supply level, mycorrhizal and non-mycorrhizal tomatoes did not differ significantly (Table 2). Seven days after inoculation with *A. solani*, the remaining non-mycorrhizal tomato plants had lost between 13 and 22% of their dry matter, as many chlorotic leaves were shedded. Tomatoes with mycorrhiza suffered much less, thus resulting in higher amounts of dry weight after infection with early blight, though this difference was only significant at the 0 P level due to high variability of the data (Fig. 2b).

The dry weight of mycorrhizal and non-mycorrhizal tomatoes was quantified before and after inoculation (Fig. 2c and d). Before inoculation with *A. solani*, dry matter of plants was slightly higher the more phosphate was added, and non-mycorrhizal tomatoes were generally larger than mycorrhizal ones even if these differences were rarely significant (see Fig. 2c). The P-content in mg g^{-1} dry weight in shoots before infection was nearly at the same level for all treatments (Fig. 2e), only the treatments 0 P + AMF and 3 P contained significantly lower phosphate than plants of treatment 24 P. As expected, mycorrhizal colonisation was significantly reduced the more phosphate was added (Fig. 2f), this reduction could explain the described interactions.

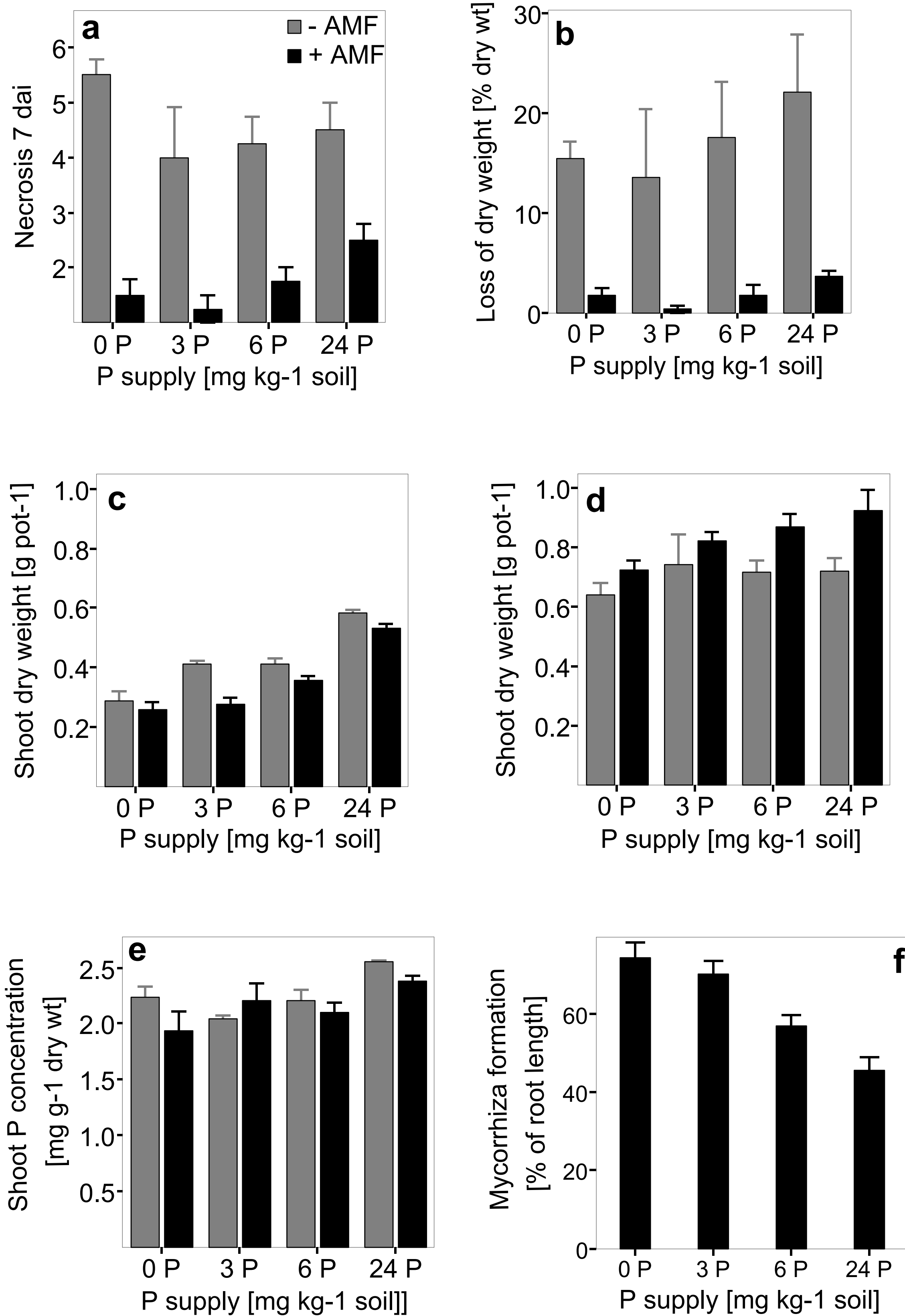

Figure 2: Leaf necrosis (a), necrosis-induced dry wt loss (b), shoot dry wt (c, d), shoot P concentration (e), and mycorrhiza formation (f) in mycorrhizal (+ AMF) and non-mycorrhizal (- AMF) tomato plants grown at 4 levels of phosphate (P) supply. Shoot dry weights in (c) were recorded just before inoculation with *Alternaria solani* on different plants while

all other data were recorded 7 days after inoculation (dai). Columns represent arithmetic means and bars are standard errors (*n* = 3 for (c) and otherwise 4). Experiment 2.

