Effect of Arbuscular Mycorrhizal Fungi and *Fusarium oxysporum* f. sp. *lycopersici* on Tomato and/or its Exudation in Single and Intercropping Settings

Dissertation

submitted by

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Abstract

Arbuscular mycorrhizal fungi (AMF) are the most prevalent type of mycorrhizal fungi and form a mycorrhizal symbiosis with a wide range of vascular plants including many important crop species. Apart from improved plant nutrition, AMF are reputed to control a number of plant diseases, especially soil-borne diseases. This is of high significance in the field of sustainable agriculture, where the input of fertilizers and chemical plant protectants is reduced or even absent.

In this work the focus was laid on the role of AMF in the control of the soil-borne fungus *Fusarium oxysporum* f. sp. *lycopersici* (*Fol)* in tomato. Thereby, different tomato varieties and different intercropping partners were assessed. Additionally, root exudation and its role in disease development was selected as one specific area of interaction and investigated in greater detail.

For the intercropping studies an inoculum consisting of six different *Glomus* species was tested against *Fol* with tomato (cv. Kremser Perle) intercropped with either leek, cucumber, basil, fennel or tomato itself. Arbuscular mycorrhizal root colonization of tomato was clearly affected by its intercropping partner. Furthermore, bioprotective effects of AMF resulting in the decrease of *Fol* disease severity and/or compensation of plant biomass were evident. However, these effects depended on the intercropping partner. Leek and basil proved to be beneficial intercropping partners of tomato.

Furthermore, wild-type, old and modern tomato cultivars alone were compared in their interactions with AMF (*Glomus mosseae*) and *Fol*. The varieties differed in their susceptibility to AMF and *Fol*. The cultivars Yellow Pearshaped, Rheinlands Ruhm and Supersweet had the highest *Fol* infection rates. The same cultivar dependency was observed for the bioprotective effect of AMF, which were evident in *Lycopersicon peruvianum*, Kremser Perle and Marmande.

Additionally, alterations in root exudation of tomato mediated by *G. mosseae* and *Fol* were investigated. AMF inoculation increased the germination rate of *Fol* in total exudates, whereas, the simultaneous inoculation of AMF and *Fol* decreased the germination rate of *Fol* in total exudates. The GC-MS analyses revealed an AMF-dependent increase of sugars and decrease of organic acids, mainly glucose and malate. Furthermore, an increase of chlorogenic acid in root exudates of tomato

plants inoculated with AMF and *Fol* was found with HPLC analyses, an effect, which could be shown for the first time. In subsequent single compound *in-vitro* assays citrate and chlorogenic acid were identified as possible candidates for the reduction of *Fol* germination rate in the AMF+*Fol* treatment, since they proved inhibition at concentrations naturally occurring in the rhizosphere.

Kurzfassung

Die arbuskuläre Mykorrhiza (AM) ist die häufigste Form der Mykorrhiza und bildet eine sogenannte Symbiose mit einer Vielzahl an krautigen Pflanzen, unter denen sich auch viele Kulturpflanzen befinden. Neben einer erhöhten Nährstoffversorgung sind auch Biokontrolleffekte, besonders im Bezug auf bodenbürtige Krankheitserreger, bekannt. Diesen Eigenschaften kommt eine hohe Bedeutung im Bereich der nachhaltigen Landwirtschaft zu, die den Einsatz mineralischer Düngung und des chemischen Pflanzenschutzes ablehnt.

In dieser Arbeit wurde der Schwerpunkt auf die Rolle der AM in der Kontrolle des bodebürtigen Pathogens *Fusarium oxysporum* f. sp. *lycopersici* (*Fol*) an der Tomate gelegt. Dabei wurden unterschiedliche Tomatensorten und Mischkulturpartner und zusätzlich noch die Wurzelexsudation und ihre spezielle Rolle in der Krankheitsentwicklung untersucht.

Für die Mischkulturuntersuchungen der Tomate mit Lauch, Gurke, Basilikum, Fenchel und Tomate wurde die Wirkung eines Inokulums, welches aus sechs unterschiedlichen *Glomus* Arten bestand, gegen *Fol* getestet. Dabei wurde festgestellt, dass der Mischkulturpartner den Mykorrhizierungsgrad der Tomate deutlich beeinflusste. Zusätzlich wurden Biokontrolleffekte durch AM festgestellt, die zu einer Reduktion der *Fol* Befallsstärke und/oder einer Kompensation der pflanzlichen Biomasse führten. Diese Effekte waren jedoch Mischkulturpartner-spezifisch. Lauch und Basilikum erwiesen dabei sich als günstige Mischkulturpartner für die Tomate.

Weiters wurden Tomatenwildtypen, alte und moderne Tomatensorten in deren Interaktionen mit AM (*Glomus mosseae*) und *Fol* verglichen. Sie unterschieden sich in ihrer Anfälligkeit für AM als auch für *Fol*, dabei erwiesen sich die Sorten Yellow Pearshaped, Rheinlands Ruhm und Supersweet als die anfälligsten. Auch bei den beobachteten Biokontrolleffekten durch AM bei *Lycoerpersicon peruvianum*, Kremser Perle und Marmande konnte eine Sortenabhängigkeit festgestellt werden.

Zusätzlich wurden Veränderungen in der Wurzelexsudation der Tomate hervorgerufen von *G. mosseae* und *Fol* untersucht. Durch AM Inokulation wurde die Sporenkeimung von *Fol* erhöht, während die gleichzeitige Inokulation mit AM und *Fol* zu einer Reduktion der Sporenkeimung führte. In den GC-MS Analysen konnte eine

AM-abhängige Zunahme an Zuckern, hauptsächlich Glucose, und eine Abnahme an organischen Säuren, hauptsächlich Malat, festgestellt werden. Weiters konnte mittels HPLC zum ersten Mal ein Anstieg an Chlorogensäure in Wurzelexsudaten von Tomatenpflanzen, die mit AM und *Fol* inokuliert wurden, festgestellt werden. In darauffolgenden *in-vitro* Tests mit Einzelkomponenten konnten Citrat und Chlorogensäure als mögliche Kandidaten für die Reduktion der Sporenkeimung in der AM+*Fol* Variante identifiziert werden, da sie auch bei Konzentrationen, die natürlich in der Rhizosphäre vorkommen, zu einer Reduktion der *Fol* Sporenkeimung führten.

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1. Introduction

Soil-borne pathogens impose particular challenges on the field of plant protection. Usually their propagules remain infectious in the soil for several seasons and the soil itself, as their environment with its numerous contributing variables still remains to be a “blackbox” to some extent. Furthermore, the ban on chemical soil desinfectants like methyl bromide and in general, the growing awareness on health and environmental issues and the increase of organic production make it necessary to search for alternatives in the control of soil-borne pathogens. As for many other cases, nature itself offers promising mechanisms to deal with these particular challenges.

Disease resistance in plants still remains to be one of the most efficient ways to control plant diseases. Apart from genetic traits also microorganisms can mediate resistance. In this work the focus was laid on the role of arbuscular mycorrhizal fungi (AMF) in the control of the soil-borne fungus *Fusarium oxysporum* f. sp. *lycopersici* in tomato. Thereby, different tomato varieties and different intercropping partners were assessed. Additionally, root exudation and its role in disease development was selected as one specific area of interaction and investigated in greater detail.

1.1 *Fusarium oxysporum* f. sp. *lycopersici*

1.1.1 The pathogen

Fusarium oxysporum Schlechtend.:Fr. f.sp. *lycopersici* (Sacc.) W. C. Snyder & H. N. Hans. (*Fol*) is the causal agent of Fusarium wilt in tomato. This fungal agent belongs to the Ascomycota and produces macroconidia, microconidia and chlamydospores (Figure 1). Microconidia are formed on monophialides, do not have septations and are either oval or elliptical in shape (5-12 x 2.2-3.5 µm) (Huang et al. 2012; Jones 1991). Macroconidia are formed in sporodochia and are three- (27-46 x 3-5 µm) to five-septate (35-60 x 3-5 µm). Chlamydospores are thick-walled and can stay viable for several years (Figure 1). To date 3 physiological races have been reported. Race 1 was discovered at the beginning of the 20th century. Race 2 was first reported 1961 in Florida (Huang et al. 2012; Jones 1991). In 1982, another race was discovered. Race 3 overcame resistance genes introgressed in tomato cultivars and had a better overseasoning ability than race 1 and 2 (Huang et al. 2012), consequently, imposing new challenges to disease control.

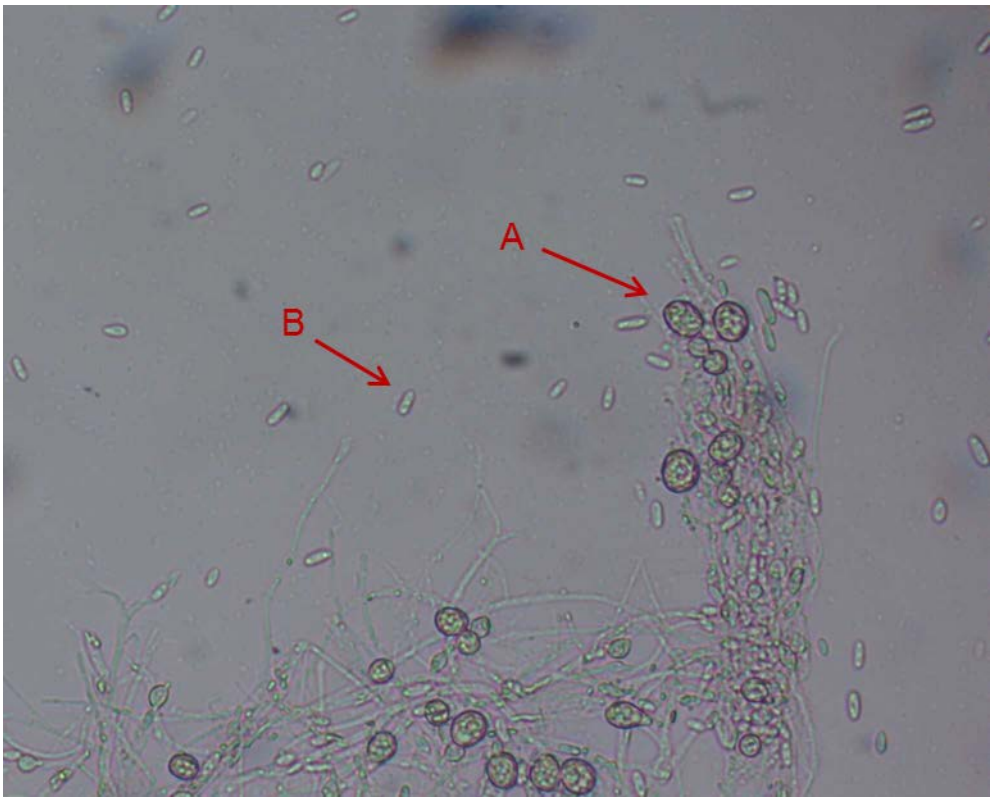

Figure 1. Chlamydospores (A) and microconidia (B) of *Fol*.

1.1.2 Epidemiology and symptoms

Fusarium wilt is a warm-weather disease and thrives optimally at a temperature of 28°C (min 20°C, max 34°C). The pathogen prefers sandy, acidic soils. Additionally, factors like soil moisture approaching field capacity, short day length and low light intensity are conducive for Fusarium wilt development (Huang et al. 2012; Jones 1991). Low nitrogen and phosphorus and high potassium content in fertilizers can increase the susceptibility of plants to Fusarium wilt (Huang et al. 2012; Jones 1991).

Fol either directly penetrates root tips or enters the roots via wounds or openings formed by lateral root development (Huang et al. 2012). After penetration the mycelium enters the vessel system and moves upwards to the crown. In the vessels microconidia germination occurs and causes clogging of the vessels. As a direct consequence water transport can be interrupted leading to wilting or even death of the plant (Huang et al. 2012).

The typical symptoms of Fusarium wilt include stunting of young seedlings, yellowing of older leaves and brownish discoloration of the vessel system (Figure 2) (Huang et

al. 2012; Jones 1991). Once infection of the roots has occurred root rot is apparent (Huang et al. 2012). Early symptoms on older plants like yellowing of leaves on typically one side of the plant can become apparent from blossoming to fruit maturation. With increase of disease severity also leaves of the other side start yellowing. In general yellowing progresses upwards and is accompanied by wilting, which becomes more severe and finally results in collapse of the plant (Huang et al. 2012; Jones 1991). Fruit infection occasionally occurs resulting in fruit rot and drop (Huang et al. 2012). Fruits show vascular tissue discoloration as well (Jones 1991).

Figure 2. Vessel discoloration caused by *Fusarium oxysporum* f.sp. *lycopersici*.

Dissemination can occur via seed, tomato stakes, soil, transplants, farm machinery (Huang et al. 2012; Jones 1991) and infected plants on the field via aerial dissemination (Huang et al. 2012).

1.1.3 Control

The best way to control Fusarium wilt is the use of resistant cultivars. Currently, 3 resistance genes, *I*, *I2* and *I3*, are available against race 1, race 2 and race 3, respectively (Huang et al. 2012). However, single-point mutations may result in a breakdown of resistance against race 3 (Takken and Rep 2010). This will probably lead to the emergence of race 4 (Huang et al. 2012).

Apart from resistant cultivars cultural control can also be involved in strategies against *Fol*. This can include the raise of the soil pH to 6.5-7.0 (Jones 1991). Furthermore, the use of nitrate nitrogen in fertilizers is better for controlling Fusarium wilt than ammonium nitrogen. Soil solarization, however, only applicable in warmer areas, and a 5- to 7-year crop rotation can reduce losses greatly (Huang et al. 2012; Jones 1991).

Soil fumigation with methyl bromide has been used for a long time but is phased out since several years. Stricter laws and the demand for more organically produced tomatoes promote biological control against *Fol*. Until now fungal as well as bacterial antagonists are available. This includes fungi and bacteria such as non-pathogenic *F. oxysporum*, *F. solani*, *Gliocladium virens*, *Trichoderma harzianum*, *Bacillus subtilis*, *Burkholderia cepacia* and *Pseudomonas fluorescens* (Larkin and Fravel 1998 ; Larena et al. 2002; Shishido et al. 2005; Ramamoorthy et al. 2002).

1.2 Arbuscular mycorrhizal fungi

1.2.1 Biology

The arbuscular mycorrhizal (AM) association with plants developed more than 430 million years ago simultaneously with the first colonization of the terrestrial environment by plants (Smith and Read 2008; Stürmer 2012). Arbuscular mycorrhzial fungi form a mutualistic symbiosis with 80 % of all vascular plants (Smith and Read 2008) and probably were involved in the colonization of land by plants, when no true roots had been developed yet. A "mutualistic symbiosis" implies benefits for both partners. With regard to the AM–plant interaction this is mainly characterized by a bidirectional nutrient transfer (Smith and Read 2008). The fungus provides the host plant with minerals like phosphorus, nitrogen, copper and zinc and in return receives plant-derived C (i.e. hexoses) (Smith and Read 2008). Apart from nutritional benefits AMF also offer bio-protective effects (Azcón-Aguilar et al. 2002; Singh et al. 2000; St-Arnaud and Vujanovic 2007; Whipps 2004; Xavier and Boyetchko 2004).

Since 2001 AMF no longer belong to the Zygomycota and are classified into their own phylum, the Glomeromycota, based on a combination of genetic and phenotypic characters and fossil record (Stürmer 2012; Schüßler et al. 2001). The term "arbuscular" derives from the tree-like highly branched structures, the arbuscules (Figure 3 and Figure 4), which are formed within the plant cell while invaginating the plasma membrane. At this plant–fungal interface nutrient transfer of mainly minerals takes place, whereas the highest sugar exchange takes place at the hyphal–plant interface (Smith and Read 2008).

AMF enter the roots via appressoria and the intraradical mycelium colonizes the root cortex. Spores, infected root fragments and hyphae serve as inoculum sources. Arbuscules can be formed within 2 days from penetration on. Depending on the

environmental conditions thick-walled storage units (i.e. vesicles) containing mainly lipids can be formed within the roots as well (Figure 3). They play a crucial role in root fragments serving as inoculum (Smith and Read 2008).

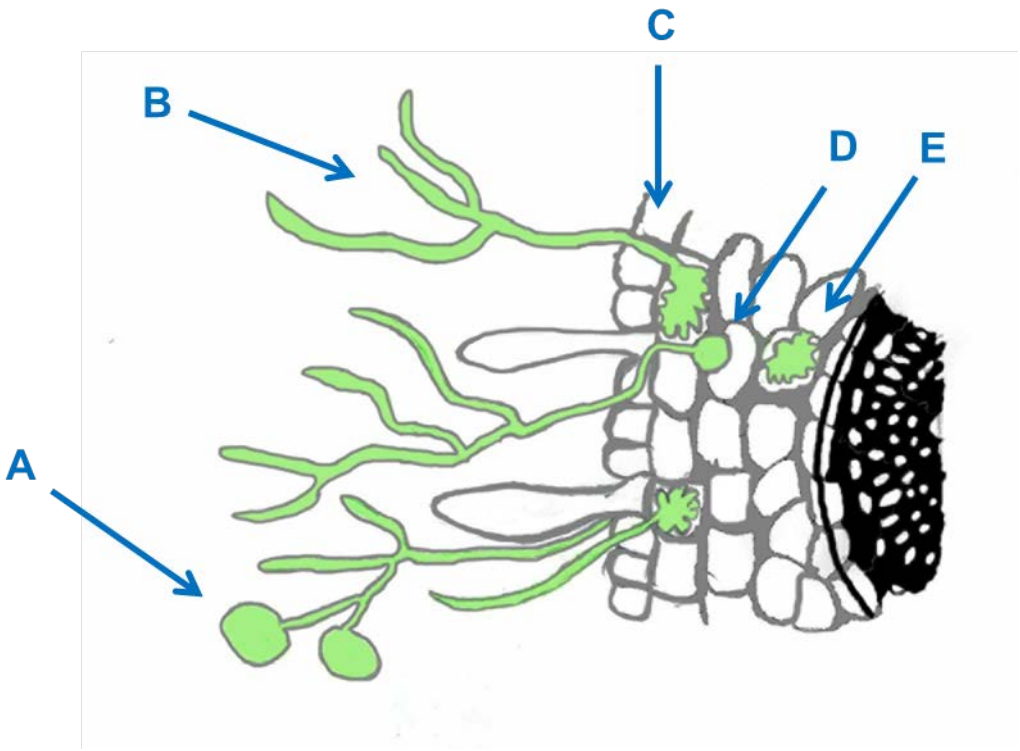

Figure 3. Schematic illustration of a root cross-section colonized by AMF. A=spores, B=extraradical hyphae, C=intraradical hyphae, D=vesicle, E=arbuscule.

