# Interaction between maize root-associated microorganisms and the Western Corn Rootworm

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***To my parents… again.***
***More than ever.***

*“There are men who struggle for a day, and they are good.*
*There are men who struggle for a year, and they are better.*
*There are some who struggle many years, and they are better still.*
*But there are those who struggle all their lives, and these are the indispensable ones.”*

*Life of Galileo* by Bertolt Brecht

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# Chapter I

## General introduction and thesis outline

## The Western Corn Rootworm*, Diabrotica virgifera virgifera* LeConte: origin, spread in the United States and economical impact

The Western Corn Rootworm (WCR), *Diabrotica virgifera virgifera* LeConte (Coleoptera: Chrysomelidae) is known as one of the most devastating maize pests in North America. In 1868 LeConte first described the species reported in Kansas on a wild gourd.

Several evidences suggested that WCR was originating in Central America and co-evolved with *Cucurbitaceae* host plants prior to a shift onto graminaceous species. This theory is supported by the fact that WCR adults feed compulsively on *Cucurbitaceae* plants containing cucurbitacins B and E, implying an original co-evolutionary association between those plants and the diabroticite insect (Metcalf, 1979; Metcalf & Lampman, 1991; Tallamy *et al*., 2005).

The Western Corn Rootworm was first recognized as a maize pest in Colorado in 1909 (Gillette, 1912). By 1949 the WCR distribution expanded eastward across the western maize-growing areas reaching the Atlantic Coast already in 1980 (Metcalf, 1983). The high WCR spread rate was aided by farming practices such as the maize monoculture and the massive and repeated use of cyclodiene insecticides, which determined the development of a widespread resistance associated to higher beetle mobility (Metcalf, 1983).

Nowadays, the range of activity of the WCR in the United States covers 30 million acres (120,000 km$^2$) of corn (Fig. 1) causing per year about $ 1 billion in crop losses and control costs (Rice 2004, Sappington *et al.*, 2006).

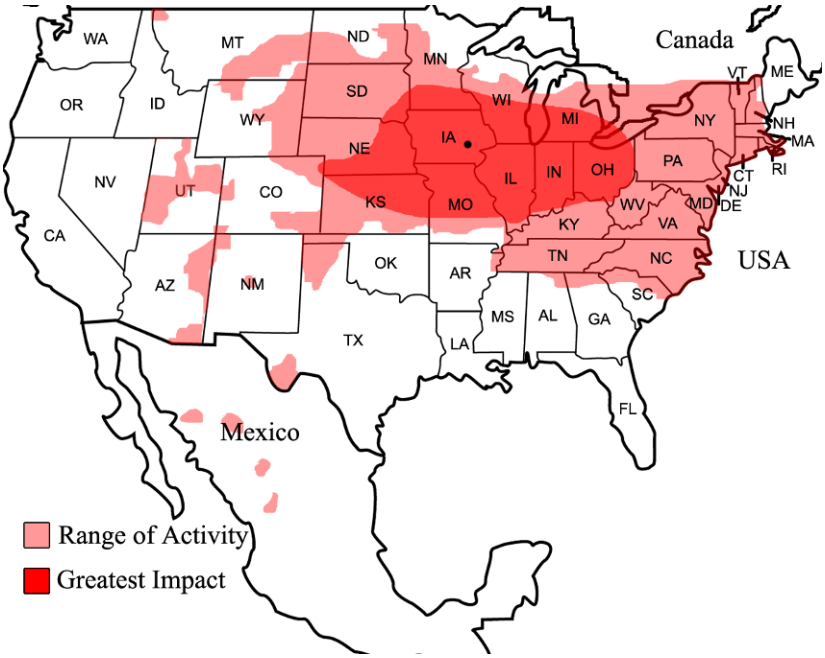


Fig. 1 Geographical distribution of Western Corn Rootworm, *Diabrotica virgifera virgifera* LeConte in North America in 2009. The range of WCR activity is shown in light red and the greatest impact in dark red. Picture downloaded from the website of Purdue University (http://extension.entm.purdue.edu/wcr/).