6.5 Discussion

The presence of mycorrhiza in tomato roots led to significantly lower infection-levels of *Alternaria solani* than observed in non-mycorrhizal plants in two separate experiments. Bioprotection of AM colonised plants against soil-borne pests like nematodes and various root diseases is commonly observed (Cordier *et al.*, 1996; Dugassa *et al.*, 1996; Boedker *et al.*, 1998; Vaast *et al.*, 1998; Slezak *et al.*, 2000; Elsen *et al.*, 2001). Some studies have shown mycorrhizal protection of tomato plants against the root pathogens *Erwinia carotovora* and *Pseudomonas syringae* (García-Garrido & Ocampo, 1988; 1989). In contrast, susceptibility to leaf pathogens is often higher in mycorrhizal than in non-mycorrhizal plants (Schönbeck & Dehne, 1979; Lindermann, 1994; Dugassa *et al.*, 1996). Dehne (1982) suggests that the systemic influence of AMF may be attributed to enhanced nutrition, plant growth and physiological activity of mycorrhizal plants, and therefore, with increased levels of assimilates, such plants can serve as improved nutrient sources for plant parasitic organisms. Shaul *et al.* (1999) provide an alternative mechanism by explaining the increased disease severity by the suppression of the plant defence response by AMF shortly after the early events of root colonisation. However, mycorrhiza may induce qualitative or quantitative changes in plant performance that could compensate higher disease susceptibility. Hence, although mycorrhiza formation in barley resulted in increased leaf infection by *Blumeria graminis* f. sp. *hordei*, mycorrhizal plants suffered less than non-mycorrhizal plants in terms of reductions in grain number, ear yield and thousand-grain weight (Gernns *et al*., 2001).