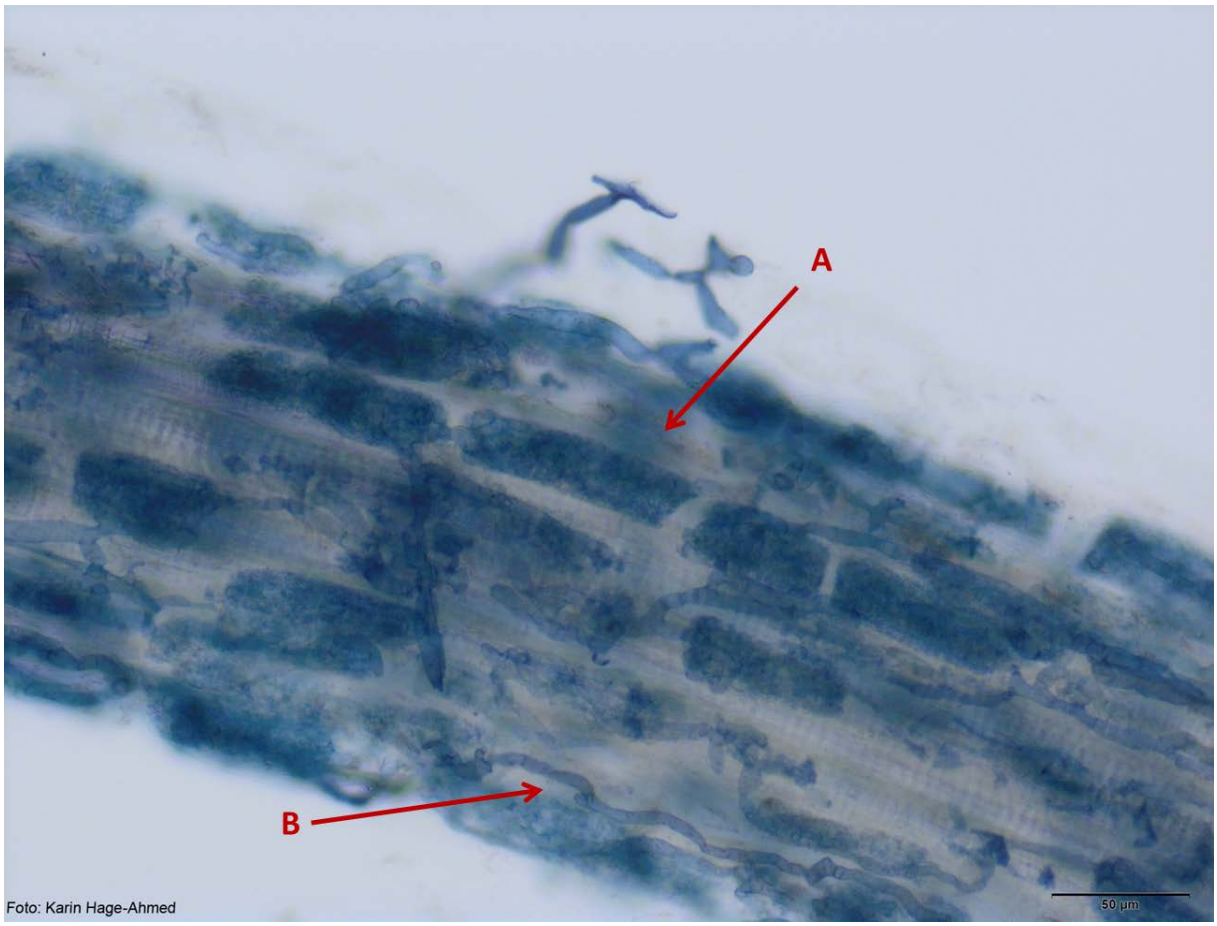

Figure 4. Fennel roots colonized by AMF. A=arbuscule, B=intraradical hyphae.

Different AM species and strains have in general a wide host range, however, certain colonization preferences and host specificity (Smith and Read 2008), which are regulated by a sophisticated cross-talk between fungus and host plant (Kiers et al. 2011; Vierheilig 2004), are evident. Consequently, the composition of AM fungal communities has an impact on plant community structures and diversity by altering inter- or intra-specific competitive situations (Schroeder-Moreno and Janos 2008; Smith and Read 2008; van der Heijden et al. 2003). In return fungal community structures are influenced by their host plants (Bever 2002; Smith and Read 2008). As a consequence, highly dynamic feedback situations are created.

1.2.2 Arbuscular mycorrhiza and the control of soil-borne diseases

AMF colonize many crop species apart from members of the Brassicaceae and Chenopodiaceae family (Smith and Read 2008). In addition to nutritional benefits AMF can also control soil-borne diseases (Azcón-Aguilar et al. 2002; Singh et al. 2000; St-Arnaud and Vujanovic 2007; Whipps 2004; Xavier and Boyetchko 2004). As

reviewed by Whipps (2004) the following modes of action are involved in disease control by AMF:

(i) Direct competition or inhibition

This includes competition for carbon sources such as assimilates and root exudates; furthermore, competition for infection sites or space on roots can occur. Exudates of roots and AMF can be involved in pathogen inhibition as well.

(ii) Enhanced or altered plant growth, nutrition and morphology

This category includes mechanisms like increased nutrient uptake, alleviation of abiotic stress, alteration in root morphology, hormonal changes and compensation of damaged plant tissue.

(iii) Biochemical changes associated with plant defence mechanisms and induced resistance

This includes the formation of structural defence barriers and the production of defence-related proteins, phenolics and phytoalexins.

(iv) Development of an antagonistic microbiota of bacteria and fungi

Although bio-protective effects of AMF are evident no plant protectant on mycorrhizal basis is available on the market. They are sold as plant growth promoters instead. This is due to limitations of efficacy of AMF caused by the indigenous microbial community, agricultural practices, host plant specificity, regulation events of the host plants etc. (Whipps 2004; Xavier and Boyetchko 2004). Furthermore, large scale production is complicated due to the obligate biotrophicity of AMF (Xavier and Boyetchko 2004). All these challenges make the registration process for AMF as plant protectants difficult. However, AMF always need to be seen in a broader context, also considering interactions with rhizosphere organisms and their host plants as suggested by Sikora (1997) to exploit their whole potential.

1.3 Root exudates and rhizosphere interactions

1.3.1 The rhizosphere

According to Bais et al. (2006) the rhizosphere is defined as "the soil zone that surrounds and is influenced by the roots of plants". The rhizosphere is a very dynamic area where root growth, exudate production and community development of

macrobiota and microbiota occurs (Bertin et al. 2003). Furthermore, root-insect, root-root and root-microbe interactions take place causing dynamic feedback situations. This also includes interactions between soil-borne pathogens and their host plants. Important elicitors for these interactions are root exudates (Bais et al. 2006; Nelson 1990).

1.3.2 Root exudates

Root exudates contain a diverse array of different compounds such as ions, water, enzymes, mucilage, primary and secondary metabolites (Bais et al. 2006), which can be soluble or volatile (Nelson 1990). According to their molecular weight root exudates can be separated in low-molecular and high-molecular weight compounds. The first group comprises sugars and simple polysaccharides (such as arabinose, fructose, glucose, maltose, mannose, oligosaccharides), amino acids (such as arginine, asparagine, aspartic, cysteine, cystine, glutamine), organic acids (such as acetic, ascorbic, benzoic, ferulic, malic acid), phenolic compounds, alcohols and aldehydes (Bais et al. 2006; Bertin et al. 2003; Nelson 1990). At the same time these low-molecular weight compounds contribute to much of the diversity of root exudates (Bais et al. 2006) and particularly the phenols have a special influence on growth and development of surrounding plants and soil microorganisms (Bertin et al. 2003). On the contrary high-molecular weight compounds such as flavonoids, enzymes, fatty acids, growth regulators, nucleotides, tannins, carbohydrates, steroids, terpenoids, alkaloids, polyacetylenes and vitamins are less diverse but often compose a larger proportion of the root exudates by mass (Bais et al. 2006; Bertin et al. 2003). However, root exudation is a very dynamic process and, consequently, root exudate composition is influenced by factors such as plant species, age and physiological state of the plant, soil pH, soil moisture, temperature and presence of microorganisms (Bais et al. 2006; Bertin et al. 2003; Nelson 1990; Jones 1998).

Root exudates are released by living root hairs and actively growing primary and secondary roots. There were 3 major ways proposed how the event of release actually takes place (Bertin et al. 2003):

(i) Diffusion

Low-molecular weight organic compounds such as amino acids, sugars, carboxylic acids and phenols are released via a passive process along steep concentration gradients between the cytoplasm of root cells (mM)

and the soil (µM). Membrane permeability depends mainly on the physiological state of the plant and the polarity of the compounds to be exuded.

(ii) Anion channel

Under specific stress conditions like nutritional deficiency or Al toxicity diffusion through membranes is not possible. In such situations anion channels can mediate the release of specific carboxylates.

(iii) Vesicle transport

High-molecular weight compounds are exuded through vesicle transport. This includes the transport of mucilage polysaccharides and ectoenzymes (acid phosphatase, peroxidase). Phenols and phytosiderophores are also stored and released using vesicles and vesicle transport.

1.3.3 Root exudates in the context of *Fusarium oxysporum* f. sp. *lycopersici* and AMF

It has been shown previously that AMF reduced adverse effects of *Fol* when co-inoculated with this pathogen (Akköprü and Demir 2005; Dehne and Schönbeck 1979). Apart from improved plant nutrition mechanisms like changes in root and vessel system, mycorrhizosphere effect and induced systemic resistance are involved in these effects (Akköprü and Demir 2005; Dehne and Schönbeck 1979; Whipps 2004). However, to date it is not known whether AMF inoculation alters the root exudation in such a way that *Fol* propagules are affected.

The germination rate of microconidia of *Fol* is influenced by root exudates. Plant species, plant age and different *F. oxysporum* strains in the rhizosphere influence these effects (Steinkellner et al. 2005, 2008). In recent years negative effects of root exudates of AM tomato plants on zoospores of *Phytophthora nicotianae* (Lioussanne et al. 2008) and *Meloidogyne incognita* infection (Vos et al. 2012) were reported. On the contrary, the germination rate of *Fol* is increased in root exudates of mycorrhizal plants compared to non-mycorrhizal ones (Scheffknecht et al. 2006; Scheffknecht et al. 2007). However, until now the compounds contributing to these effects on germination rate of *Fol* are not known in detail.

1.4 Intercropping

Intercropping implies the simultaneous cultivation of two or more plant species or varieties. Monoculture and narrow crop rotation are usually factors which favor disease development. On the contrary, intercropping promotes diversity and among other benefits like increased yield and quality reduces the impact of pest and diseases (Sullivan 2004; Ratnadass et al. 2012; Vandermeer 1989). One mechanism involved in disease control is allelopathic suppression of soil-borne pathogens mediated by root exudates (Hao et al. 2010; Yu 1999; Ratnadass et al. 2012). Therefore, intercropping with special stress on root exudate-mediated effects can be one strategy in plant protection in sustainable agriculture.

1.5 Hypotheses

In Chapter 2 three publications will be presented, which pursue the following hypotheses:

I) Hypothesis concerning plant, AMF and disease development:

   a. In intercropping settings the AMF colonization rate of tomato is changed
   b. The intercropping system itself has effects on *Fol* disease severity
   c. AMF application in intercropping systems has effects on *Fol* disease severity
   d. Root and shoot weights of tomato are affected by AMF and/or *Fol* in different intercropping systems
   e. AMF colonization rate depends on the tomato cultivar
   f. *Fol* disease severity depends on the tomato cultivar.
   g. Bio-protective effects of AMF against *Fol* depend on the tomato cultivar

II) Hypothesis concerning root exudation and its effects on *Fol*:

   a. AMF and/or *Fol* inoculation changes root exudate composition of tomato
   b. AMF and/or *Fol* inoculation affects *Fol* development in root exudates in *in-vitro* experiments
   c. Single compounds identified in chromatographic analyses exhibit different effects on *Fol* development in *in-vitro* assays.

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2. Publications

Hage-Ahmed K, Krammer J, Steinkellner S (2013) The intercropping partner affects arbuscular mycorrhizal fungi and *Fusarium oxysporum* f. sp. *lycopersici* interactions in tomato. Mycorrhiza. doi:10.1007/s00572-013-0495-x

In this study an inoculum consisting of six different *Glomus* species was tested against *Fusarium oxysporum* f. sp. *lycopersici* with tomato (cv. Kremser Perle) intercropped with either leek, cucumber, basil, fennel or tomato itself. Arbuscular mycorrhizal root colonization of tomato was clearly affected by its intercropping partner. Furthermore, bioprotective effects of AMF resulting in the decrease of *Fol* disease severity and/or compensation of plant biomass were evident. However, these effects depended on the intercropping partner.

Steinkellner S, **Hage-Ahmed K**, Garca-Garrido JM, Illana A, Ocampo JA, Vierheilig H (2012) A comparison of wild-type, old and modern tomato cultivars in the interaction with the arbuscular mycorrhizal fungus *Glomus mosseae* and the tomato pathogen *Fusarium oxysporum* f. sp. *lycopersici*. Mycorrhiza. 2012; 22(3):189-194

In Steinkellner et al. (2012) wild-type, old and modern tomato cultivars were compared in their interactions with AMF and *Fol*. The varieties differed in their susceptibility to AMF and *Fol*, which was not linked to their cultivar age.

Hage-Ahmed K, Moyses A, Voglgruber A, Hadacek F, Steinkellner S (2013) Alterations in root exudation of intercropped tomato mediated by the arbuscular mycorrhizal fungus *Glomus mosseae* and the soil-borne pathogen *Fusarium oxysporum* f. sp. *lycopersici*. J Phytopathology. **accepted**

In this study alterations in root exudation of tomato mediated by *G. mosseae* and *Fol* were investigated. AMF inoculation impacted on the relative amounts of sugars and organic acids in tomato root exudates. Furthermore, an increase of chlorogenic acid in root exudates of tomato plants inoculated with AMF and *Fol* was found. In *in-vitro* assays citrate and chlorogenic acid proved inhibition against *Fol* at concentrations naturally occurring in the rhizosphere.

2.1 The intercropping partner affects arbuscular mycorrhizal fungi and *Fusarium oxysporum* f. sp. *lycopersici* interactions in tomato

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ORIGINAL PAPER

The intercropping partner affects arbuscular mycorrhizal fungi and *Fusarium oxysporum* f. sp. *lycopersici* interactions in tomato

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Abstract Arbuscular mycorrhizal fungi (AMF) and their bioprotective aspects are of great interest in the context of sustainable agriculture. Combining the benefits of AMF with the utilisation of plant species diversity shows great promise for the management of plant diseases in environmentally compatible agriculture. In the present study, AMF were tested against *Fusarium oxysporum* f. sp. *lycopersici* with tomato intercropped with either leek, cucumber, basil, fennel or tomato itself. Arbuscular mycorrhizal (AM) root colonisation of tomato was clearly affected by its intercropping partners. Tomato intercropped with leek showed even a 20 % higher AM colonisation rate than tomato intercropped with tomato. Positive effects of AMF expressed as an increase of tomato biomass compared to the untreated control treatment could be observed in root as well as in shoot weights. A compensation of negative effects of F. oxysporum f. sp. lycopersici on tomato biomass by AMF was observed in the tomato/leek combination. The intercropping partners leek, cucumber, basil and tomato had no effect on *F. oxysporum* f. sp. *lycopersici* disease incidence or disease severity indicating no allelopathic suppression; however, tomato co-cultivated with tomato clearly showed a negative effect on one plant/pot with regard to biomass and disease severity of *F. oxysporum* f. sp. *lycopersici*. Nonetheless, bioprotective effects of AMF resulting in the decrease of F. oxysporum f. sp. lycopersici disease severity were evident in treatments with AMF and *F. oxysporum* f. sp. *lycopersici* co-inoculation. However, these bioprotective effects depended on the intercropping partner since these effects were only observed in the tomato/leek and tomato/basil combination and for the better developed plant of tomato/tomato. In conclusion, the effects of the intercropping partner on AMF colonisation of tomato are of great interest for crop plant communities and for the influences on each other. The outcome of the bioprotective effects of AMF resulting in the decrease on *F. oxysporum* f. sp. *lycopersici* disease severity and/or compensation of plant biomass does not depend on the degree of AM colonisation but more on the intercropping partner.