**Introduction of *Diabrotica virgifera virgifera* into Europe and present situation**

In 1992 the Western Corn Rootworm (WCR), *Diabrotica virgifera virgifera* LeConte, was detected for the first time in Europe near the Belgrade International Airport (Baca, 1993). The origin of its introduction remains unknown. However, the study of the genetic variability based on the microsatellite regions of both American and European WCR populations revealed that the homogenous population that extends from the Corn Belt to the East Coast of North America represents the original source of the WCR European population. Furthermore, genetic analysis showed that the several European outbreaks were caused not only by an intercontinental redistribution of the pest, but also by a repeated transatlantic introduction of the insect from North America (Kim & Sappington, 2005; Miller *et al.,* 2005; Ciosi *et al.*, 2008).

Once in Europe, the WCR had infested approximately 400.000 $Km^2$ throughout former Yugoslavia and neighboring countries by the end of 2003 (Kiss *et al.*, 2005). Up to 2011, WCR has been identified in 21 European countries including: Serbia, Hungary, Croatia, Romania, Bosnia and Herzegovina, Bulgaria, Italy, Slovakia, Switzerland, Ukraine, Austria, Czech Republic, France, Great Britain, the Netherlands, Belgium, Poland, Germany and Greece (Michaelakis *et al.*, 2010). However, economic losses have been reported only in Serbia, in some bordering areas in Croatia, Hungary, Romania, in small areas in Bosnia-Herzegovina, in Bulgaria and in North Italy (Fig. 2). So far, the other European countries have succeeded to border the initial hotbed of infection thanks to the timely protective and eradication measures implemented at European level since 2003 (Decision 2003/766/EC; Decision 2006/564/EC; EC Recommendation 2006/565/EC). In the other regions where WCR populations are already established and the pest has become a feature of the agro-ecosystem, the eradication measures are useless and an integrative pest management for WCR has to be developed still.

The rapid spread rate of the WCR in Europe may be attributed to three main factors: (i) species traits such as the quick adaptation of the WCR to new environmental conditions, a high reproductive rate (one female produces 100 to 1000 eggs) and long-distance flight capacity (beetles can fly even over 100 km/day); (ii) insufficient number of natural enemies or competing species able to keep the WCR populations below the maize economic damage threshold; (iii) human activities responsible for accidental pest spreading by land, air and water transports.

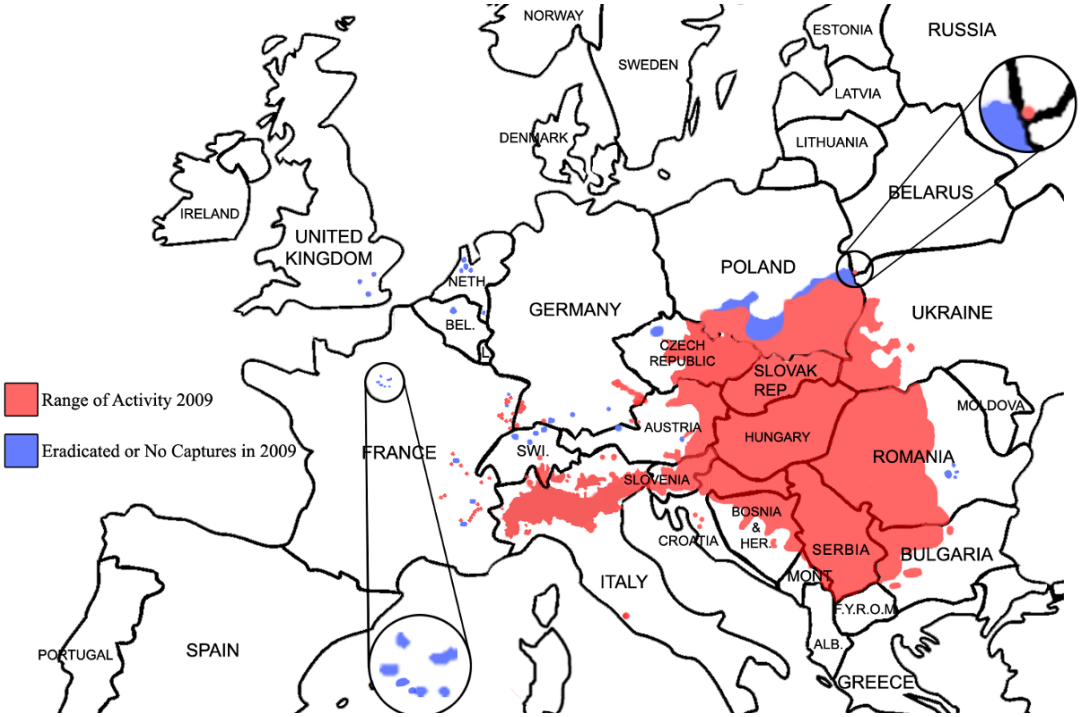


Fig. 2 Geographical distribution of the Western Corn Rootworm, *Diabrotica virgifera virgifera* LeConte in Europe in 2009. In red is highlighted the range of WCR activity while in blue the eradicated areas. Picture downloaded from the website of Purdue University (http://extension.entm.purdue.edu/wcr/).