Alternaria fungi are saprophytes and facultative parasites with necrotrophic nature that may explain why they require a weakened, stressed or senescent host plant for infection (Rotem, 1994). Rotem *et al.* (1990) showed for *Alternaria macrospora* that the disease severity of cotton plants grown in cool regime, causing delayed aging, was lower than for those grown under hot conditions (causing accelerated aging). This demonstrates that physiological rather than chronological age governs the age-conditioned susceptibility. AM fungi constitute a sink for the carbon

resources of the host and mycorrhizal plants require improved production of assimilates (Drüge & Schönbeck, 1992; Smith & Read, 1997; Gernns *et al.*, 2001). As carbon costs can be as high as 20% (Jakobsen & Rosendahl, 1990; Douds *et al.*, 2000; Graham, 2000), competition for carbon between hosts and AM fungus can be strong. This competition can result in a growth reduction of mycorrhizal plants compared to non-mycorrhizal ones, especially under light deficiency or other photosynthesis-limiting condition. An age delaying effect of AMF on senescence has been observed in barley (Gernns *et al.*, 2001; West, 1995) and in roots of herbaceous plants (Gavito *et al.*, 2001; Lingua *et al.*, 2002). In our experiments, the tomatoes with AMF did not show a positive growth effect due to mycorrhization but actually a slight growth depression in terms of fresh and dry matter before inoculation with early blight. This was expected, based on previous studies in our laboratory (Burleigh *et al.*, 2002; Smith *et al.*, 2004). The mycorrhizal tomato plants could have been physiologically younger than non-mycorrhizal ones and therefore probably harder to infect by *Alternaria solani*. Alternatively, the competition for carbon compounds could be a cause of pathogen's depression in mycorrhizal plants as the growth of both symbiotic and pathogenic organisms depends on host photosynthates (Azcón-Aguilar *et al.*, 2002).

The nutritional status can be an important factor influencing the disease susceptibility of plants. Our experiments demonstrated that additional P did not increase early blight symptoms, only reduced formation of mycorrhiza. This reduced mycorrhiza formation also reduced the mycorrhiza-induced resistance effects for plants of the treatment 24 P + AMF of experiment 2. The phosphate concentration of mycorrhizal tomato was not increased by *Glomus intraradices*. Smith *et al.* (2004) could show that nearly 100% of the P uptake of mycorrhizal tomatoes happened via AMF and not directly through the roots as the direct P uptake pathway seemed to be inactivated. The nutritional status of the mycorrhizal tomatoes was not improved because the indirect P uptake via AM fungus did not occur in addition to but instead of the direct uptake. Thus, the increase in resistance in the treatments with AMF cannot be explained by a better P supply. Other nutrients as nitrogen, potassium or zinc were not measured during the studies, as only surplus supplies of N are known to reduce early blight symptoms (Thomas, 1948; Vintal *et al.*, 1999).

An early mycorrhizal inoculation, previous to pathogen attack, has been shown to be a successful practice to increase disease tolerance/resistance in economically important crop species mainly for those involved in horticultural and fruit production systems (Jaizme-Vega *et al.*, 1997; Pinochet *et al.*, 1998). In tomato plants infected with *Phytophthora parasitica*, only a well-established mycorrhizal colonization could protect plants (Cordier *et al.*, 1996) and bioprotection by *Glomus mosseae* against *Aphanomyces euteiches* was shown to depend on a fully established symbiosis with presence of arbuscules (Slezak *et al.*, 2000). In our first experiment mycorrhizal colonisation was as high as 79% with no difference due to phosphate levels. Percentage of mycorrhizal infection in our second study did depend significantly on the P supply but still reached around 50% in the treatment with maximum P addition (see Fig. 2), showing that the colonisation with AMF was well developed and sufficient for bioprotection of the plants. The decrease of root colonisation due to additional P could be an explanation for the lack of resistance induction in the treatment with high P supply.

Different organisms can stimulate plants and activate either pathogen-induced SAR or rhizobacteria-mediated ISR. Parallel to ISR, mycorrhizal fungi interact with the host plant's roots and influence the whole plant including the above-ground parts. ISR is effective against necrotrophic pathogens and it seems possible that similar mechanisms reduce susceptibility of mycorrhizal plants towards necrotrophic leaf pathogens like *Alternaria solani*.