Keywords AM fungi · *Fusarium oxysporum* f. sp. *lycopersici* · Intercropping · Solanum lycopersicum · Biological control

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Introduction

Arbuscular mycorrhizal fungi (AMF) are the most prevalent type of mycorrhizal fungi and form a mycorrhizal symbiosis with a wide range of vascular plants including many important crop species (Smith and Read 2008). Apart from improved plant nutrition, AMF are reputed to control a number of plant diseases, especially soil-borne diseases (Azcón-Aguilar et al. 2002; Whipps 2004; Singh et al. 2000; Xavier and Boyetchko 2004; St-Arnaud and Vujanovic 2007; Newsham et al. 1995). This is of high significance in the field of sustainable agriculture, where the input of fertilisers and chemical plant protectants is reduced or even absent. Furthermore, it is known that AMF have an impact on plant community structure and diversity by altering inter- or intraspecific competitive situations (Smith and Read 2008; van der Heijden et al. 2003; Schroeder-Moreno and Janos 2008). In return, however, arbuscular mycorrhizal (AM) fungal community structure can be influenced by the host plants (Smith and Read 2008; Bever 2002). These effects are well described for grassland communities; for

crop species, only Schroeder-Moreno and Janos (2008) have reported similar effects. For the application of intercropping in combination with AMF, these feedback matters need to be kept in mind and need to be tested for each intercropping arrangement separately. Thus, further research is necessary for the application to crop species, especially with regard to intercropping arrangements with the aim of improved plant performance, which also implies lower infection rates caused by diseases. Plant species diversity could make a significant contribution to the reduction of plant diseases (Ratnadass et al. 2012). This would reduce the use of chemical pesticides and therefore reduce adverse effects on humans and the environment.

Fusarium oxysporum f. sp. *lycopersici* is a soil-borne fungus, which invades the plants through the roots and causes wilting in tomato which can result in severe yield losses. Apart from the environmental issues mentioned before, chemical control of soil-borne pathogens is difficult to impossible, thus, giving a further strong reason for alternative methods of disease control.

It has been shown previously that AMF reduced adverse effects of *F. oxysporum* f. sp. *lycopersici* in tomato when co-inoculated with this pathogen (Dehne and Schönbeck 1979; Akköprü and Demir 2005). Apart from improved plant nutrition mechanisms like changes in root and vessel system, mycorrhizosphere effects and induced systemic resistance are involved in these effects (Akköprü and Demir 2005; Dehne and Schönbeck 1979; Whipps 2004). However, this bioprotective effect depends on the AM fungal identity (Sikes et al. 2009), making it crucial to choose the proper AMF isolates. Furthermore, it has been shown that allelopathic suppression by root exudates can occur in intercropping arrangements, like for tomato and Chinese chive against Pseudomonas solanacearum (Yu 1999) and for watermelon and rice against *Fusarium oxysporum* f. sp. *niveum* (Hao et al. 2010). A combination of the bioprotective effects of AMF and intercropping partners can be considered as a new potential strategy against soil-borne pathogens and would be of high significance for sustainable agriculture.

In our work, we focused on tomato (*Solanum lycopersicum* L.) intercropped with leek (*Allium porrum* L.) and basil (*Ocimum basilicum* L.), commonly known as stimulating species, and fennel (*Foeniculum vulgare* (L.) Mill.) and cucumber (*Cucumis sativus* L.), known as species with adverse effects on tomato, in combination with a commercially available AMF inoculum. We hypothesised that the intercropping partners of tomato can have conducive, adverse or neutral effects on AMF and tomato wilt caused by *F. oxysporum* f. sp. *lycopersici*. The aim was to investigate (1) the AMF colonisation rate of tomato in intercropping settings, (2) the influence of AMF application in intercropping systems on *F. oxysporum* f. sp. *lycopersici* disease severity and (3) the effects of AMF and/or *F. oxysporum* f. sp. *lycopersici* in different intercropping systems on root and shoot weights of tomato.

Material and methods

Plant and fungal material

Tomato (*S. lycopersicum* L. cv. Kremser Perle), leek (*A. porrum* L. cv. Golem), fennel (*F. vulgare* (L.) Mill. cv. Fino), basil (*O. basilicum* L. cv. Genovese) and cucumber (*C. sativus* L. cv. Aztec F1) were used as crop plants. All seeds were surface-sterilised by soaking in 50 % household bleach ('Dan Klorix', 3.8 % NaOCl) for 10 min and rinsed afterwards three times with autoclaved distilled water. Seeds were transferred to pots filled with autoclaved perlite (Granuperl S 3–6, Knauf Perlite GmbH, Vienna, Austria) and incubated in a growth chamber (York International) with a 16-h light (light intensity 296 μmol m^{-1} s^{-1}) and 8-h dark photoperiod at 24 °C. The perlite was irrigated with tap water. Seeds of leek and fennel were pre-cultivated 6 weeks, and seeds of tomato and basil 3 weeks, before transplanting. Due to the rapid growth, cucumber seeds were used in the below described plant bioassay without pre-cultivation.

F. oxysporum f. sp. *lycopersici* (*F. oxysporum* f. sp. *lycopersici*007) was cultivated for 2 weeks at 24 °C in darkness on Czapek Dox Agar (Duchefa Biochemie, Haarlem, The Netherlands). For plant inoculation, a microconidial suspension was prepared by flooding the F. oxysporum f. sp. lycopersici colonies with sterile, distilled water and gently rubbing with a Drigalski spatula. Thereafter, the conidial suspension was filtered through three layers of cheese cloth (fleece filters, 20–150 μm pore diameter, Laporte, Wels, Austria) and adjusted to a final concentration of 10^5 microconidia ml^{-1}.

For AMF plant inoculation, a commercially available inoculum (Symbivit®, Zivojin Rilakovic, Guntramsdorf, Austria) was used. This inoculum contains at least 80,000 spores l^{-1} and comprises six *Glomus* species (*Glomus etunicatum, Glomus microagregatum, Glomus intraradices, Glomus claroidium, Glomus mosseae and Glomus geosporum*).

Plant bioassay

Pre-cultivated plantlets of tomato, basil, leek and fennel and cucumber seeds were transferred to pots (volume 1,183 cm^{-3}) filled with an autoclaved (20 min at 121 °C) mixture of sand, soil and expanded clay (1:1:1, v/v/v). The plants were cultivated as a dual culture system with the following plant combinations: tomato/tomato, tomato/basil,

tomato/cucumber, tomato/fennel and tomato/leek. The experimental set-up included four different treatments for each dual culture: (1) *F. oxysporum* f. sp. *lycopersici*, (2) AMF, (3) *F. oxysporum* f. sp. *lycopersici* and AMF and (4) a control without *F. oxysporum* f. sp. *lycopersici* and without AMF. For each treatment three replicates comprising 6 pots each were used, giving 72 pots per plant combination and a total of 360 pots.

For the AMF treatment, 4 ml of the AMF inoculum were added to the planting hole at the potting procedure. *F. oxysporum* f. sp. *lycopersici* was applied to the plant roots by dipping the roots for 5 min in a microconidial suspension (10^5 microconidia ml^{-1}) before plants were transferred to the pots. For the AMF + *F. oxysporum* f. sp. *lycopersici* treatment, both inocula were added as mentioned above.

The plants were grown in a random design in a greenhouse for 11 weeks and were irrigated according to their moisture requirements with a nutrient solution (Steinkellner et al. 2005).

After 11 weeks, the plants were gently removed from the substrate und washed thoroughly under tap water. Root and shoot fresh weights were determined. Plants of the tomato/tomato combination were separated into two groups per pot according to their root weights to assess intraspecific effects.

Disease incidence was calculated according to the following formula:

$$\text{Disease incidence} \quad \frac{\text{Number of infected plants}}{\text{Total number of plants}} \times 100$$

F. oxysporum f. sp. *lycopersici* disease severity was determined by measuring the amount of vessel discolouration of the stem in relation to the total stem length (length of infected stem [cm]/total length of stem [cm]). Leaf symptoms were not evident at this plant stage and were therefore not considered for disease severity assessment. For confirmation of *F. oxysporum* f. sp. *lycopersici* infection, segments of 2-cm length starting upwards the shoot basis were dipped in 70 % ethanol, flamed and put into Petri dishes containing potato dextrose agar amended with antibiotics to prevent bacterial growth according to Steinkellner et al. (2011). The determination of *F. oxysporum* f. sp. *lycopersici* was done according to Nelson et al. (1983) by visual and microscopic analyses.

Defined root segments of 1-cm length, starting 2 cm down the shoot, were used for determining the degree of mycorrhization. The root segments were cleared by boiling for 4 min (tomato, cucumber, leek and fennel) and 5 min (basil), respectively, in 10 % KOH and afterwards rinsed three times with tap water. Roots were stained by boiling for 3 min in a 5 % ink–vinegar solution (Vierheilig et al. 1998). The percentage of root colonisation was determined according to the method of McGonigle et al. (1990).

Statistical analyses

AM colonisation rate was rank-transformed and analysed by using one-way ANOVA and Bonferroni's test. Data of disease severity were analysed by Kruskal–Wallis and Mann–Whitney U test. Since neither root nor shoot weights met the homogeneity assumption of variance, even after transformation, a two-way ANOVA could not be applied. Therefore, data were analysed by one-way ANOVA (cross-checked with Welch's ANOVA) and Tamhane's test. Correlation analyses were based on Spearman's rho. All statistical analyses were performed using PASW Statistics 18.

Results

AM colonisation

The intercropping partner significantly influenced the AM colonisation levels of tomato plants ($F_{8,151} = 18.761$, $P < 0.0001$) (Fig. 1). Tomato plants intercropped with leek showed a 20 % higher colonisation level of the roots than tomato co-cultivated with tomato, whereas, tomato intercropped with fennel showed a 13 % lower AM colonisation level. Tomato intercropped with cucumber and basil, respectively, did not reveal any differences in the AM colonisation level compared to the tomato/tomato (TT) combination. Within the TT combination, tomato plants grown in one pot did not show any differences in their AM colonisation levels. Tomato and basil plants in the tomato/basil (TB)

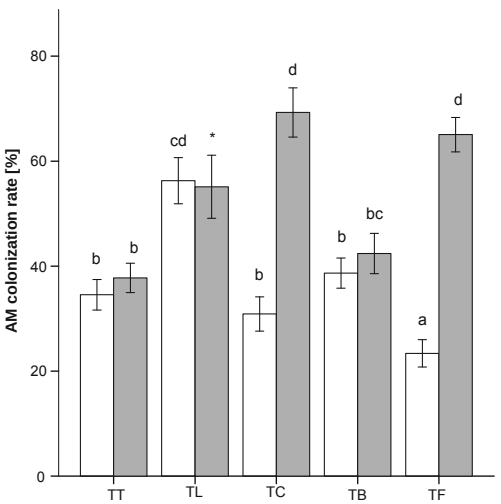

Fig. 1 AM colonisation rate (%) of different plant species in different intercropping combinations (mean ± standard error). T = tomato, L = leek, C = cucumber, B = basil, F = fennel; empty bars represent the tomato plants and grey bars the corresponding intercropping partner. Plants were only inoculated with AMF. Different letters indicate significant difference according to ANOVA and Bonferroni's test ($P < 0.05$). * excluded from statistical analysis

combination did not show any significant differences in their AM colonisation rates, whereas for tomato and cucumber in the tomato/cucumber (TC) combination, cucumber had a 2.3-fold higher AM colonisation level than tomato. Furthermore, fennel in the tomato/fennel (TF) combination had a 2.7-fold higher colonisation level than the tomato plants. The AM colonisation level of leek (55.11 ± 6.0) in the tomato/leek (TL) combination was not considered for ANOVA due to its high variance caused by the small root system of the leek plants. However, AM colonisation level was similar to the one of tomato in the TL combination. AM colonisation levels are only shown for the AMF treatment since *F. oxysporum* f. sp. *lycopersici* did not show an influence on the AM colonisation levels compared to the AMF treatment alone. Plants of the control and *F. oxysporum* f. sp. *lycopersici* treatment were also checked for AM colonisation but did not show any presence of AMF.

Assessment of *F. oxysporum* f. sp. *lycopersici* disease incidence and disease severity

F. oxysporum f. sp. *lycopersici* disease incidence and disease severity are presented in Table 1. For the TT combination, means of all plants from pots are given. Also, tomato plants of one pot were separated according to their root weights (see also Table 2) to see intraspecific effects and presented as Tomato_1 (T_1) and Tomato_2 (T_2). When considering all plants, the disease incidence of TT was 80.6 and 52.9 % in the *F. oxysporum* f. sp. *lycopersici* and AMF + *F. oxysporum* f. sp. *lycopersici* treatments, respectively, so that disease incidence was 35 % less in the AMF + *F. oxysporum* f. sp. *lycopersici* treatment than in the *F. oxysporum* f. sp. *lycopersici* treatment. In the other dual cultures, AMF + *F. oxysporum* f. sp. *lycopersici* also showed lower disease incidences than the *F. oxysporum* f. sp. *lycopersici* treatment alone. The plant combinations had no impact on *F. oxysporum* f. sp. *lycopersici* disease incidence. With regards to root weights of the tomato plants of one pot, disease incidence in the TT combination of T_1 was reduced in the AMF + *F. oxysporum* f. sp. *lycopersici* treatment (37.5 %) and, consequently, showed almost 50 % less disease incidence than in the *F. oxysporum* f. sp. *lycopersici* treatment (72.2 %).

Disease severity within the *F. oxysporum* f. sp. *lycopersici* treatment ranged between 14.0 and 16.2 % and between 4.4 and 14.32 % within the AMF + *F. oxysporum* f. sp. *lycopersici* treatment. The plant combinations had neither in the *F. oxysporum* f. sp. *lycopersici* ($\chi^2_{(4)} = 0.412$, $P = 0.981$) nor in the AMF + *F. oxysporum* f. sp. *lycopersici* treatment ($\chi^2_{(4)} = 0.195$, $P = 0.700$) a significant influence on *F. oxysporum* f. sp. *lycopersici* disease severity. Within the TL and TB combinations, AMF + *F. oxysporum* f. sp. *lycopersici* reduced disease severity significantly by 70 % ($P < 0.05$) and 63 % ($P < 0.05$), respectively. In the other plant combinations, the AMF + *F. oxysporum* f. sp. *lycopersici* treatment also tended to show lower disease severity. Within T_1, *F. oxysporum* f. sp. *lycopersici* showed higher disease severity than AMF + *F. oxysporum* f. sp. *lycopersici* ($P < 0.05$). Within T_2, disease severity was similar between *F. oxysporum* f. sp. *lycopersici* and AMF + *F. oxysporum* f. sp. *lycopersici* ($P = 0.703$). Within the *F. oxysporum* f. sp. *lycopersici* ($P < 0.05$) as well as in the AMF + *F. oxysporum* f. sp. *lycopersici* ($P < 0.05$) column, T_1 had significantly lower disease severity than T_2. There was no significant correlation between disease severity and AM colonisation rate ($P = 0.778$, $R^2 = 0.0008$).

Table *1* *F. oxysporum* f. sp. *lycopersici* disease incidence and disease severity (%) in the different plant combinations (mean ± S.E.)

Dual culture	Disease incidence (%)		Disease severity (%)	
	F. oxysporum f. sp. *lycopersici*	AMF + *F. oxysporum* f. sp. *lycopersici*	*F. oxysporum* f. sp. *lycopersici*	AMF + *F. oxysporum* f. sp. *lycopersici*
Tomato/tomato[a]	80.6	52.9	16.2±2.1 A a	14.32±3.8 A a
Tomato/leek	66.7	44.4	14.3±3.4 B a	4.4±1.5 A a
Tomato/cucumber	81.3	55.6	15.8±3.9 A a	7.0±2.2 A a
Tomato/basil	72.2	55.6	14.2±2.9 B a	5.3±1.7 A a
Tomato/fennel	66.7	55.6	14.0±2.8 A a	6.9±2.0 A a
Tomato/tomato (T_1)[b]	72.2	37.5	11.4±2.4 B x	3.8±1.3 A x
Tomato/tomato (T_2)[c]	88.8	66.7	21.0±3.0 A y	23.7±6.4 A y

Different letters indicate significant differences, small letters among columns (Kruskal–Wallis test, $P < 0.05$), capital letters among rows (Mann–Whitney U test, $P < 0.05$).

[a] Data were calculated for both plants/pot

[b] Data were calculated for the stronger tomato plant/pot according to the root weight

[c] Data were calculated for the weaker tomato plant/pot according to the root weight

Table 2 Root weights (in grams) of tomato plants grown in a dual culture system (mean ± S.E.)

Dual culture	Treatment				
	Control	*F. oxysporum* f. sp. *lycopersici*	AMF	AMF + *F. oxysporum* f. sp. *lycopersici*	
Tomato/tomato (T_1)[a]	6.36±0.44 (A) cd	5.76±0.41 (A) c	7.11±0.48 (B) bc	4.90±0.36 (A) bc	$F_{(3,66)} = 4.549$ $P<0.01$
Tomato/tomato (T_2)[b]	3.71±0.24 (B) a	2.42±0.26 (A) a	4.42±0.39 (B) a	2.45±0.35 (A) a	$F_{(3,67)} = 9.713$ $P<0.0001$
Tomato/leek	7.03±0.22 (A) d	5.87±0.46 (A) c	8.47±0.46 (B) d	6.06±0.39 (AB) c	$F_{(3,67)} = 8.947$ $P<0.0001$
Tomato/ cucumber	5.14±0.46 (B) abc	2.70±0.35 (A) ab	5.42±0.54 (B) ab	3.55±0.47 (AB) ab	$F_{(3,66)} = 7.095$ $P<0.0001$
Tomato/basil	7.15±0.36 (B) d	5.57±0.40 (A) c	8.90±0.27 (B) d	6.79±0.73 (AB) c	$F_{(3,68)} = 8.424$ $P<0.0001$
Tomato/fennel	4.80±0.15 (A) b	4.32±0.41 (A) bc	6.23±0.26 (B) b	5.42±0.22 (AB) c	$F_{(3,68)} = 9.006$ $P<0.0001$
	$F_{(5,101)} = 16.695$ $P<0.0001$	$F_{(5,100)} = 15.735$ $P<0.0001$	$F_{(5,101)} = 17.336$ $P<0.0001$	$F_{(5,101)} = 12.823$ $P<0.0001$	

Different letters indicate significant differences; capital letters among rows, i.e. plant combinations and letters among columns, i.e. treatments (ANOVA and Tamhane's test $P<0.05$)

[a] Data were calculated for the stronger tomato plant/pot according to the root weight

[b] Data were calculated for the weaker tomato plant/pot according to the root weight

Effect of intercropping, AMF and *F. oxysporum* f. sp. *lycopersici* on tomato growth

Plant growth of tomato in the different plant combinations and treatments was assessed by root and shoot weights.