**WCR life cycle and damage**

The Western Corn Rootworm, *Diabrotica virgifera virgifera* LeConte, has one generation per year (univoltine species). The larvae hatch in mid-May or early June and they reach the corn roots led by the emission of volatiles from corn seedlings as well as carbon dioxide released by decaying organic matter in the soil and living plant roots (Branson, 1982; Hibbard & Bjostad, 1988). The mobility of the larvae in the soil is about 12-18 inches before they starve to death. WCR larvae are present in the field until the end of July and they pass through three growth stages commonly referred to as the first, second, and third instars. Newly hatched larvae feed primarily on root hairs and small root tissues. Third instars tunnel through root tips to the plant base, and feed on the larger roots to the plant stalk. The larval development takes three weeks to complete. At maturity, the third instars leave the roots, form an earthen cell, and pupate. One week to 10 days later, the adults emerge from the soil and start feeding corn foliage and developing kernels in absence of corn silks, pollen, and ear tips. The beetles remain active in the field for about 75-85 days throughout August until the arrival of the first lethal frost. During their life, the adults feed, mate, and lay in the soil their eggs, which is the overwintering stage of the WCR life cycle (Fig. 3). Ovideposition starts in mid- to late summer (Shaw *et al.*, 1978; Levine & Oloumi-Sadeghi, 1991), and traditionally the females lay the eggs (ca. 400 per each) at a depth of 5 to 10 cm near the base of maize plants. Since the late 1980s, in the USA a WCR variant with a new egg-laying behavior has been observed. The eggs of this variant are deposited in soybean fields and hatch the following year in maize crop (O'Neal *et al.*, 1999; Onstad *et al.,* 2001; Levine *et al.*, 2002).

It is clear that the life cycle of *Diabrotica virgifera virgifera* LeConte may have destructive consequences on the maize plants. The main damage is caused by larval feeding on the roots. Extensive root injury may, in fact, alter the water and nutrient uptake reducing plant growth and grain yield (Godfrey *et al.*, 1993; Urías-López & Meinke, 2001). Moreover, the larval feeding may drastically compromise the stability of the maize plants which may results in bent stalks (goose necking) and lodging (Fig. 4a and 4b). The main yield losses are due to the difficulties in mechanical harvesting of injured maize plants. Larval feeding may also facilitate infection by root and stalk-rot fungi with consequential further damages.

Western corn rootworm adults, feeding on leaf tissues, may cause the “window pane” symptoms reported in Fig. 5a. A substantial silk feeding (adult density higher than 5 beetles per plant) can significantly interfere with the maize pollination which may result in the reduction of the grain production (Levine & Oloumi-Sadeghi, 1991). However, usually the economic impact of beetles is not highly relevant because peak of adults in the field often does not coincide with the pollination. In addition, the feeding of the pollen does not compromise the large amount of pollen released within the field, while the feeding of the ear may create a dangerous opportunity for disease-causing pathogens to enter the plant (Fig. 5b).