We can conclude that mycorrhization of tomato roots can induce a reduced susceptibility to the necrotrophic leaf pathogen *Alternaria solani*. This effect is indirectly influenced by the supply with phosphate that reduced mycorrhizal development and led to reduced resistance. The effect resembles the rhizobacteria-mediated induced systemic resistance that is also induced by associated organisms and is also effective against necrotrophic pathogens.

6.6 Acknowledgements

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6.7 References

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7 SUMMARY

The objective of this work was to study the pathosystem tomato – *Alternaria solani* with regard to several new plant protection strategies. Agents and symbionts were tested alone and in combinations to find environmentally friendly but effective methods of early blight control. The included agents and symbionts were: (i) systemic acquired resistance, induced by ASM; (ii) plant restorative *Spirulina platensis*; (iii) growth-promoting fungus *Piriformospora indica*; (iv) symbiotic arbuscular mycorrhizal fungi; and (v) minimum quantities of the chemical fungicide Maneb.

The protective activity against *Alternaria* was assessed either on whole plants or on leaf discs. Whenever root symbionts were included, experiments with whole plants were necessary, otherwise leaf discs were preferred, especially since this allowed to simultaneously examine the influence of leaf age on susceptibility. Interaction between ASM and Maneb, as well as between *Piriformospora* and *Alternaria*, were studied *in vitro* on artificial medium.

The main results of the work are:

- A new method to induce sporulation of *Alternaria* on axenic medium has been developed, which is easily integrated into the normal process of cultivation. The new technique yields high amounts of spores and requires no more than a switch from the normally used PDA medium to the nutrient-poor DA medium. Ten days before spore harvest, one has to change back to PDA.

The age of host leaves emerged as one important parameter determining the degree of susceptibility to *Alternaria*. Old leaves exhibit higher levels of disease severity than young leaves.

The application of *Alternaria* toxins causes only minor disease symptoms. This ensures that observed disease severity after inoculation with spore suspension is not generated solely by the contained toxins.

- *Spirulina platensis*, which is suspected to have plant restorative potential, has no diminishing activity on early blight disease. To the contrary, if sprayed, *Spirulina* has even an enhancing effect. Soil drenching *Spirulina* shows a less pronounced degree of infection. In tomato cultures, therefore, *Spirulina platensis* should not be used as plant restorative.

- ASM has a distinct fungitoxic efficacy with respect to *Alternaria*. *In vitro* experiments showed that – if in direct contact with the fungus – ASM unfolds its toxicity and inhibits fungal growth.

  Application of the resistance inducer ASM via soil drench increases the susceptibility to early blight infection. This clear counter-protective effect might be due to strong phytotoxicity of root-absorbed ASM.

  Application of ASM via spraying reduced the susceptibility to early blight in some instances but increased it in others. This inconsistency may be explained by the duality of ASM toxicity, which harms on one hand the fungus (fungitoxicity) and on the other the host (phytotoxicity). In sprayed form both effects can occur in parallel with unpredictable outcome. A successfully established SAR, which is assumed to benefit necrotrophic pathogens as *Alternaria* may be a third contributing factor.

- It turned out to be impractical to pre-protect tomato plants by small quantities of Maneb until SAR is established. A satisfactory protection can only be achieved with amounts of Maneb close to the recommended dose.

  The mode of interaction between ASM and Maneb depends substantially on the particular *Alternaria* isolate. Different isolates seem to have different levels of resistance against the two agents, resulting in synergistic, additive or antagonistic modes of interaction.

- *Piriformospora indica* has a measurable growth-promoting effect on tomato plants, which levels out approximately 50 days after germination. The strength of growth promotion is different for different cultivars.

  Under certain circumstances, *Piriformospora* has a suppressing effect on *Alternaria* growth. *In vitro* experiments showed that *Piriformospora* cultures inhibit growth of *Alternaria* if the pathogen is added one week later.