Root effects

Root weights for the treatments with *F. oxysporum* f. sp. *lycopersici* and/or AMF application within the different plant combinations are shown in Table 2. The factor 'intercropping partner' had in each treatment a significant effect on tomato root weights (for p values, see Table 2). Root weights of T_1 and T_2 of the TT combination in the control treatment differed significantly, indicating an intraspecific effect of tomato plants. Plants of T_2 had almost 50 % less weight than the ones from T_1. TL and TB showed the highest tomato root weights, whilst TC and TF ranged between the lowest and the highest root weights. These trends were also seen in the *F. oxysporum* f. sp. *lycopersici*, AMF and AMF + *F. oxysporum* f. sp. *lycopersici* treatments.

The factor 'treatment' had in each dual culture a significant influence on root weights (for p values, see Table 2). For T_2 within the TT treatment, *F. oxysporum* f. sp. *lycopersici* as well as AMF + *F. oxysporum* f. sp. *lycopersici* significantly reduced the root weight compared to the control treatment. For T_1, this effect could not be observed. A significant reduction of the root weights in the F. oxysporum f. sp. lycopersici treatment compared to the control could also be observed in TC. The AMF treatment over all plant combinations showed similar root weights as the corresponding control treatments, apart from TL, TF and T_1 of TT which showed an increase in root weights. AMF + *F. oxysporum* f. sp. *lycopersici* over all plant combinations did not show a change in root weights compared to the *F. oxysporum* f. sp. *lycopersici* or control treatment, apart from AMF + *F. oxysporum* f. sp. *lycopersici* of T_2, where the root weight was reduced compared to the control. There was no significant correlation between root weight and AM colonisation rate ($P=0.113$, $R^2=0.012$); however, root weight was negatively correlated with disease severity ($P<0.0001$, $R^2=0.233$).

Shoot effects

Shoot weights of tomato plants from the different plant combinations are shown in Table 3. The factor 'intercropping partner' had a significant effect on tomato shoot weights in each treatment (for p values see Table 3). Within the control treatment, T_2 of the TT combination showed around 40 % less shoot weight than T_1. The highest shoot weights were reached by tomato plants of the TB and TL combinations (increases up to 320 and 180 %, compared to T_2 and T_1, respectively). The shoot weights of the tomato plants of the TC and TF combinations ranged between 11.49 and 13.

06 g and were therefore higher than the shoot weights of T_2 (6.23 g).

The factor 'treatment' had a significant influence on shoot weights in each dual culture (for p values, see Table 3). In the TT combination, shoot weights of T_1 showed a significant increase of 70–80 % in the AMF and AMF + *F. oxysporum* f. sp. *lycopersici* treatments, compared to the control and *F. oxysporum* f. sp. *lycopersici* treatment. A similar picture can be seen within the TL and TF combinations, where the shoot weights increased up to 200–230 % and 170–210 %, respectively, compared to the control and *F. oxysporum* f. sp. *lycopersici* treatment. In T_2 of the TT combination, the *F. oxysporum* f. sp. *lycopersici* treatment reduced the shoot weights of the plants compared to the control and the AMF treatment. On the other hand, AMF as well as AMF + *F. oxysporum* f. sp. *lycopersici* increased the shoot weights as compared to the control and the F. oxysporum f. sp. lycopersici treatment in T_1 of the TT combination, the TL and the TF combinations. In T_2 of the TT combination, the TC and TB combinations, AMF increased the shoot weights compared to the *F. oxysporum* f. sp. *lycopersici* treatment, whereas the AMF + *F. oxysporum* f. sp. *lycopersici* treatment did not change the shoot weights compared to the *F. oxysporum* f. sp. *lycopersici* treatment. There was a low positive correlation between shoot weight and AM colonisation levels ($P<0.01$, $R^2=0.040$); however, shoot weight was negatively correlated with disease severity ($P<0.0001$, $R^2=0.200$).

Discussion

Mycorrhizal symbiosis with its aspects of biofertilization and bioprotection is of special interest in the context of sustainable agriculture. The present work assessed the impact of AMF in an intercropping system with the main focus on the performance of tomato (cv. Kremser Perle). As far as AM root colonisation of tomato is concerned, this was clearly affected by the intercropping plant partners. Tomato intercropped with leek showed a 20 % higher colonisation level than tomato intercropped with tomato. Fennel on the other hand decreased the colonisation level of tomato by 13 %. Leek had a similar colonisation level (55.11 %) as its intercropping partner tomato. However, the AM colonisation values of leek had a very high standard deviation, probably due to the rather small root system leading to a small amount of material for AM colonisation assessment. The well-established AM symbiosis in leek stimulated the colonisation of tomato, an effect that is concordant with data reported by Cavagnaro et al. (2004), and additionally, is in line with the frequent use of leek as nurse plants in experimental set-ups (Smith and Read 2008). However, a high AM colonisation level of the intercropping partner does not necessarily imply an increase in colonisation of the other one, as can be seen in the results with cucumber and fennel.

The small size of the rooting system of leek and as a consequence a faster colonisation of the root system and

Table 3 Shoot weights (in grams) of tomato plants grown in a dual culture system (mean ± S.E.)

Dual culture	Treatment				
	Control	*F. oxysporum* f. sp. *lycopersici*	AMF	AMF + *F. oxysporum* f. sp. *lycopersici*	
Tomato/tomato (T_1)[a]	10.79±0.83 (A) b	10.11±0.70 (A) bc	19.17±1.32 (B) b	16.66±1.60 (B) bc	$F_{(3,66)} = 15.279$ $P<0.0001$
Tomato/tomato (T_2)[b]	6.23±0.53 (B) a	4.15±0.28 (A) a	10.22±1.08 (C) a	8.21±1.49 (ABC) a	$F_{(3,67)} = 7.398$ $P<0.0001$
Tomato/leek	18.67±0.72 (B) c	14.10±1.20 (A) cd	31.73±1.31 (C) d	28.71±2.32 (C) d	$F_{(3,67)} = 30.005$ $P<0.0001$
Tomato/ cucumber	11.49±0.66 (A) b	7.95±1.22 (A) ab	16.72±1.50 (B) b	12.62±1.94 (AB) ab	$F_{(3,66)} = 6.293$ $P=0.001$
Tomato/basil	19.05±0.86 (A) c	16.85±1.21 (A) d	27.94±1.96 (B) d	21.77±2.11 (AB) cd	$F_{(3,68)} = 8.789$ $P<0.0001$
Tomato/fennel	13.06±0.58 (A) b	11.15±0.80 (A) bc	23.78±1.67 (B) bc	21.96±1.77 (B) c	$F_{(3,66)} = 23.058$ $P<0.0001$
	$F_{(5,101)} = 48.299$ $P<0.0001$	$F_{(5,100)} = 22.359$ $P<0.0001$	$F_{(5,101)} = 26.616$ $P<0.0001$	$F_{(5,100)} = 15.169$ $P<0.0001$	

Different letters indicate significant differences; capital letters among rows, i.e. plant combinations and letters among columns, i.e. treatments (ANOVA and Tamhane's test $P<0.05$)

[a] Data were calculated for the stronger tomato plant/pot according to the root weight

[b] Data were calculated for the weaker tomato plant/pot according to the root weight

development of hyphal networks, which are apart from spores and root fragments, of high significance in AM root colonisation (Smith and Read 2008) might be the reason for the increased colonisation rates compared to intercrops like tomato, cucumber, basil and fennel. Furthermore, putative colonisation preferences could have more impact when larger root systems are available. The colonisation preference observed for cucumber of the AMF used in the present work is consistent with the findings of Kubota and Hyakumachi (2004), who tested cucumber as well as tomato in soils of different vegetation sites with native AMF species and found a clear colonisation preference of AMF for cucumber; however this is not in an intercropping setting with different AMF. The importance of intercropping or individual settings can be seen in the work of Cavagnaro et al. (2004), who reported that mycorrhizal responsiveness of the tomato variety 76R to *Glomus coronatum* depended on the presence or absence of a mycorrhiza-defective tomato mutant derived from cv. 76R (i.e. a setting with interspecific competition). It therefore appears probable that the outcome of an AMF–plant interaction in intercropping settings cannot be predicted from individual settings. The significance of the intercropping partner for AM colonisation levels is clearly shown in the present study on tomato.

The investigation of the influence of AMF and/or *F. oxysporum* f. sp. *lycopersici* on growth of intercropped tomato showed that the intercropping partners of tomato impacted on root and shoot weight. Most striking was the reduction of 50 and 40 %, respectively, in the root and shoot weights of one tomato plant/pot in the TT control treatment compared to the other tomato plant. AMF did not change the effects of the intercropping partner on these growth parameters compared to the control treatment. Root and shoot weight increases were AMF-dependent and not intercropping partner-dependent. Schroeder-Moreno and Janos (2008) found that AMF had negative effects on the root as well as on the shoot weights of chilli, maize and zucchini grown in intraspecific density settings. Negative effects on tomato root or shoot weights due to AMF could not be observed in the present work. However, lowest density here was two and not three plants, as in the work of Schroeder-Moreno and Janos (2008). With regards to interspecific competition, van der Heijden et al. (2003) clearly showed that the AMF species can influence the outcome of a competitive situation between plants. Working with *Brachypodium pinnatum* and Prunella vulgaris, these authors observed that the way in which the two plants coexisted depended on the *Glomus* isolate inoculated. In the present work, six different *Glomus* species were inoculated together; it would be interesting to test different AMF inocula separately in the same intercropping setting to find out more about such dynamics.

When assessing the influence of AMF inoculation in intercropping systems on *F. oxysporum* f. sp. *lycopersici* disease severity in tomato, a bioprotective effect was observed. This bioprotective effect resulted in a reduced disease severity in AM plants of the T_1, TL and TB treatments. Looking on T_1, TL and TF, AMF + *F. oxysporum* f. sp. *lycopersici*-treated plants produced more shoot biomass compared to the *F. oxysporum* f. sp. *lycopersici*-treated plants. Thus, mycorrhization enhances the tolerance of the tomato plants to the pathogen. Positive effects of AMF, expressed as an increase in biomass compared to the control treatment, could be observed for root as well as shoot weights. For roots, these effects could be observed for the AMF treatment in T_1 of TL and TF, whilst for shoots, the positive effects on biomass could be observed for AMF as well as AMF + *F. oxysporum* f. sp. *lycopersici* treatments in T_1 of TT, TL and TF combinations. *F. oxysporum* f. sp. *lycopersici* clearly reduced and AMF clearly increased shoot biomass for T_2 and TL. Consequently, the positive effects of AMF co-inoculation with F. oxysporum f. sp. lycopersici on plant biomass are shown in shoot weights and not in reduced disease incidence. Compensation by AMF for the negative effects of *F. oxysporum* f. sp. *lycopersici* on plant biomass could particularly be reached with leek as intercropping partner. Thus, a well-chosen intercropping partner like leek and basil can allow expression of a bioprotective effect of AMF, even when the symbiosis is not established before pathogen inoculation. A different experimental set-up, like in Dehne and Schönbeck (1979), where AMF were applied 6 and 9 weeks, respectively, before *F. oxysporum* f. sp. *lycopersici* inoculation might have led to more positive effects. However, a simultaneous inoculation in an intercropping setting appears to be more comparable to natural conditions where concurrent activity of AMF and *F. oxysporum* f. sp. *lycopersici* will not be uncommon.

When tomato was intercropped with tomato, one intercropping partner turned to be the ‘stronger’ and the other the ‘weaker’ one, which also clearly affected the disease incidence and disease severity. Thus, keeping tomato in a competitive situation with itself has adverse effects on the biomass of one of the intercropping partners. The ‘weaker’ partner also showed significantly higher *F. oxysporum* f. sp. *lycopersici* disease severity and no positive effects of AMF inoculation. Therefore, we conclude that the positive effects of AMF on disease severity are limited in competitive situations, and that the intercropping partner affects the positive effects of AMF on tomato plants with regard to *F. oxysporum* f. sp. *lycopersici* disease severity. Results indicate that leek and basil are candidates for further intercropping set-ups, where further questions like fruit yield and fruit quality of tomatoes should be assessed and also the impact of pre-colonisation by AMF of intercropping partners could be investigated.

To summarise, tomato intercropped with different species had no effect on *F. oxysporum* f. sp. *lycopersici* disease incidence or disease severity indicating no allelopathic sup- pression. However, tomato intercropped with tomato clearly showed negative effects on one plant/pot with regard to biomass and disease severity of *F. oxysporum* f. sp. *lycopersici*. Furthermore, crucial effects of the intercropping partner on AMF colonisation of tomato were found, which is of great interest in crop plant communities and the in- fluences on each other. However, the outcome of the AMF effects on *F. oxysporum* f. sp. *lycopersici* disease severity and/or plant biomass did not depend on the degree of AM colonisation but more on the intercropping partner.

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2.2 A comparison of wild-type, old and modern tomato cultivars in the interaction with the arbuscular mycorrhizal fungus *Glomus mosseae* and the tomato pathogen *Fusarium oxysporum* f. sp. *lycopersici*

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A comparison of wild-type, old and modern tomato cultivars in the interaction with the arbuscular mycorrhizal fungus *Glomus mosseae* and the tomato pathogen *Fusarium oxysporum* f. sp. *lycopersici*

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Abstract The effect of the arbuscular mycorrhizal symbiosis (AM) varies in plant cultivars. In the present study, we tested whether wild-type, old and modern tomato cultivars differ in the parameters of the AM interaction. Moreover, the bioprotective effect of AM against the soilborne tomato pathogen *Fusarium oxysporum* f. sp. *lycopersici* (Fol) was tested in the different cultivars. Ten tomato cultivars were inoculated with the arbuscular mycorrhizal fungus (AMF) *Glomus mosseae* alone or in combination with Fol. At the end of the experiment, AM root colonization, Fusarium infection, and the plant fresh weight was determined. The tomato cultivars differed in their susceptibility to AMF and Fol, but these differences were not cultivar age dependent. In all the cultivars affected by Fol, mycorrhization showed a bioprotective effect. Independent of the cultivar age, tomato cultivars differ in their susceptibility to AMF and Fol and the bioprotective effect of mycorrhization, indicating that the cultivar age does not affect the AM parameters tested in this study.

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Keywords Arbuscular mycorrhiza · Bioprotection · *Fusarium oxysporum* f. sp. *lycopersici* · *Glomus mosseae* · Tomato cultivars

Introduction

Arbuscular mycorrhizal fungi (AMF) colonize roots of more than 80% of vascular plants and form a symbiotic association, the arbuscular mycorrhizal symbiosis (AM), improving the nutritional status of the host plant and enhancing its tolerance to biotic and abiotic stress (Smith and Read 2008). The effect of the AM varies in the interaction with their hosts and this variation can be observed even at the plant cultivar level (recently reviewed by Estaún et al. 2010).

Most data on mycorrhizal dependence of plant cultivars are available with cereals (Azcón and Ocampo 1981; Lackie et al. 1987; Kapulnik and Kushnir 1991; Vierheilig and Ocampo 1991a, b; Hetrick et al. 1992, 1993; Kirk et al. 2008; Castellanos-Morales et al. 2011). In wheat, it was suggested that depending on the age of the cultivar, the degree of AM colonization and the responsiveness varies (Hetrick et al. 1993). Compared to modern cultivars, old wheat cultivars showed higher degrees of root colonization and were more responsive to AM colonization; however, in a more recent study, this could not be confirmed (Kirk et al. 2008).

Apart from improving the nutritional status of the host plant, the mycorrhizal symbiosis provides bioprotection against a number of soilborne fungal pathogens (St-Arnaud and Vujanovic 2007). *Fusarium oxysporum* f. sp. *lycopersici* (Fol) is the cause of a severe wilt disease and is an important

pathogen in tomato cultures (Jones 1991); however, data on the interaction of AMF with Fol are scarce. Recently, the germination of microconidia, which are efficient propagules of Fol for the infection of tomato plants, has been shown to be stimulated by tomato root exudates (Steinkellner et al. 2005, 2008a, b). Interestingly root exudates of plants colonized by AMF have been shown to exhibit a different effect on microconidia germination of Fol than root exudates of non-mycorrhizal plants (Scheffknecht et al. 2006, 2007). Moreover, co-inoculation of tomato with AMF and Fol resulted in a certain bioprotection (Dehne and Schönbeck 1979; Akköprü and Demir 2005).

To our knowledge, no comparison of tomato cultivars has been performed yet for the AM interaction and the bioprotective effect of AM against Fol. In the present work, we tested whether in wild-type, old, and modern tomato cultivars, these parameters differ.

Materials and methods

Plant and fungal materials

Ten tomato cultivars (Table 1) representing heirloom (old) tomatoes, modern cultivars, and wild types were selected. Tomato seeds were surface sterilized by soaking in 50% household bleach (Dan Clorix, 3.8% NaOCl) for 10 min. After three rinses with tap water, the seeds were transferred to pots containing autoclaved perlite (Granuperl S 3-6, Knauf Perlite GmbH, Vienna, Austria) and incubated in a growth chamber (York International) with a 16-h light (light intensity 296 mol m^{-1} s^{-1}) and 8-h dark photoperiod at 24°C for 2 weeks. The plants were irrigated with tap water during this germination period.

Fol isolate Fol007 (race 2) was subcultured for 2 weeks at 24°C in darkness on Czapek Dox Agar before using it for plant inoculation. A suspension containing microconidia was obtained by flooding the colonies with sterile, distilled water; separating the microconidia from the mycelium using a trigalski spatula; and filtering the resulting suspension through three layers of filter paper. The concentration of the microconidia suspension was adjusted to 10^5 microconidia ml^{-1}.

For the inoculation of the plants with the AMF *Glomus mosseae* (BEG 12), a commercially available inoculum was purchased from BIORIZE/Agrauxine (Quimper, France).