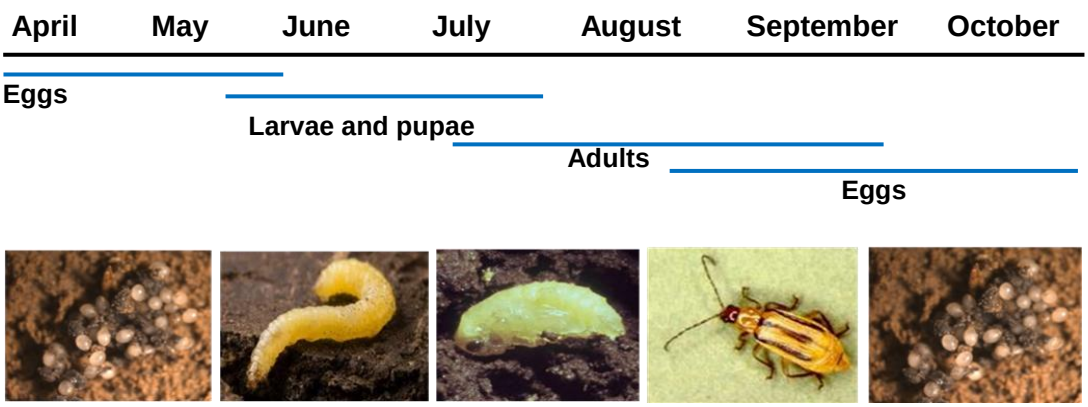


Fig.3. Life cycle of *Diabrotica virgifera virgifera* LeConte.

(a) (b)

Fig. 4 (a) Maize root system damaged by WCR larval feeding (http://www.forestryimages.org/images/768x512/0725088.jpg); (b) Goose necking and lodging caused by larvae feeding on roots (http://www.lfl.bayern.de/ips/blattfruechte_mais/38310/bild_4_maislager_2klein.jpg).

(a) (b)

Fig. 5 (a) WCR beetle feeding on a maize leaf leaving a window-pane appearance (http://passel.unl.edu/Image/siteImages/CRWWindowPaneLG.jpg); (b) WCR beetles feeding on maize kernels and promoting fungal infections (http://www.lfl.bayern.de/ips/blattfruechte_mais/30839/bild_4_k_fer_k_rnerfra_klein.jpg).

**Current pest management options and limitations**

For large-scale farming operations the main options in controlling the Western Corn Rootworm include the chemical control, the crop rotation and the use of transgenic plants.

The chemical control can be done via soil insecticides or insecticidal seed treatments as a protection against larval damage (Mayo & Peters 1978). Foliar insecticides instead, are often used for adult beetle suppression to protect the ears from silk feeding and to reduce the number of eggs laid at the end of the maize-growing season (Pruess *et al.*, 1974). So far, the major problems the farmers have to deal with are the high costs of the treatments and their potential impacts on non-target organisms. Moreover, a successful control of the pest requires the development of an accurate management plan according to the active ingredient and on a high number of variable factors such as larval population level, timing of application, physical and chemical composition of the soil, weather conditions and cropping practices (Gerber, 2003). In addition, the repeated use of pesticides can provide high selective pressure, which can lead to chemical resistance in the WCR populations, resulting in poor control of the pest and increasing insecticide application rate and further control costs (Meinke *et al.*, 1998; Wright *et al*., 1999).

Another strategy, widely used in the past in the United States (U.S.) for managing the western corn rootworms is the crop rotation. Corn rotated annually with soybeans was, in fact, not susceptible to rootworm larval damage as WCR adults laid eggs exclusively in cornfields and larvae hatched in soybeans starved to death. Unexpectedly, the intensive annual rotation of corn with soybeans caused in the U.S. the selection of an existing, but rare, WCR variant with reduced egg-laying fidelity to maize field (Onstad *et al.*, 2001, Levine *et al.*, 2002). As a consequence of rotation resistance, farmers have experienced, since 1995, economic losses caused by WCR larval injury to first-year maize. However, in Europe, where only the WCR wild type is present, the best management option remains, up to now, the crop rotation.

Over the past decade, the development of the crop biotechnology offers new potential control option against WCR. In the U.S. *Diabrotica*-resistant transgenic maize expressing the *cry(3Bb1)* gene from the bacterium *Bacillus thuringiensis kumamotoensis* (Bt maize) has been introduced in 2003 (Vaughn *et al*. 2005; Hellmich, 2008). The advantages of the Bt technology include a broad spectrum of