  Piriformospora seems to have – in addition to its growth-enhancing effect – protective potential against *Alternaria* infections. The mode of action of this ability to reduce the plant matter losses is yet unclear.

- Nitrogen and phosphorus deficient growth conditions weaken the host plants. This tends to decrease the defence capability against early blight, a characteristic feature of interactions between hosts and necrotrophic pathogens.

- Arbuscular mycorrhizal fungi are an effective bioprotectant against *Alternaria* in tomato. It has been demonstrated in this work that mycorrhizal tomatoes have a significantly lower level of disease severity than non-mycorrhizal plants. The mechanism seems to be similar to ISR, which is also induced by associated organisms in the roots and effective against necrotrophic pathogens.

  It is well-known that P surplus hinders an optimal colonisation of host roots by AMF. Therefore, a restricted P supply is advantageous if the mycorrhizal protection method is exploited against *Alternaria* infection. Since mycorrhiza itself ensures a sufficient P availability for the host plant, no P deficiency will occur.

8 DEUTSCHE KURZFASSUNG

8.1 Einleitung

Zielsetzung der vorliegenden Arbeit war die Untersuchung des Pathosystems Tomate – *Alternaria solani* sowie die Entwicklung einer Pflanzenschutzstrategie, die systemische induzierte Resistenz, Pflanzenstärkungsmittel, symbiotische Pilze und, falls notwendig, minimale Mengen chemischer Fungizide kombiniert, um die Dürrfleckenkrankheit langfristig und auf umweltfreundliche Weise zu kontrollieren.

8.2 Das Pathosystem Tomate - *Alternaria solani*

Tomaten (*Solanum lycopersicum* L., Syn. *Lycopersicon esculentum* Mill.) gehören zu den wichtigsten Fruchtgemüsen für die menschliche Ernährung und werden auf praktisch allen Kontinenten angebaut.

Alternaria solani (im Folgenden *Alternaria*) ist der Erreger der Dürrfleckenkrankheit und befällt außer Tomaten noch weitere Mitglieder der *Solanaceen* sowie Pflanzen anderer Familien. An Tomaten verursacht *Alternaria* Stängelgrundfäule, Blattflecken, Stängelläsionen und Fruchtfäule. Tomatensämlinge können durch befallenes Saatgut oder Substrat absterben. Typische Symptome der Dürrfleckenkrankheit sind dunkle Flecken mit konzentrischen Sporenringen, die von aufgehelltem Blattgewebe umgeben sind. Der Ertrag wird sowohl indirekt durch die Zerstörung des Laubes als auch direkt durch befallene und fleckige Früchte (nach zu hoher Sonneneinstrahlung) verringert, so dass komplette Ertragsausfälle möglich sind (Rotem, 1994).

8.3 Induzierte Resistenz

Die Nutzung induzierter Resistenz könnte eine Alternative zum chemischen Pflanzenschutz bieten. Dabei wird zwischen zwei Arten induzierter Resistenz unterschieden: (a) die systemisch induzierte Resistenz oder SAR, und (b) die von Rhizobakterien induzierte systemische Resistenz, kurz ISR.

SAR wird in Pflanzen durch eine Vielzahl von Auslösern aktiviert, darunter nekrotisierende Pathogene, biotischer oder abiotischer Stress, sowie chemische Induktoren wie Salze, ungesättigte Fettsäuren und subletale Mengen an Herbiziden (Kuć, 1982; Oostendorp *et al.*, 2001). Dabei wird die Pflanze bildlich gesprochen in einen Zustand erhöhter Alarmbereitschaft versetzt, so dass sie schneller und effektiver auf angreifende Pathogene reagieren kann. Diese Abwehr besteht in den meisten Fällen aus Papillenbildung, um das Eindringen von Pathogenen in einzelne Zellen zu verhindern, und aus der beschleunigt ablaufenden hypersensitiven Reaktion (HR), bei der die angegriffene Zelle unter Produktion verschiedener Sauerstoffradikalen spontan abstirbt.