Plant bioassay

After 2 weeks, the plantlets (plant growth stage 11, BBCH scale) were transferred into pots containing an autoclaved (20 min at 121°C) mixture of sand, soil, and expanded clay (1:1:1, v/v/v). The plants were grown in a random design in the greenhouse under natural conditions. The transplanted, inoculated plants were cultivated further for 11 weeks. According to their moisture requirements, they were watered every second day with a low concentration nutrient solution (Steinkellner et al. 2005). If necessary, during the days in between, plants were watered with tap water.

The experimental set-up included (1) a control treatment without AMF and without Fol; (2) an AMF treatment; (3) a Fol treatment; and (4) a combined AMF–Fol treatment. Each treatment comprises eight plants.

For the AMF treatment, 4 ml of the AMF inoculum were added directly into the planting hole, to the roots of each plantlet, when the plantlets were transferred to the sterile substrate mixture. To inoculate the plantlets with Fol, the roots were dipped in a microconidial suspension for 5 min. Water was used for mock inoculation. For the combined AMF–Fol treatment, the root dip method as described above was used; afterwards, 4 ml of the AMF inoculum was added.

Eleven weeks after transplanting, the plants were removed from the substrate, gently washed under running tap water to preserve the root system, and dried between folded paper towels. The Fusarium infection was determined visually according to Wellman (1939). Afterwards, roots and shoots were separated and the fresh weights were

Table 1 Tomato cultivars selected to represent various cultivar ages

Cultivar	Type	Cultivar age	Reference source
Lycopersicon hirsutum	Wild type		Arche Noah
Lycopersicon peruvianum	Wild type		Arche Noah
Yellow Pearshaped	Heirloom (old)	Older than 1900	Austrosaat
Kremser Perle	Heirloom (old)	Older than 1900	Austrosaat
Marmande	Heirloom (old)	Older than 1900	Austrosaat
Rheinlands Ruhm	Heirloom (old)	Older than 1960	Austrosaat
Vitamina	Heirloom (old)	Older than 1960	Arche Noah
Apero F1	Modern cultivar	Younger than 1970	Austrosaat
Myriade F1; F2	Modern cultivar	Younger than 1970	Austrosaat
Supersweet F1	Modern cultivar	Younger than 1970	Austrosaat

F1 resistance to Fol, race 1; F2 resistance to Fol, race 2 (used in the present study); Arche Noah The Austrian Seed Savers Association, Schiltern, Austria; Austrosaat Österreichische Samenzucht- u. Handels-Aktiengesellschaft, Vienna, Austria

Table 2 AM colonization percentage (means ± standard error) of tomato cultivars inoculated with AMF (G. mosseae) and co-inoculated with the AMF and Fol (*F. oxysporum* f. sp. *lycopersici*)

Cultivar means with different letters indicate a statistically significant difference following the LSD test (p=0.0000). There were no significant differences between the Fol and the AMF and Fol treatment (p=0.7321). The interactions between cultivar and treatment were significant (p=0.0071)

Cultivar	AMF	AMF and Fol	Mean
L. hirsutum	19.75 (±3.88)	9.38 (±1.46)	14.56 (±2.05) b, c
L. peruvianum	14.00 (±2.75)	17.86 (±2.64)	15.78 (±2.12) b, c
Yellow Pearshaped	14.25 (±2.69)	10.00 (±10.00)	13.22 (±2.63) b, c
Kremser Perle	27.00 (±1.39)	17.25 (±2.68)	22.13 (±2.05) d, e
Marmande	2.63 (±1.69)	4.63 (±1.34)	3.63 (±2.05) a
Rheinlands Ruhm	11.25 (±2.93)	22.20 (±3.81)	15.39 (±2.28) b, c
Vitamina	9.75 (±2.99)	11.38 (±2.68)	10.56 (±2.05) b
Apero	22.33 (±1.90)	26.88 (±4.71)	24.97 (±2.20) e
Myriade	17.38 (±3.05)	19.25 (±1.75)	18.31 (±2.05) c, d
Supersweet	13.13 (±3.06)	8.00 (±3.14)	10.71 (±2.12) b
Mean	15.22 (±0.93)A	14.63 (±1.01)A	

determined. To confirm the infection with Fol, segments of 2-cm length starting upwards the shoot basis were dipped in 70% ethanol, flamed, and put into Petri dishes containing potato dextrose agar (containing 10 mg/l Streptomycinsulfat and 10 mg/l Chloramphenicol to prevent bacterial growth). The presence of Fol was determined by visual and microscopic analysis of the morphology of the mycelium and the conidia based on Nelson et al. (1983). The degree of mycorrhization was estimated on defined fresh root segments of 1-cm length, starting 2 cm down the shoot. The roots were cleared by boiling for 4 min in 10% KOH, rinsed three times with tap water, and stained by boiling for 4 min in a 5% ink (Sheaffer black) according to the method of Vierheilig et al. (1998). Thereafter, the percentage of root colonization was determined according to the counting procedure of McGonigle et al. (1990).

Statistical analysis

The effects of cultivars and treatments were determined by two-factorial analysis of variance. Analysis of variance was done after a variance check by the Levene's test. Mean values were compared using Fisher's least significant difference ($p<0.05$). These analyses were performed using appropriate standard statistical methods (Statgraphics Plus 5.0).

Results

The percentage of AM root colonization was determined in roots of plants co-inoculated with the AMF and Fol (AMF/Fol) and in the roots of plants inoculated only with the AMF (Table 2). The examination of the roots of the control plants and the Fol-inoculated plants for a possible contamination with AMF was negative.

AM root colonization levels differed between the cultivars. One cultivar that is older than 1900 showed the highest (Kremser Perle), whereas another one that is older than 1900 showed the lowest levels of AM root colonization (Marmande) and the modern cultivar Apero (younger than 1970) showed relatively high AM root colonization levels. All other cultivars including the two wild-type tomatoes (*Lycopersicon hirsutum* and *Lycopersicon peruvianum*) showed intermediate levels of AM root colonization.

Looking over all the cultivars, none of the cultivars co-inoculated with Fol affected the percentage of AM root

Table 3 Fusarium infection (diseases index, means ± standard error) of tomato cultivars inoculated with Fol (*F. oxysporum* f. sp. *lycopersici*) and co-inoculated with AMF (*G. mosseae*) and Fol

Columns within a cultivar with different lowercase letters indicate a statistically significant difference following the LSD test (p = 0.0000). There were no significant differences between the Fol and the AMF and Fol treatment (p=0.1875)

Cultivar	Fol	AMF and Fol	Mean
L. hirsutum	0.38 (±0.26)	0.13 (±0.13)	0.25 a
L.peruvianum	2.13 (±1.86)	0.00 (±0.00)	1.06 a
Yellow_Pearshaped	12.00 (±2.04)	11.75 (±2.16)	11.88 e
Kremser Perle	2.63 (±0.50)	1.38 (±0.38)	2.00 a, b, c
Marmande	4.86 (± 0.72)	2.86 (±0.77)	3.86 b, c
Rheinlands_Ruhm	7.00 (±1.76)	7.25 (± 2.36)	7.13 d
Vitamina	1.88 (±0.44)	1.25 (±0.53)	1.56 a, b
Apero	1.86 (±0.35)	2.75 (±0.31)	2.31 a, b, c
Myriade	0.00 (±0.00)	0.00 (±0.00)	0.00 a
Supersweet	5.00 (±2.22)	3.13 (±1.75)	4.06 c

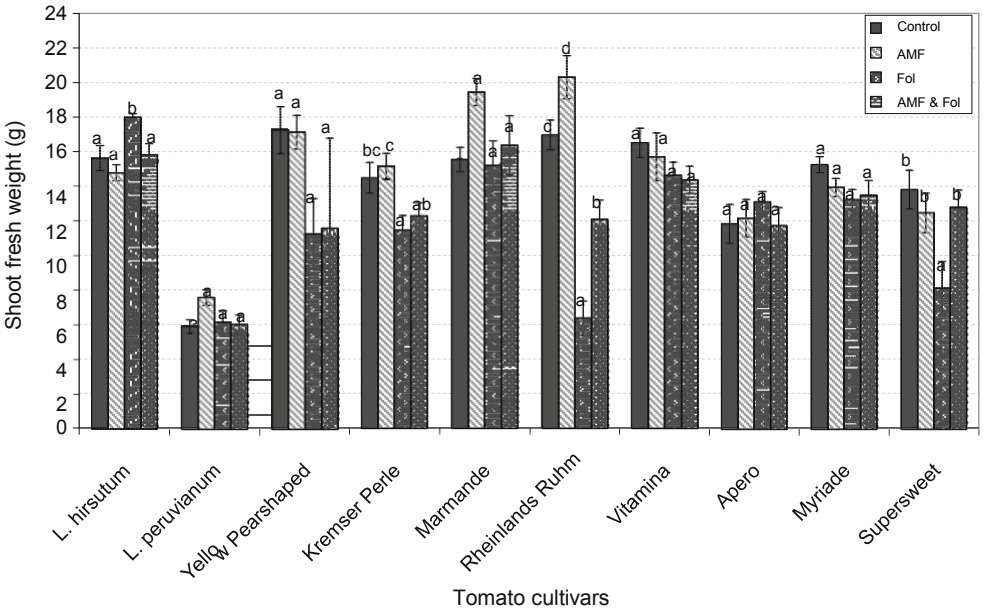

Fig. 1 Shoot fresh weight of uninoculated tomato cultivars and cultivars inoculated with *F. oxysporum* f. sp. *lycopersici* (Fol) and/or the AMF G. mosseae (Means ± SE). Columns within a cultivar with different lowercase letters indicate a statistically significant difference following the LSD test ($p<0.05$)

colonization compared to the "AMF only" treatment. Comparing the percentage of AM root colonization within a cultivar in the AMF/Fol treatment and the "AMF only" treatment, co-inoculation with Fol reduced the percentage of AM root colonization in L. hirsutum and Kremser Perle, whereas an increase could be observed in Rheinlands Ruhm.

The level of Fusarium infection (disease index) was determined in the roots of plants co-inoculated with Fol and the AMF and in the roots of plants inoculated only with Fol (Table 3). The control plants and the AMF-inoculated plants did not show any contamination with Fol.

Fusarium infection differed between the cultivars. Infection was highest in the cultivar Yellow Pearshaped (older than 1900), followed by the cultivars Rheinlands Ruhm (older than 1960) and Supersweet (younger than 1970). All other cultivars showed a lower Fusarium infection. The cultivar Myriade did not show any signs of Fusarium infection.

Looking over all the cultivars, none of the cultivars co-inoculated with AM were affected by Fusarium infection compared to the "Fol only" treatment. Comparing the Fusarium infection within a cultivar in the AMF/Fol treatment and the "Fol only" treatment, co-inoculation with AMF reduced the Fusarium infection in L. peruvianum, Kremser Perle, and Marmande.

Inoculation with AMF and/or Fol affected the shoot and root fresh weight of only a few cultivars. AM inoculation

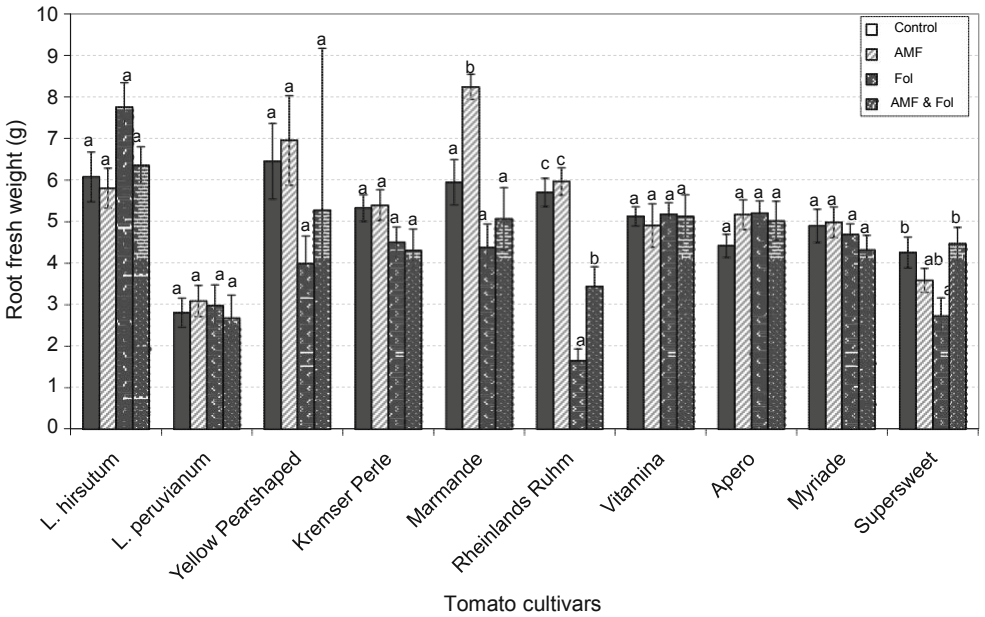

Fig. 2 Root fresh weight of uninoculated tomato cultivars and cultivars inoculated with *F. oxysporum* f. sp. *lycopersici* (Fol) and/or the AMF G. mosseae (means ± SE). Columns within a cultivar with different lowercase letters indicate a statistically significant difference following the LSD test ($p<0.05$)

alone showed an effect with Rheinlands Ruhm (older than 1960) by increasing the shoot fresh weight (Fig. 1) and with Marmande (older than 1900) by increasing the root fresh weight (Fig. 2). Fol inoculation alone reduced the shoot and root fresh weight of only two cultivars (Rheinlands Ruhm and Supersweet) (Figs. 1 and 2), whereas in these two cultivars co-inoculation of AMF and Fol had a positive effect on the shoot and root fresh weight, when compared to the Fol only treatment (Figs. 1 and 2).

Discussion

The effect of AMF varies in the interaction with their hosts, and this variation can be observed even at the plant cultivar level (Estaún et al. 2010). In our study, when looking at AM root colonization, we found a large variability between the tested tomato cultivars, confirming data on the variability of AM root colonization between cultivars from other plant families such as legumes, cereals, and trees (Estaún et al. 2010).

In the tested tomato cultivars, these differences in AM root colonization could not be linked with the age of the cultivar as found in wheat (Hetrick et al. 1993). In the old tomato cultivars (older than 1900), we detected the highest and the lowest levels of AM root colonization, and the modern cultivar Apero also showed high levels of AM root colonization. All other tomato cultivars showed similar intermediate levels of AM root colonization, thus confirming the data with wheat and barley (Kirk et al. 2008; Castellanos-Morales et al. 2011) that the age of the cultivar is not linked with the level of AM root colonization.

In several studies, it has been reported that not only the level of AM root colonization varies between the cultivars but also the growth response (see review Estaún et al. 2010). We found a growth response by mycorrhization in two of the ten tested cultivars showing that in the used experimental system, a positive mycorrhizal growth effect can become evident; however, in our system, no growth effect could be observed in most cultivars.

As Fol is an important pathogen in tomato cultures (Jones 1991), through plant breeding, tomato cultivars have been obtained showing resistance to different Fol races (see Table 1). In our study, cultivars' resistant to certain Fol races had similar levels of AM root colonization as cultivars not resistant to Fol, showing that resistance to Fol does not affect AM root colonization.

Tomato cultivars differ in their susceptibility to Fol and this susceptibility also depends on the Fol race (Jones and Crill 1974; Abawi and Barker 1984; Gao et al. 1995; Larkin and Fravel 2002). In our experiment with Fol race 2, independent of the cultivar age, a high and low susceptibility to Fol was observed indicating that the cultivar age is not linked with the susceptibility to Fol.

In all the cultivars affected by Fol, co-inoculation of cultivars with AMF and Fol resulted in a reduced Fusarium infection and/or an increased plant growth, corroborating a bioprotective effect of mycorrhization against Fol, as reported before (Dehne and Schönbeck 1979; Akköprü and Demir 2005). Looking at our data, it seems that this bioprotective effect of mycorrhization is not linked with the age of the cultivar.

To summarize, independent of the cultivar age, tomato cultivars differ in their susceptibility to AMF and Fol and the bioprotective effect of mycorrhization.

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2.3 Alterations in root exudation of intercropped tomato mediated by the arbuscular mycorrhizal fungus *Glomus mosseae* and the soil-borne pathogen *Fusarium oxysporum* f. sp. *lycopersici*

Alterations in root exudation of tomato

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Alterations in root exudation of intercropped tomato mediated by the arbuscular mycorrhizal fungus *Glomus mosseae* and the soil-borne pathogen *Fusarium oxysporum* f. sp. *lycopersici*

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Abstract

Arbuscular mycorrhizal fungi (AMF) can controls oil-borne diseases such as *Fusarium oxysporum* f. sp. *lycopersici* (*Fol*). Root exudates play an important role in plant–microbe interactions in the rhizosphere, especially, in the initial phase of these interactions. In this work we focus on I) elucidating dynamics in root exudation of *Solanum lycopersicum* L. in an intercropping system due to AMF and/or *Fol*, II) its effect on Fol development *in-vitro* and III) the testing of the root exudate compounds identified in the chromatographic analyses in terms of effects on fungal growth in *in-vitro* assays.

GC-MS analyses revealed an AMF-dependent increase of sugars and decrease of organic acids, mainly glucose and malate. In the HPLC analyses an increase of chlorogenic acid was evident in the combined treatment of AMF and *Fol*, which is to our knowledge the first report about an increase of chlorogenic acid in root exudates of AM plants challenged with *Fol* compared to plants inoculated with AMF only, clearly indicating changes of root exudation due to AMF and *Fol*. Root exudates of AMF tomato plants stimulate the germination rate of *Fol*, whereas, the co-inoculation of AMF and *Fol* leads to a reduction of spore germination.

In the *in-vitro* assays citrate and chlorogenic acid could be identified as possible candidates for the reduction of *Fol* germination rate in the root exudates of the AMF+*Fol* treatment since they proved inhibition at concentrations naturally occuring in the rhizosphere.

Keywords: arbuscular mycorrhizal fungi, *Glomus mosseae*, *Fusarium oxysporum*, root exudates, tomato, spore germination

Introduction

Arbuscular mycorrhizal fungi (AMF) form a mutualistic symbiosis with about 80 % of all herbaceous plants (Smith and Read 2008) and exhibit positive effects, such as plant growth promotion and control of soil-borne diseases (Azcón-Aguilar et al. 2002; Singh et al. 2000; St-Arnaud and Vujanovic 2007; Whipps 2004; Xavier and Boyetchko 2004). According to Whipps (2004) the following modes of action are involved in disease control by AMF: '(*i*) direct competition or inhibition; (*ii*) enhanced or altered plant growth, nutrition and morphology; (*iii*) biochemical changes

associated with plant defence mechanisms and induced resistance; and (*iv*) development of an antagonistic microbiota'. These attributes make AMF valuable for concepts of sustainable agriculture in connection with the control of soil-borne diseases.

Fusarium oxysporum f. sp. *lycopersici* (*Fol*), a soil-borne pathogen, which can cause severe yield losses in tomato plants due to wilting and yellowing of leaves, can be affected by AMF (Akköprü and Demir 2005; Dehne and Schönbeck 1979) resulting in a bioprotective effect due to changes in the root and vessel system. Root exudates and their multiple components (e.g. sugars, organic acids, amino acids, phenolic compounds) play an important role in plant–microbe interactions in the rhizosphere, especially, in the initial phase of these interactions (Nelson 1990; Bais et al. 2006; Straney et al. 2002; Bertin et al. 2003). The germination rate of *Fol* is influenced by root exudates as well (Steinkellner et al. 2005). Nevertheless, the potential alteration in root exudation patterns due to AMF and its putative effect on *Fol* development are not satisfactorily understood. For biochemical changes in tomato plants colonized by AMF and challenged with pathogens data are already available demonstrating systemic effects (Pozo et al. 2002; López-Ráez et al. 2010a; López-Ráez et al. 2010b). In recent years negative effects of root exudates of AM tomato plants on zoospores of *Phytophthora nicotianae* (Lioussanne et al. 2008) and *Meloidogyne incognita* infection (Vos et al. 2012) were reported, also indicating a substantial contribution of root exudates in these plant–pathogen–symbiont interactions.

As reviewed by Jones et al. (2004) and Vierheilig (2004) root exudates of mycorrhizal and non-mycorrhizal plants affects soil-borne pathogens differently. This has also been verified for the tomato pathogen *Fol*. The spore germination rate of *Fol* differs in root exudates of mycorrhizal and non-mycorrhizal host plants (Scheffknecht et al. 2006; Scheffknecht et al. 2007). However, until now the factors contributing to these effects in the germination rate of *Fol* in root exudates of AM colonized tomato plants are unknown.

For this study tomato plants were intercropped with cucumber, which is readily colonized by AMF and, consequently, provides conducive conditions for AMF. Furthermore, is has neutral effects on *Fol* infection rate. The aim of our work was I) to elucidate the dynamics in root exudation of tomato due to AMF and/or *Fol*, II) its effect on *Fol* development *in-vitro* and III) to analyse the components identified in the chromatographic analyses in terms of effects on fungal growth in *in-vitro* assays.

Material and Methods

Plant and fungal material

Seeds of tomato (*Solanum lycopersicum* L. cv. Tiny Tim, B&T World Seeds, Aigues Vives, France) and cucumber (*Cucumis sativus* L. cv. Aztec F1, Austrosaat, Vienna, Austria) were surface sterilized by soaking in 50% household bleach ("Dan Klorix", 3.8 % NaOCl) for 10 min and afterwards rinsed three times with autoclaved distilled water. Tomato seeds were transferred to pots filled with autoclaved perlite (Granuperl S 3-6, Knauf Perlite GmbH, Vienna, Austria) and incubated in a growth chamber (York International, Vienna, Austria) with a 16-h light (light intensity 296 µmol m^{-1} s^{-1}) and 8-h dark photoperiod at 24°C. The perlite was irrigated with tap water. Tomato seeds were pre-cultivated three weeks before transplanting. Due to rapid growth cucumber seeds were used without precultivation.

For plant inoculation and fungal assays microconidial suspensions of *Fusarium oxysporum* f. sp. *lycopersici* (*Fol* 007) were prepared according to Steinkellner et al. (2008).

For the arbuscular mycorrhizal fungi (AMF) plant inoculation a commercially available inoculum of *Glomus mosseae* (BEG 12, Biorize/Agrauxine, Quimper, France) was used.

Experimental set-up and exudate collection

Pre-cultivated plantlets and cucumber seeds were transferred to pots filled with an autoclaved (20 min at 121°C) mixture of sand, soil and expanded clay (1:1:1, v/v/v). The plants were cultivated in a dual culture system with one tomato and one cucumber plant per pot. The experimental set-up included the following treatments: I) *Fol*, II) AMF, III) AMF and *Fol* and IV) a control without *Fol* and AMF. Each treatment comprised 25 pots.

AMF inoculum (4 ml) was added to the planting hole during the potting procedure. *Fol* was applied to the plant roots by dipping the roots for 5 min in a microconidial suspension (1 x 10^5 microconidia ml^{-1}) before plants were transferred into the pots. For the AMF+*Fol* treatment both inocula were added as mentioned above.

Plants were grown in a random design in a greenhouse for 6 weeks and were irrigated according to their moisture requirements with a nutrient solution (Steinkellner et al. 2005).

After 6 weeks plants were gently removed from the substrate und washed thoroughly under tap water. Only tomato plants were used for further processing. Roots of the tomato plants were submerged in acetate buffer (25 mM, pH=5.5) for 6 h. After 6 hours root and shoot fresh weights were determined. Root exudates were adjusted to a concentration of 10 ml g^{-1} root weight, sterile filtrated (0.22 µm, Steriflip® vacuum filter units, Millipore, ROTH, Karlsruhe) and stored at -20°C until further processing. 1 exudate comprised 5 plants and 5 replicates (i.e. exudates) per treatment were prepared.

Fol disease incidence and severity were determined for all tomato plants and represent means of 5 exudate pools, each comprising 5 plants.

Disease severity was determined as followed: tomato plants were rated by using a modified system of Wellman (1939), whereas numerical values were assigned to 5 groups (g1=0.5,1; g2=2; g3=3,4; g4=5,6; g5=≥7). Disease severity was calculated by the following formula:

$$\text{Disease severity} = \frac{5 \times (n_{g1} + 2n_{g2} + 5n_{g3} + 10n_{g4} + 20n_{g5})}{n \text{ diseased plants}}$$

Confirmation of *Fol* infection was done according to Steinkellner et al. (2011) and Nelson et al. (1983) by visual and microscopic analyses.

Tomato root segments of 1-cm length, starting 2 cm down the shoot, were used for determining the degree of mycorrhization. Root segments were cleared by boiling for 3 min in 10 % KOH and afterwards rinsed 3 times with tap water. Root staining was done by boiling the roots for 3 min in a 5 % ink-vinegar solution (Vierheilig et al. 1998). The percentage of root colonization was determined according to the method of McGonigle et al. (1990).

Leaf samples were pooled per exudate, dried at 35° C and used for phosphorus analysis according to Nell et al. (2009) and Gericke and Kurmies (1952).

Chemical analyses

Tomato root exudates were fractionated on an Amberlite XAD-1180 (Fluka Chemie GmbH, Buchs, Switzerland) to hydrophilic and lipophilic compounds. The hydrophilic compounds were obtained by eluting with MQ water, lipophilic compounds by elution with absolute ethanol.

For GC-MS analyses the hydrophilic fraction was hydrolyzed by 10% HCl for three hours at room temperature. Further analyte derivatisation followed recommended procedures (Kanani et al. 2008). Dried samples were dissolved in 50 µl of a solution of methoxyamine hydrochloride (Sigma Aldrich, Schnelldorf, Germany) in pyridine (20 mg ml^{-1}). After incubation at room temperature for 18 h, 50 µl *N*-methyl-*N*-TMS-trifluoroacetamide (MSTFA, Thermo Scientific, Pittsburgh, PA) were added for derivatisation into trimethylsilyl ethers and esters. 0.5 µl of this solution were injected into an AutoSystem XL gas chromatograph (Perkin Elmer Inc., Waltham, MS) in the splitless mode. The temperature of the Programmed Temperature Vaporization (PTV) injector was raised from 100 to 300 °C in 5 min and then dropped to 250 °C. The temperature gradient started at 70 °C and rose to 300 °C at a constant rate of 3°C min^{-1}. A ZB-5ms (25 m x 0.25 mm, 0.25 µm film thickness; Phenomenex, Torrance, CA) column was used, the helium follow rate was 0.8 ml min^{-1}. The Autosystem gas chromatograph was coupled to a TurboMass quadrupol mass spectrometer (Perkin Elmer, Waltham MS). The transfer line temperature was set to 280 °C, the ion source to 200 °C, the filament emission to 70 eV. The mass spectrometer was run in the total ion current (TIC) mode from 40 to 620 amu. Experimental time was 100 minutes.

The obtained chromatograms were integrated with Turbomass 4.1.1 (Perkin Elmer Inc., Waltham, MS) and the peak areas of measured compounds where converted to relative amounts (% of the total peak area of every chromatogram). Mass spectra were tentatively identified by comparison with commercial (NIST 08, National Institute of Standards and Technology, Gaithersburg, MD) and non-commercial databases (Kopka et al. 2005) and grouped into substance classes.

For HPLC-UV analyses of lipophilic analytes dried exudates were diluted in methanol (Chromasolv grade, 10 mg ml^{-1}). The analyses were carried out using a Dionex Summit HPLC (Dionex, Sunnyvale, USA) equipped with a photodiode array detector

and a Famos autosampler (LC Packings, Amsterdam, Netherlands). The column used was a Phenomenex Synergi Max C12 (150 x 2 mm, Phenomenex, Torrance, CA). The particle size of the stationary phase was 5 µm. The column oven was adjusted to 40 °C and the flow rate was adjusted to 0.2 ml min^{-1}. Solvent A was prepared as follows: water/methanol/*o*-phosphoric acid (9:1:0.5, v/v/v), solvent B was pure methanol. The solvent gradient started with 100 % of solvent A for 2 minutes and subsequently changed linearly to 100 % of solvent B within 98 minutes. Finally, this concentration was held for further 10 minutes. Five µl of each sample were injected. The UV-spectra were recorded from 220–590 nm; the signal wavelength was set to 229 nm.

Peak areas are expressed as % of the total peak of a chromatogram. Wherever possible, tentative structure assignment of structures was carried out by comparison of UV spectra with those in an in-house library of standards and reference compounds.

Fungal growth assays

Spore germination assays of *Fol* in tomato root exudates were prepared as followed: aliquots of 500 µl of root exudates or acetate buffer (25 mM, pH=5.5) were mixed with 100 µl of a *Fol* conidial suspension (1 x 10^7 microconidia ml^{-1}) in 24-well-culture plates (Greiner bio-one, Nr. 662160, Frickenhausen, Germany) and incubated at 24 °C in the dark while shaking (200 rpm) for 20 h. After 20 h 200 spores in each well were checked under the microscope for germination. The tests were run in triplicates.

For spore germination and mycelial development assays of *Fol* in selected root exudate compounds Czapek Dox broth composition (Singleton et al. 1992) was modified as followed: 3 mg L^{-1} $NaNO_3$, 1 mg L^{-1} K_2HPO_4, 0.5 mg L^{-1} KCl, 0.5 mg L^{-1} $MgSO_4$ x 7 H_2O and 0.01 mg L^{-1} $FeSO_4$ x 7 H_2O. All components were mixed in acetate buffer (25 mM, pH=5.5). This mixture was used as the standard medium. Based on the chemical analyses for the single compound testing the following compounds were used: glucose (α-D(+)-glucose monohydrate, ROTH, Karlsruhe, Germany), fructose (D-fructose Alfa Aesar GmbH & Co KG, Karlsruhe, Germany), citrate (citric acid monohydrate, ROTH, Karlsruhe, Germany), malate (DL-malic acid,

Merck Chemicals, Darmstadt, Germany), succinate (succinic acid, Sigma-Aldrich, Schnelldorf, Germany), tryptophan (DL-tryptophan), cinnamic acid (trans-cinnamic acid), protocatechuic acid (3,4-dihydroxybenzoic acid, Alfa Aesar GmbH & Co KG, Karlsruhe, Germany), chlorogenic acid and salicylic acid (Fluka Chemie GmbH, Buchs, Switzerland). The following concentrations were prepared: 0.003, 0.03, 0.3, 3 and 30 mM. Due to the limits of solubility no 30 mM concentration were prepared for tryptophan, cinnamic acid, chlorogenic acid and salicylic acid. Non-polar compounds were dissolved in DMSO before mixing with the standard medium. Polar compounds were directly dissolved in the standard medium. pH was adjusted to 5.5 with 10 M NaOH.

For the spore germination assays aliquots of 175 µl standard medium or single compound solution and 35 µl of conidial spore suspension (10^7 microconida ml^{-1}) were used per well of 96-well plates (NUNCLON™ Δ Surface, F 96 MicroWell™ Plates, NUNC™ Brand Products, Denmark). For each compound and concentration 3 wells were used. Assays were run in triplicates. Plates were incubated for 24 h in the dark at 24 °C on a rotary shaker (200 rpm), after incubation 200 spores per well were checked under the microscope for germination.

Mycelial growth was determined by OD (optical density) measurements (Broekaert et al. 1990). 96-well plates were prepared like the spore germination assay but instead of 3 wells 6 wells were loaded. 4 of them were filled with living spores, 2 of them served as blanks and contained no spores. This assay was also run in triplicates. Plates were incubated in the dark at 24°C on a rotary shaker (200 rpm), OD measurements (λ=600 nm, FLUOstar Omega, BMG LABTECH GmbH) were carried out every 24 h on 4 consecutive days.

Statistical analyses

Data were analysed by one-way ANOVA and Bonferroni's post-hoc test (total exudates) or Tukey's post-hoc test. Homogeneity of variance was tested with Levene's test, when necessary data were transformed. All statistical analyses were performed using PASW Statistics 18 (Version 18.0.0, IBM).

Results

Plant growth, AMF colonization and disease assessment

AMF increased the root weights of tomato compared to the *Fol* and AMF+*Fol* treatment (Table 1). However, compared to the control no differences could be detected. Shoot weights were not affected by the application of *Fol*, AMF or AMF+*Fol*. Disease incidence and disease severity of *Fol* did not differ between the *Fol* and the AMF+*Fol* treatment. AMF colonization rates were equal in the AMF and AMF+*Fol* treatment. Treatments without *Fol* or AMF did not show any *Fol* infection or AMF colonization. P levels did not show any significant difference between treatments.

GC-MS analyses of hydrophilic root exudate analytes

In the polar fraction of the root exudates sugars and organic acids had the highest peak areas ranging between 32-66 % and 29-54 %, respectively (Fig. 1). Amino acids with 1-13 % had the lowest relative peak area in the polar fraction of the root exudates. Within the organic acids the AMF and AMF+*Fol* treatments had around 40 % less organic acids than the control and the *Fol* treatment. In the sugars the AMF and AMF+*Fol* treatments had around 100 % more relative amount of sugar than the control treatment.

Glucose, fructose, malate, citrate and succinate were selected according to their prevalence in the analysed root exudates and are presented in Fig. 2. Glucose was more prevalent in exudates of the AMF and AMF+*Fol* treatment compared to the control and *Fol* treatment. For malate the situation appeared to be inverted, showing more prevalence in the control and *Fol* treatment. Fructose shows a high prevalence in the exudates (24-48 %), whereas citrate and succinate showed a lower prevalence compared to malate and glucose.

HPLC–UV analyses

In the non-polar fraction of the root exudates 5 main compounds could be identified (Fig. 3), namely, tryptophan, protocatechuic acid, chlorogenic acid, saylicylic acid and cinnamic acid. The relative peak area ranged from 0.3 to 7.1 %. Chlorogenic acid was the only compound where a difference between the different treatments could be

detected. The relative amount of chlorogenic acid was significantly increased in the AMF+*Fol* treatment by almost 200 % compared to the control treatment.

Fungal growth assays

Spore germination rates of *Fol* in root exudates of the different treatments were tested and ranged between 59 and 79 % (Fig. 4). *Fol* in root exudates of the AMF+*Fol* treatment exhibited a 10 % and 20 % lower germination rate than *Fol* in the control and AMF treatments, respectively.

Furthermore, the influence of different chemical compounds found in the root exudates on the germination rate of *Fol* was tested (Table 2; Table 3). Germination rates were normalized to the standard medium (34 % ± 0.7 (mean ± S.E.)). Glucose and fructose increased *Fol* germination rate compared to the standard medium (Table 2). For glucose concentrations from 0.3 mM to 30 mM increased the germination rate significantly, for fructose concentrations from 3 to 30 mM also led to a significant increase compared to the standard medium, however, the effects of glucose were higher with 26 to 36 % increase compared to fructose with around 10 % increase. The organic acids on the contrary had neutral to negative effects on the spore germination rate. Malate and succinate showed neutral effects apart from the highest concentration (30 mM), where malate also showed a reduction of the spore germination rate to 60 %. Citrate reduced the germination rate apart from the 0.03 mM concentration.

The germination rate of *Fol* was either not or negatively affected by the selected compounds of the non-polar fraction (Table 3). At 30 mM concentration protocatechuic acid reduced the spore germination to 53 % and cinnamic acid at 3 mM to 25 %. A lower reduction to 80 % could be observed in chlorogenic acid at a concentration of 0.003 mM. Salicylic acid reduced spore germination at the 0.003, 0.3 and 3 mM concentration.

To test the growth of *Fol* within 96 h in the different chemical compounds OD measurements were carried out. Living spores of *Fol* caused discoloration in the samples of protocatechuic acid and chlorogenic acid mixed with living spores and were therefore not comparable. Malate, citrate, succinate and salicylic acid did not show any differences in growth compared to the standard medium (data not shown).