Salizylsäure, ein Hauptbestandteil des SAR-Signalweges, und deren Derivate ASM (Acibenzolar-S-methyl, Wirkstoff des kommerziell genutzten Resistenzinduktors Bion®) und INA (2,6-Dichlorisonikotinsäure) können sowohl über die Blätter als auch über die Wurzeln von Pflanzen aufgenommen werden und SAR auslösen. Dabei wird ein über mehrere Wochen andauernder Schutz gegenüber Virosen, Bakterien, Pilzen sowie einigen tierischen Schaderregern erreicht.

Im Gegensatz zu SAR entsteht ISR in Erwiderung auf die Besiedlung der Pflanzenwurzeln durch bestimmte Kulturen von wachstumsfördernden Rhizobakterien. In den meisten Fällen verläuft das Auslösen der systemischen Resistenz über einen Jasmonat/Ethylen-empfindlichen Signalweg (van Loon *et al*, 1998; Pieterse *et al.*, 2001). Es wird weitgehend angenommen, dass SAR hauptsächlich gegen biotrophe Pathogene schützt, während ISR wirksam gegen nekrotrophe Schaderreger ist (Thomma *et al.*, 1998; 2001; Tierens *et al.*, 2002).

8.4 Symbiotische Pilze

Symbiotische Pilze, wie arbuskuläre Mykorrhizapilze (AMF) und *Piriformospora indica*, besiedeln die Rhizosphäre von Pflanzenwurzeln und können dramatische Auswirkungen auf den Pflanzenstatus und das -wachstum ihres Wirtes haben.

Arbuskuläre Mykorrhizapilze der Ordnung *Glomales* bilden mit terrestrischen Pflanzen die sogenannte Mykorrhiza. Diese Symbiose hat für die Wirtspflanze den Vorteil, dass ihre Versorgung mit dem meist wachstumslimitierenden Makronährstoff Phosphat verbessert wird. Aber auch andere Nährstoffe wie beispielsweise Zink werden vom Pilz verfügbar gemacht und zur Pflanze transportiert, und auch die Wasseraufnahme wird erhöht. Zusätzlich reagieren viele Pflanzen mit einer Wachstumssteigerung auf die Bildung der Mykorrhiza und ihre Toleranz gegenüber abiotischem Stress, wie Versalzung oder Schwermetallbelastung des Bodens, wird erhöht. Die Bildung von Mykorrhiza schützt die Pflanzen auch gegen verschiedene Wurzelkrankheiten und Nematoden, während die Wirkung auf Blattkrankheiten nicht eindeutig ist. Die AM Pilze hingegen sind vollständig auf die Versorgung mit Kohlenhydraten durch die Wirtspflanzen angewiesen, damit sie ihren Lebenszyklus vollenden und Sporen bilden können.

Piriformospora indica (im Folgenden *Piriformospora*) wurde erst kürzlich aus westindischem Wüstenboden isoliert. Der Pilz besiedelt eine weites Wirtsspektrum aus vielen Pflanzenfamilien und fördert das Pflanzenwachstum, unter anderem durch die Mobilisierung von Phosphaten und deren Translokation. Im Gegensatz zu AMF kolonisiert *Piriformospora* auch *Brassicaceen* wie beispielsweise *Arabidopsis thaliana* und lässt sich auf künstlichem Nährboden vermehren.