Glucose and fructose showed an increase of OD values at the 3 and 30 mM concentration (Fig. 5a, b). All concentrations of tryptophan (Fig. 5c) showed the same effects on mycelial growth of *Fol* until 48 h. Afterwards the growth of *Fol* in the 0.3 and 3 mM solution was increased compared to the standard medium and to the other concentrations. With cinnamic acid the picture is different. In the 3 mM solution *Fol* showed no growth within the first 24 hours. After 24 hours *Fol* started to grow but the OD levels were clearly lower than in all other treatments.

Discussion

This study was initiated to elucidate dynamics in root exudation of tomato in an intercropping system due to AMF and/or *Fol* and their possible effects on *Fol*. Chemical analyses of the polar fraction of the root exudates identified organic acids and sugars as the main substance classes in tomato root exudates of the cultivar "Tiny Tim" in a tomato-cucumber intercropping setting. These substance classes comprised compounds like glucose, fructose, malate, citrate and succinate, compounds which were already reported in other experiments with tomato root exudates (Kamilova et al. 2006; Lioussanne et al. 2008). In our study AMF application alone as well as in combination with *Fol* led to a reduction of organic acids and an increase in sugar content (Fig. 1), in particular the organic acid malate and the sugar glucose (Fig. 2) (sucrose could not be measured due to hydrolyzing the samples).

Organic acids like malate and citrate have a crucial role in plant nutrition since they can mobilize inorganic P and are therefore readily exuded by plants under P deficiency (Jones 1998). AMF are strongly connected to an improved P nutrition of plants due to their ability to provide P to their host plants. In this respect it is tempting to assume that an increased P supply shifts the profuse exudation of organic acids to a redundant and physiologically costly event, since at the same time this increased P supply by AMF comes with plant-derived C (i.e. hexoses) as a trade-off. However, studies on decreased malate concentrations due to AM inoculation are rare and even in a recent work no relationship between root P concentration and the reduced amount of organic acids in root exudates could be shown (Ryan et al. 2012). This is in line with our findings where a reduction of malate exudation in AMF treatments

was not accompanied by changes in plant P contents (Table 1). We conclude that the reduction of malate in root exudates is linked to AMF symbiosis and its impact on alteration of host physiology and not due to P effects.

As already mentioned before AMF receive hexoses as a trade-off for P supply. Apart from receiving the plant-derived assimilates in the hyphal–plant interface AMF also use exuded metabolites for the germination and growth of AMF during pre- and postcolonization events (Graham et al. 1981). The increased levels of glucose in the exudates of all AMF treatments could be used by the AMF spores for the presymbiotic growth phase (Bücking et al. 2008), since AMF colonization rate was still at 6 % and further colonization might still occur. To date reports about elevated sugar levels in root exudates are scarce and have been linked to low P in the substrate (Tawaraya et al. 2012; Graham et al. 1981), which could not be confirmed in our work since the plants received sufficient P supply.

Apart from beneficial effects on AMF elevated levels of sugars can attract plant-growth-promoting bacteria (Sood 2003) as well as plant pathogens (Nelson 1990), indicating putative effects on rhizosphere microorganisms. Lioussanne et al. (2008), by contrast, found no AMF-induced differences in glucose content of root exudates, however, working with transformed tomato roots grown under gnotobiotic conditions and with *G. intraradices.*

AMF colonization rates of 6 % appeared to be low in our experiments but, however, in a previous study of Scheffknecht et al. (2006) stimulating effects on *Fol* microconidia germination were already evident at an AMF colonization rate of 4 %. Scheffknecht et al. (2006) studied root exudates challenged only with AMF. In our experiments a *Fol* treatment and a simultaneous application of AMF and *Fol* was added to the experimental set-up. AMF applied alone to the plants stimulated *Fol* germination rate, which is in accordance with Scheffknecht et al. 2006, but in the AMF+*Fol* treatment germination rate of *Fol* was reduced compared to the control and the *Fol* treatment (Fig. 4). Increased germination in the AMF exudate coincides with higher amounts of glucose in the root exudates; glucose addition to the medium also increased the spore germination and mycelial growth rate of *Fol* when the single compound was tested (Table 2; Fig. 5a), which is in accordance with Steinberg et al. (1999). This increase was observed at a glucose concentration of 0.3 mM, which represents a very realistic concentration in the rhizosphere (Jones 1998).

Furthermore, Ruan et al. (1995) propose two pathways for the stimulation of germination, either by nutrients like sugars and amino acids or by phenolic compounds in *F. solani* f. sp. *pisi* and f. sp. *phaseoli*. However, an increase in germination and growth of *Fol* induced by glucose does not necessarily indicate an advantage for the pathogen with regard to host infection processes. The increased amounts of glucose can explain the development of the spore germination in root exudates of AM plants but for the AMF+*Fol* treatment, where germination rate was reduced, the situation is different, although the relative amount of glucose in the exudates was increased as well. Root exudates consist of many different compounds with different effects on microorganisms in the rhizosphere. Therefore, we assume that in root exudates of AMF+*Fol* plants other compounds had stronger effects than glucose that resulted in decreased spore germination.

In the single compound assays several compounds reduced spore germination. Among these were the organic acids malate and citrate (Table 2) and also phenolic acids like chlorogenic, cinnamic, salicylic and protocatechuic acid (Table 3). The latter ones were suggested to contribute to plant defense in tissues (Bennett and Wallsgrove 1994) and were also reported to be influenced in their prevalence in tomato roots by AM symbiosis (López-Ráez et al. 2010a). Priming of the immune system of the plant leads to a systemic protection against pathogens in combination with changes in the secondary metabolism as reviewed by Jung et al. (2012). It is quite tempting to assume that priming might also be involved in the relative increase of the amount of chlorogenic acid in root exudates of AMF+*Fol* tomato plants (Fig. 3), however, further studies involving detailed metabolic profiling need to be done to explore this assumption further. Nevertheless, to our knowledge this is the first report on an increase of chlorogenic acid in root exudates of AM plants challenged with a pathogen compared to plants inoculated with AMF only, clearly indicating changes of root exudation due to AMF and *Fol*. Thus, chlorogenic acid could be a candidate for the reduction of spore germination. However, in the single compound assay only the 0.003 mM concentration caused a clear reduction of spore germination. Taking a closer look at the single compound assays, also organic acids like malate and citrate and need to be taken into consideration for the reduction of spore germination. In the rhizosphere concentrations of 1 µM to 1 mM are realistic (Jones 1998) depending on the chemical compound. Organic acids and sugars account for the major components in root exudates and are therefore available in larger quantities in the

rhizosphere. Citrate already inhibited *FoI* spore germination at a concentration of 0.003 mM which is a more realistic concentration in the rhizosphere than the effective concentration of malate with 30 mM. However, citrate was not significantly increased in the AMF+*FoI* treatment, although a slight tendency was seen.

For citrate and malate the observed effects were proportional to their concentrations, whereas for the chlorogenic acid negative effects were only evident at a low concentration. This might appear inconclusive in the beginning. But when considering that phenolics are available in less quantities in root exudates and the findings of previous studies demonstrating that on the one hand low concentrations of flavonoids had more antimicrobial activity against *FoI* than higher concentrations (Steinkellner et al. 2007) and on the other low concentrations had more stimulatory effects on *F. solani* germination than higher concentrations (Ruan et al. 1995) the picture is different. Regarding realistic concentrations in the rhizosphere negative effects of protocatechuic acid and cinnamic acid on *FoI* at 30 mM and 3 mM, respectively, are not transferable to the rhizosphere. Salicylic acid effects appeared to be more of a random nature. In conclusion, citrate and chlorogenic acid as well are good candidates for the reduction of *FoI* germination rate in the root exudates of the AMF+*FoI* treatment since they proved inhibition at concentrations naturally occuring in the rhizosphere.

Apart from spore germination rates also the mycelial development was tested, which is also essential for a successful colonization of roots. Effects on spore germination were not necessarily the same as on mycelial development. Apart from negative effects of certain phenolic acids and positive effects of sugars also positive effects of tryptophan on mycelial development were evident (Fig. 5c), which are debatable in the context of rhizosphere concentrations.

Nevertheless, due to analytical limits the available analytical data do not represent all possible analytes that might be present in the root exudates. Possibly, such analytes, which were not detected by the applied analytical methods, may also contribute to the observed effects.

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Fig. 1 Relative peak area (TIC) of GC–MS analyses grouped into substance classes and treatments (mean ± S.E., *n*=3). Different letters within substance classes indicate significant differences (ANOVA, *P* <0.05, Tukey's test).

Fig. 2 Relative peak area (TIC) of selected compounds from GC–MS analyses (mean ± S.E., *n*=3). Different letters indicate significant differences within compounds (ANOVA, *P* <0.05, Tukey's test).

Fig. 3 Relative peak area (TIC) of HPLC–UV analyses (mean ± S.E., *n*=3). Different letters indicate significant differences within compounds (ANOVA, *P* <0.05, Tukey's test).

Fig. 4 Spore germination rate of *Fol* in root exudates after 20 h (mean ± S.E., *n*=5). Different letters indicate significant differences (ANOVA, *P* <0.05, Bonferroni's post-hoc test).

Fig. 5 OD_{600} of glucose (a), fructose (b), tryptophan (c) and cinnamic acid (d) with different concentrations.

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Table 1

Tomato plant growth parameters, *Fol* infection parameters, AMF root colonization and P concentration of leaves (mean ± S.E.).

	Control	*Fol*	AMF	AMF+*Fol*
Root fresh weights [g]	1.87 ± 0.18 ab[a]	1.24 ± 0.17 a	2.06 ± 0.18 b	1.39 ± 0.14 a
Shoot fresh weights [g]	3.08 ± 0.33 a	2.51 ± 0.29 a	3.53 ± 0.25 a	2.67 ± 0.24 a
Disease incidence [%]	-	10.40 ± 2.71 a	-	5.60 ± 2.40 a
Disease severity	-	1.80 ± 0.67 a	-	1.00 ± 0.42 a
AMF colonization rate [%]	-	-	6.10 ± 0.90 a	6.90 ± 1.30 a
Leaf P concentration [mg g^{-1} dry weight]	2.45 ± 0.24 a	3.00 ± 0.17 a	3.00 ± 0.18 a	2.80 ± 0.07 a

[a] Different letters indicate significant differences (ANOVA, P <0.05, Tukey's test).

Table 2

Germination rate of *Fol* relative to standard growth medium [%] in chemical compounds from the hydrophilic fraction of the root exudates with different concentrations.

Compound	Germination rate relative to standard [%]					
		Concentration [mM]				
	Standard	0.003	0.03	0.3	3	30
Glucose	100 a[a]	99.22 ± 3.80 a	108.78 ± 3. 89 ab	126.44 ± 5.20 bc	133.22 ± 5. 12 c	136.22 ± 4. 19 c
Fructose	100 ab	94.33 ± 4.00 a	93.00 ± 3.61 a	101.89 ± 3.70 ab	113.44 ± 3. 52 b	111.89 ± 3. 68 b
Succinate	100 ab	105.22 ± 4.85 b	107.33 ± 3.78 b	103.00 ± 4.70 b	92.33 ± 2. 03 ab	86.57 ± 4.49 a
Citrate	100 c	74.44 ± 4.89 ab	86.11 ± 4.22 bc	79.00 ± 3.28 b	74.67 ± 5. 81 ab	59.44 ± 3.73 a
Malate	100 b	81.89 ± 5.87 b	81.00 ± 4.71 b	84.33 ± 4.08 b	85.56 ± 3.28 b	60.11 ± 5.15 a

[a] Different letters indicate significant differences within a compound (n=9, ANOVA, P <0.05, Tukey's test).

Table 3

Germination rate of *FoI* relative to standard growth medium [%] in chemical compounds from the lipophilic fraction of the root exudates with different concentrations [mM].

Compound	Germination rate relative to standard [%]					
		Concentration [mM]				
	Standard	0.003	0.03	0.3	3	30
Protocatechuic acid	100 b[a]	94.56 ± 4.09 b	89.56 ± 4.30 b	90.33 ± 4.04 b	95.78 ± 3.00 b	52.78 ± 4.37 a
Tryptophan	100 a	88.33 ± 4.09 a	83.56 ± 4.44 a	99.78 ± 4.04 a	100.33 ± 6.15 a	-
Chlorogenic acid	100 b	80.33 ± 5.17 a	92.22 ± 5.49 ab	92.33 ± 3.81 ab	105.11 ± 5.13 b	-
Cinnamic acid	100 b	101.11 ± 6.06 b	96.33 ± 4.22 b	83.56 ± 5.39 b	24.78 ± 6.04 a	-
Salicylic acid	100 b	84.78 ± 7.47 a	96.67 ± 8.25 ab	97.44 ± 8.11 a	87.00 ± 5.79 a	-

[a] Different letters indicate significant differences within a compound (n=9, ANOVA, P <0.05, Tukey's test).

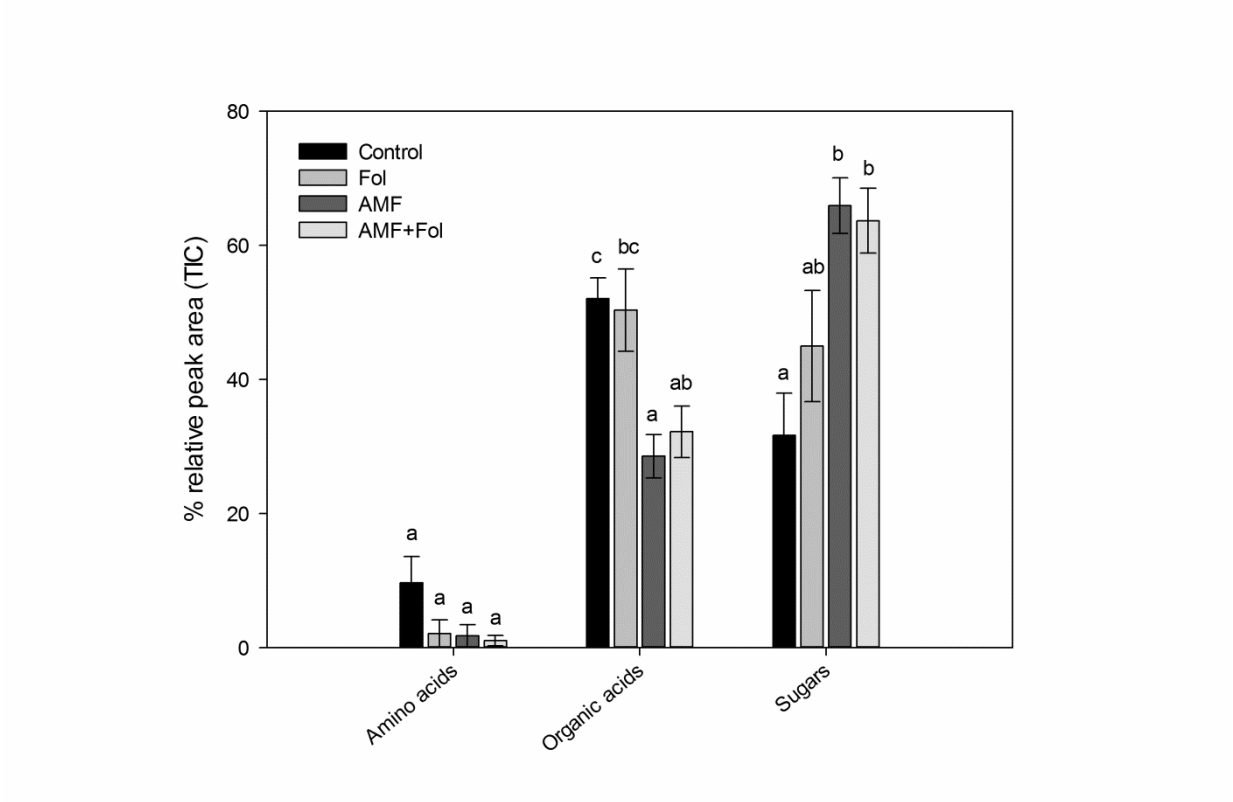
80
60
40
20
0
% relative peak area (TIC)
Control
Fol
AMF
AMF+Fol
a
a
a
a
c
bc
a
ab
a
ab
b
b
Amino acids
Organic acids
Sugars

Fig. 1

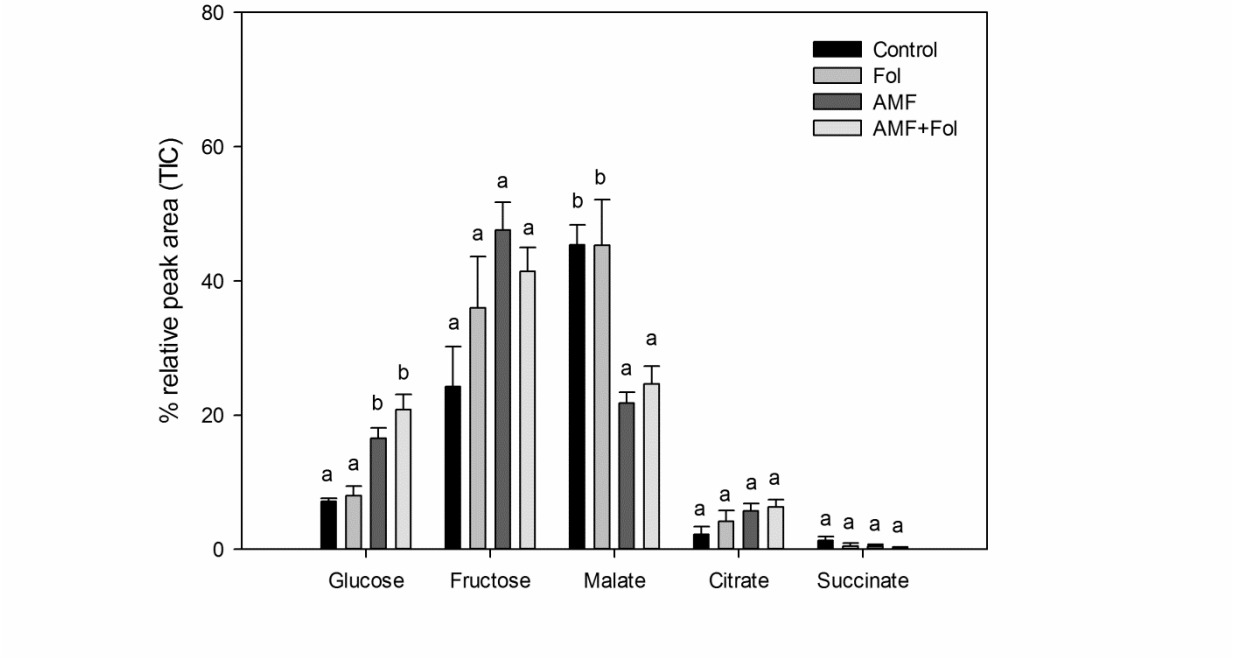
80
60
40
20
0
% relative peak area (TIC)
Control
Fol
AMF
AMF+Fol
a
a
b
b
a
a
a
a
b
b
a
a
a
a
a
a
a
a
Glucose
Fructose
Malate
Citrate
Succinate