8.5 Methoden

Zur Überprüfung der einzelnen Pflanzenschutz-Methoden wurden verschiedene Experimente verwendet: (a) Versuche mit ganzen Pflanzen, beispielsweise bei Studien mit den symbiotischen Pilzen, (b) Versuche mit Blattscheiben, die immer dann eingesetzt wurden, wenn auch das Blattalter eine Auswirkung auf die Wirksamkeit haben könnte, und (c) *in vitro* Versuche, mit denen Wechselwirkungen zwischen ASM und Maneb sowie direkte Interaktionen zwischen *Alternaria* und *Piriformospora* untersucht wurden. Die Ausprägung der

Alternaria Infektion wurde jeweils mit angepassten Boniturschemen geschätzt und die Daten generell mittels ANOVA und nachfolgendem Tukey Test ausgewertet. Bei den Experimenten bezüglich der Versorgung mit P und N sowie den Versuchen mit AMF wurde der Krankheitsverlauf über einen Zeitraum von mehreren Tagen bonitiert. Diese Daten wurden mit einem Ansatz für lineare gemischte Modelle analysiert, da dabei Mittelwerte über die gesamte Zeitspanne errechnet und diese dann in nachfolgenden post hoc Tests verglichen werden.

8.7 Zusammenfassung der Ergebnisse

- Wir konnten eine neue, zeitsparende Methode entwickeln, mit der die Sporulation von verschiedenen *Alternaria*-Isolaten auf künstlichem Nährboden angeregt wird. Diese Methode lässt sich problemlos in den normalen Kultivationszyklus integrieren, da nur zwei Wechsel des Nährbodens erforderlich sind. Zuerst werden die Isolate von normalem PDA-Medium auf nährstoffärmeres DA-Medium transferiert, auf dem sie nur langsam wachsen. Zehn Tage bevor Sporen benötigt werden, setzt man die Isolate wieder zurück auf PDA, dieser Wechsel im Nährstoffangebot stimuliert dann reichliche Sporenproduktion.

- Wie erwartet konnten auch wir bestätigen, dass das Auftreten der Dürrfleckenkrankheit deutlich vom Blattalter der Pflanzen beeinflusst wird. Dabei zeigen die ältesten Blätter die signifikant höchste Anfälligkeit, und die jüngsten Blätter ausgeprägte Resistenz gegenüber *Alternaria*.

- Es war geplant, die Pflanzen bzw. Blattscheiben mit einer Sporensuspension zu inokulieren. Im Hinblick darauf wurde untersucht, ob nur die in der Sporenlösung enthaltenden Toxine, ohne die Anwesenheit von *Alternaria*-Sporen, an den Pflanzen die Dürrfleckenkrankheit auslösen können. Dabei wurden nur marginale Krankheitssymptome beobachtet. Dieses Ergebnis gewährleistet, dass die bonitierte *Alternaria*-Infektion nach Inokulation mit einer Sporensuspension nicht durch die Toxine allein verursacht wird. Die Methode der Inokulation mittels Sporensuspension konnte daher beibehalten werden.

- Das Cyanobakterium *Spirulina platensis*, für das eine potentielle Wirkung als Pflanzenstärkungsmittel angenommen wird, hat keinen reduzierenden Effekt

auf die *Alternaria*-Infektion. Bei Applikation als Spray hingegen hat *Spirulina* sogar eine verstärkende Wirkung auf die Dürrfleckenkrankheit. Das Wässern der Pflanzen mit *Spirulina* resultiert in einem weniger ausgeprägten Grad der Infektion relativ zur Spraybehandlung. In Tomatenkulturen kann *Spirulina platensis* daher nicht als Pflanzenstärkungsmittel empfohlen werden.

- ASM hat eine direkte fungitoxische Wirkung auf *Alternaria*. *In vitro*-Experimente zeigten, dass ASM in direktem Kontakt mit *Alternaria* seine Toxizität entfaltet und das Pilzwachstum verhindert.
- Die Behandlung mit dem Resistenzinduktors ASM durch Wässern der Pflanzen steigert die Anfälligkeit gegenüber Dürrfleckenkrankheit. Diese einer Schutzwirkung entgegengesetzte Effekt könnte möglicherweise durch die starke Phytotoxizität des über die Wurzeln aufgenommenen ASM erklärt werden.
- Applikation von ASM als Spray reduzierte die Anfälligkeit der Tomatenpflanzen gegen *Alternaria* in einigen Fällen, und erhöhte sie in anderen. Diese Inkonsistenz könnte durch die zweifache toxische Wirkung von ASM erklärt werden: auf der einen Seite wird der Pilz geschädigt (Fungitoxizität) und auf der anderen die Wirtspflanze (Phytotoxizität). Letzteres erhöht den Stress für die Pflanzen, was besonders bei Angriffen nekrotropher Pathogene die Anfälligkeit verstärkt. Bei der Anwendung als Spray können beide Effekte mit unvorhersehbarem Ausgang auftreten. Ein dritter auftretender Faktor könnte das erfolgreiche Induzieren von SAR sein, von der angenommen wird, dass sie nekrotrophe Pathogene während der Besiedlung des Wirtes begünstigt.
- Es zeigte sich, dass es nicht möglich ist, Tomatenpflanzen mit geringen Mengen des chemischen Fungizids Maneb zu schützen, während SAR aufgebaut wird. Ein befriedigender Schutz gegen *Alternaria* kann nur durch Konzentrationen nahe an der empfohlenen Dosis erreicht werden.
- Die Art der Interaktion zwischen ASM und Maneb hängt von bestimmten *Alternaria*-Isolaten ab. Verschiedene Isolate scheinen unterschiedliche Resistenzlevel gegenüber den beiden Mitteln zu haben, was sich in synergistischen, additiven und auch antagonsitischen Interaktionen widerspiegelt.

- *Piriformospora indica* hat einen messbaren wachstumsanregenden Effekt auf Tomatenpflanzen, der sich etwa 50 Tage nach der Keimung ausgleicht. Der Grad der Wachstumssteigerung variiert zwischen den verwendeten Tomatensorten.

- Unter bestimmten Umständen hat *Piriformospora* eine unterdrückende Wirkung gegenüber *Alternaria*. *In vitro*-Experimente zeigten, dass *Piriformospora*-Kulturen das Wachstum von *Alternaria* fast vollständig hemmen, wenn das Pathogen eine Woche nach Inokulation hinzugefügt wird.

- Zusätzlich zum wachstumssteigernden Effekt scheint *Piriformospora* eine protektive Wirkung gegen *Alternaria*-Infektionen zu haben. Die Wirkungsweise dieser Fähigkeit, den Verlust an Pflanzenbiomasse zu reduzieren, ist noch unklar.

- Nitrat- und Phosphat-Mangel schwächen und erhöhen den Stress für die Tomatenpflanzen, welche dann zu erhöhter Anfälligkeit gegenüber *Alternaria* neigen. Dies ist ein charakteristisches Merkmal von Interaktionen zwischen Pflanzen und nekrotrophen Pathogenen.

- Arbuskuläre Mykorrhizapilze sind effektive "Bioschutzmittel" gegen die Dürrfleckenkrankheit in Tomaten. In dieser Arbeit wurde bewiesen, dass mykorrhizierte Tomaten ein signifikant geringeres Ausmaß an *Alternaria*-Infektion aufwiesen als nicht-mykorrhizierte Pflanzen. Der zugrundeliegende Mechanismus scheint gleich dem der ISR zu sein, welche auch durch assoziierte Organismen an den Wurzeln induziert wird und gegen nekrotrophe Pathogene wirksam ist.

- Es ist bekannt, dass ein Überangebot von Phosphat die optimale Kolonisation der Wirtswurzeln mit AMF verhindert. Daher ist eine eingeschränkte Versorgung mit Phosphat vorteilhaft, wenn der Einsatz von Mykorrhizapilzen als Pflanzenschutzmethode gegen *Alternaria* ausgenutzt werden soll. Dabei kann kein Phosphatmangel auftreten, da die Mykorrhiza eine ausreichende Phosphatversorgung der Pflanzen garantiert.

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