Fig. 2

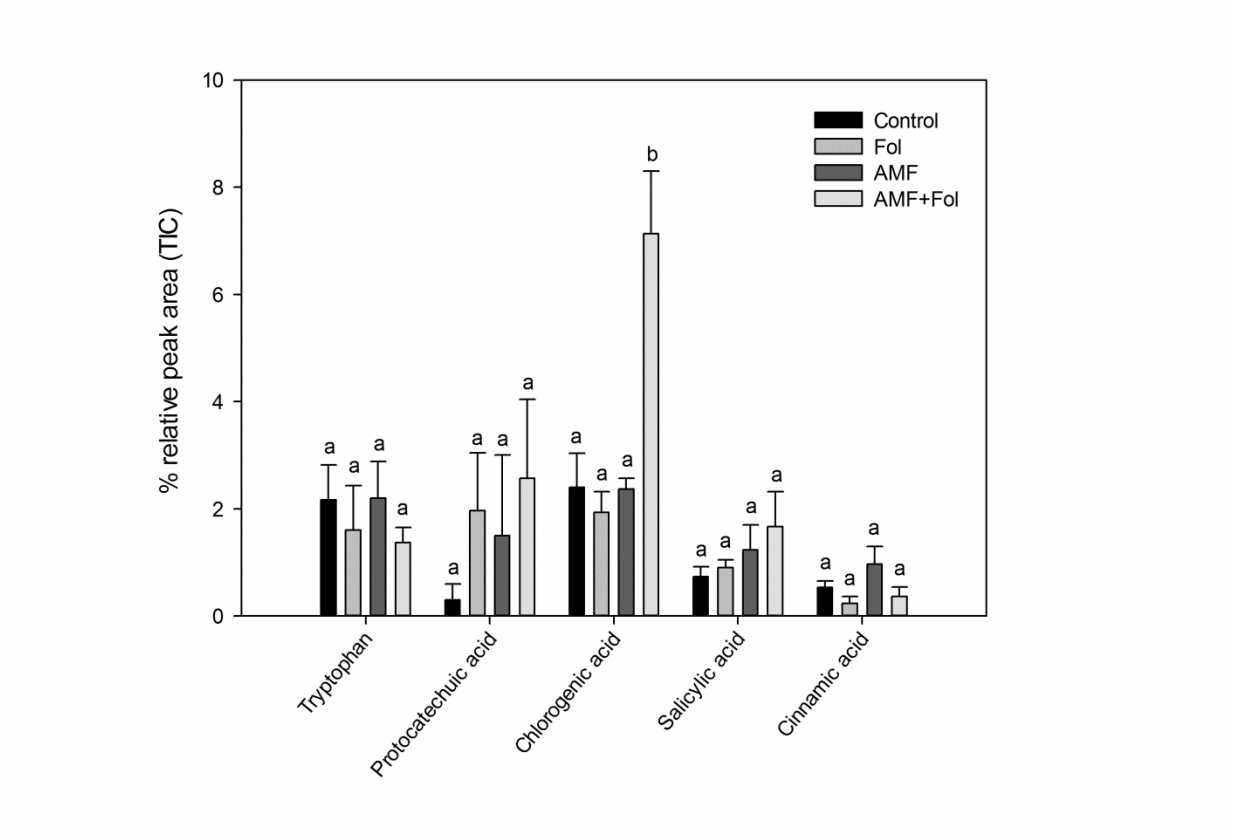
% relative peak area (TIC)
10
8
6
4
2
0
Control
FoI
AMF
AMF+FoI
a
a
a
a
a
a
a
a
a
a
a
a
a
a
a
a
a
a
b
a
a
a
a
a
Tryptophan
Protocatechuic acid
Chlorogenic acid
Salicylic acid
Cinnamic acid

Fig. 3

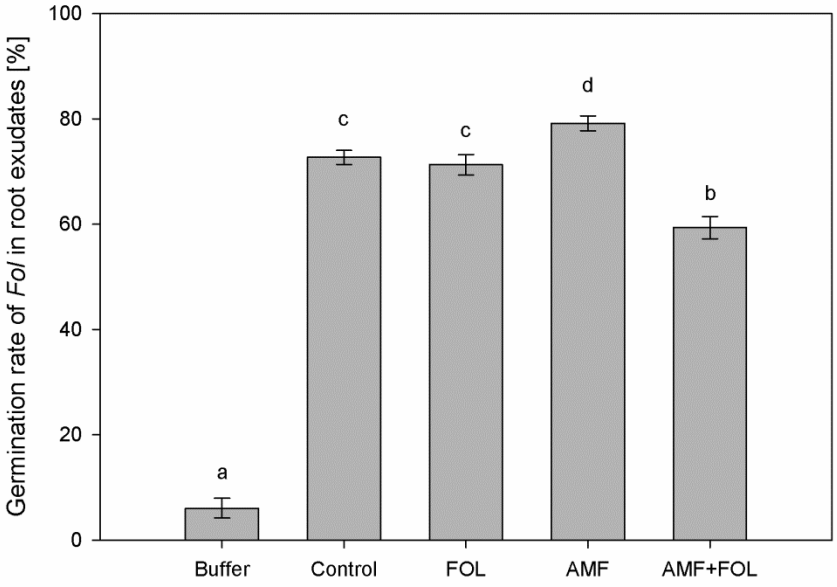
Germination rate of *Fol* in root exudates [%]
100
80
60
40
20
0
a
c
c
d
b
Buffer
Control
FOL
AMF
AMF+FOL

Fig. 4

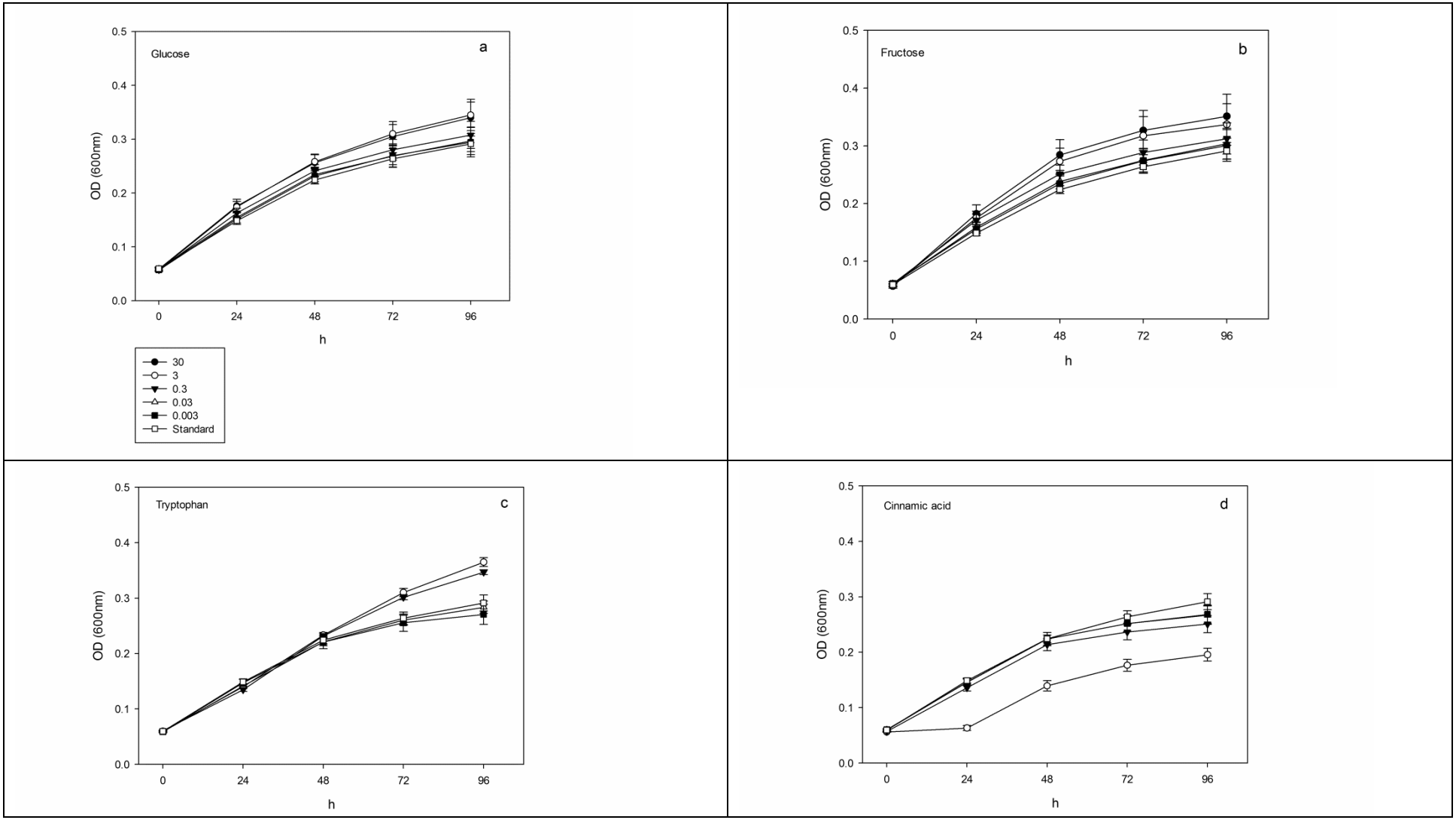
Glucose
a
OD (600nm)
h
0.0
0.1
0.2
0.3
0.4
0.5
0
24
48
72
96
30
3
0.3
0.03
0.003
Standard
Fructose
b
Tryptophan
c
Cinnamic acid
d

Fig. 5

3. Summary

In the present study effects of AMF on tomato development and *Fol* infection in different cultivation settings were investigated. This included different intercropping settings of tomato and the cultivation of different tomato cultivars alone. Additionally, root exudation and its role in disease development were investigated.

For the intercropping studies an inoculum consisting of six different *Glomus* species was tested against *Fol* with tomato (cv. Kremser Perle) intercropped with either leek, cucumber, basil, fennel or tomato itself. Arbuscular mycorrhizal root colonization of tomato was clearly affected by its intercropping partner. Tomato plants intercropped with leek showed a 20 % higher colonization rate of roots than tomato co-cultivated with tomato, whereas, tomato intercropped with fennel showed a 13 % lower AM colonisation level. Cucumber and basil as intercropping partners did not change root colonization compared to the tomato/tomato combination. As far as the *Fol* disease development is concerned, the intercropping system itself had no effect on *Fol* disease incidence or disease severity apart from the tomato/tomato combination where negative effects were evident. However, AMF application in intercropping settings had effects on *Fol* disease severity. Simultaneous inoculation of AMF and *Fol* led to a reduction of disease severity in the tomato/leek and tomato/basil combination and for the better developed plant of tomato/tomato. The application of AMF alone compared to the untreated control had positive effects on root and shoot weights. Furthermore, bioprotective effects of AMF resulting in the decrease of *Fol* disease severity and/ or compensation of plant biomass were evident for the tomato/leek, tomato/basil and the better developed plant of tomato/tomato.

For the cultivar experiment ten different tomato cultivars were inoculated with *G. mosseae* and/or *Fol*. The AM colonization rates depended on the tomato cultivar, the highest colonization rates could be observed the cultivars Kremser Perle and Apero. *Fol* infection rates also depended on the cultivar. The cultivars Yellow Pearshaped, Rheinlands Ruhm and Supersweet had the highest *Fol* infection rates, whereas, the cultivar Myriade showed no infection at all. The same cultivar dependency was observed for the bioprotective effect of AMF, which were evident in *L. peruvianum*, Kremser Perle and Marmande.

In a third experimental set-up the role of root exudates in disease development was investigated. GC-MS analyses revealed an AMF-dependent increase of sugars and decrease of organic acids, mainly glucose and malate. With the HPLC analyses a relative increase in chlorogenic acid when AMF and *Fol* were applied simultaneously could be shown for the first time. Furthermore, AMF inoculation increased the germination rate of *Fol* in total exudates, whereas, the simultaneous inoculation of AMF and *Fol* decreased the germination rate of *Fol* in total exudates. In subsequent single compound *in-vitro* assays citrate and chlorogenic acid were identified as possible candidates for the reduction of *Fol* germination rate in the AMF+*Fol* treatment, since they proved inhibition at concentrations naturally occurring in the rhizosphere. However, it is still possible that not all substances had been detected with the applied methods and other substances might had been involved as well.

In summary, these results show that AMF exhibit different effects on plant biomass and *Fol* disease development depending on the tomato cultivar and the intercropping partner. Furthermore, it could be shown that AMF and AMF+*Fol* inoculation impact on the root exudation pattern of tomato.

4. Conclusion

The mycorrhizal symbiosis is an ancient association between AMF and 80 % of all herbaceous plants. Apart from nutritional benefits for the plant also bioprotective effects are evident. It is known from ecological plant communities that AMF influence the composition of these communities and the plant composition feedbacks on the AMF community. It can be concluded from this work that also with crop plants these feedback matters can be used to influence plant health positively by using appropriate intercropping partners like leek. The use of inocula consisting of different AMF species can also be regarded as an approach that mimics more natural conditions and can compensate colonization preferences of AMF species, which can be even seen at plant cultivar level.

Apart from AMF and pathogens also other beneficial microorganisms are prevalent in the rhizosphere. The findings of this work about changes in root exudation dynamics due to AMF and *Fol* raise new questions about the effects of these dynamics on other rhizosphere inhabitants. Different multitrophic experimental set-ups could be a way to explore such putative effects and help unraveling the “hidden“ half further.

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Curriculum vitae

Personal Details

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Austrian citizenship

Education

Since Oct 2008	PhD studies in Phytopathology at the **University of Natural Resources and Life Sciences**, Vienna
Sept 2006 – June 2007	Study at **Agrarpädagogische Akademie** (teacher training college for agricultural schools) in Vienna
Oct 2000 – March 2007	Study of Agricultural Sciences at the **University of Natural Resources and Applied Life Sciences (Boku)**, Vienna, with majors in Phytopathology Crop Production Plant Biotechnology Thesis: The effect of root exudates of *Solanum lycopersicum* L. inoculated with biological control agents and/or arbuscular mycorrhizal fungi on soil-borne pathogenic fungi (written in English). At the **Institute of Plant Protection** supervised by Dr. habil. Horst Vierheilig and Ass. Prof. Dr. Siegrid Steinkellner

20th March 2006 – 26th April 2006 — Scientific internship at the „**Institut de recherche en biologie végétale**“ (**Université de Montréal**) in the lab of Prof. Marc St-Arnaud, Canada. Field of research: Attraction of root exudates of mycorrhizal plants on zoospores of *Phytophthora parasitica.*

Aug 2004 – Jan 2005 — Semester abroad at **KVL** in Copenhagen, Denmark, with emphases on plant pathogen interactions, biological control of insects with entomopathogenic fungi and co-existence of GMO and non-GMO plants in the EU

1992 – 2000 — **Bundesgymnasium Eisenstadt**, High School with stress on modern languages

Work Experience

Since Feb 2011 — Assistant at the Institute of Plant Protection, **University of Natural Resources and Life Sciences**, Vienna

Oct 2008 – Feb 2013 — Scientific staff at the Institute of Plant Protection, **University of Natural Resources and Life Sciences**, Vienna

FWF Project P20923 „Interactions of soil-borne fungi and tomato plants“

Dec 2007 – Sept 2008 — Scientific staff at the **The Austrian Agency for Health and Food Safety** in the field of Data, Statistics and Risk Assessment

Feb 2006 – June 2006 — Tutor at the Institute of Plant Protection, **BOKU**, for “Plant Protection II - Practical course”

Oct 2005 – Jan 2006	Tutor at the Institute of Plant Protection, **BOKU**, for "Plant Protection- Exercices"
March 2005 – Sept 2005	Supervision of a fertiliser field trial in sugar beet seed production for „**Österreichische Rübensamenzucht Ges.m.b.H.**" (**AGRANA**), Tulln, Austria Tasks: trial concept, implementation and statistical analysis
25th Feb – 28th Feb 2004	Co-operation at " **41. gartenbauwissenschaftliche Tagung**" (Conference in Horticultural Sciences) in Vienna, Austria
August 2003 – Sept 2003	Internship at „**Arche Noah - Gesellschaft zur Erhaltung und Verbreitung der Kulturpflanzenvielfalt**" (a society to preserve and provide diversity in cultivated plants), Schiltern und Langenlois, Austria Tasks: propagation, seed production and seed preservation
August 2002	Internship at the organic farm of **Ehrenberger-Hamader** in St. Bernhard, Austria
July 2001	Internship at **Gemüseverarbeitungs AG** (vegetable conservation) in Neusiedl/See, Austria Task: quality control