activity against different Diabrotica species (e.g. *D. virgifera virgifera*, *D. virgifera zeae*, *D. barberi*) and the conspicuous reduction of the insecticide application (Fernandez-Cornejo & Caswell, 2006) which may help to conserve beneficial arthropods (Harland, 2003). Furthermore, the toxin expressed by these maize hybrids is less likely to be affected by weather conditions, planting time, soil type or agronomic measures (Mitchell, 2002). Balanced against these potential benefits are possible drawbacks. First, genetically modified crops may have an impact on non-target species such as *Orius tristicolor* and *Chrysoperla* spp., the most common generalist predators in Midwestern U.S. maize fields (Harlan, 2003). Second, the horizontal gene transfer (or gene flow) between the transgenic crop and related plant species may cause an involuntary spread of engineered genes. In addition, the prolonged exposure to *B. thuringiensis* proteins might increase the selection pressure on the pest population and lead to the development of resistance, as has frequently occurred with chemical insecticides (Levine *et al.*, 1991; Gould, 1998; Shelton *et al.* 2002; Tabashnik *et al.*, 2003). To delay the development of resistance to Bt maize in the field, a certain percentage of conventional maize is usually grown as a "refuge" adjacent to the Bt crop. The aim is to maintain a population of WCR larvae susceptible to the Bt proteins. In this way, the mating between susceptible and resistant individuals which emerge from the refuge and the transgenic crop respectively, may originate a susceptible Bt-maize offspring. To be effective, this strategy needs a Bt-recessive resistance (rr) and a toxin concentration in plants high enough to kill resistance-heterozygous insects (Tabashnik *et al.*, 2003; Ferré *et al.*, 2008). However, the concentration of *cry(3Bb1)* expressed in Bt maize is not considered a high dose for WCR (Al-Deeb & Wilde, 2005; Oyediran, 2007), and resistance was reported to build up within three generations of selection on Bt maize in greenhouse experiments (Meihls *et al.*, 2008).

The development in the WCR populations of resistances against pest control methods described above (chemical control, crop rotation and engineered plants) paved the way for the development of resistance management strategies as a key factor in maintaining the efficiency of the different pest control options.

**Integrated pest management and resistance control strategies**

Integrated Pest Management (IPM) is an effective approach to pest management that relies on the combination of different pest control methods by the most economical means, and with the least possible hazard to people and environment.
Frequently IPM programs use natural enemies to reduce the invasive organism competitiveness with native species. A broad range of organisms with WCR antagonistic activity (e.g. microbial pathogens, nematodes, arthropod, predators, and parasitoids) are known to attack the WCR (Kuhlmann, 1998). One interesting candidate as bio-control agent against the WCR beetles is the fungus *Beauveria bassiana (Balsamo) Vuillemin,* causal agent of epizootics (Maddox & Kinney, 1989). Application of *B*. *bassiana* within field cages caused the decline of the WCR beetles by 50% at the highest rate (Mulock & Chandler, 2000).
However, the efficacy of natural enemies against WCR is often limited by the lack of formulations able to ensuring the viability, the activity and the persistence of the bio-control agent under the highly variable field conditions. For this reason biological control measures are usually part of IPM programs, where different control methods may have an additive or synergistic effect on the soil-dwelling pest. A promising strategy against WRC was recently suggested by Hiltpold *et al*. (2010). This author and his collaborators showed in field-cage tests that selected strain of *Heterorhabditis bacteriophora* in combination with maize variety releasing the volatile root signal (*E*)-β-caryophyllene reduced significantly WCR populations.
Besides the ecological aspect and the efficacy of combined different control methods, the IPM may also reduce the likelihood of pest resistance development. Recent literature reports that the combination of Bt crops with the entomopathogenic fungus *Metarhizium anisopliae* may delay the insect resistance development by reducing the number of beetles (Meissle *et al.*, 2009). Another interesting study showed that engineered corn plants expressing a dsRNAs activating the RNA interference (RNAi) pathway in WCR can be exploited to control the insect pest by silencing specific WCR genes. Also in this case, the authors suggested the use of the RNAi strategies in a pest integrated management system with Bt crop to increase the efficacy and durability of the transgenic plants (Baum *et al.*, 2007).

**Soil microorganisms and agro-ecosystem functionality**

Soil microorganisms, including bacteria, archaea, viruses, protists and fungi, are fundamental for the fertility and the functionality of all terrestrial agro-ecosystems. This is largely because they exist in enormous number of species (there are 1.5 million fungal and 4-6 x $10^{30}$ bacterial species worldwide, of which the biggest fraction occurs in the soil) (Hawkesworth, 1991; Whitman *et al*., 1998) and thereby they have an immense biomass and activity (Fuhrman, 2009).

Soil microorganisms are primarily involved in the mineralization of the organic forms of N, C, P, and S, in the nitrogen cycling (N fixation, denitrification, nitrification), in the carbon cycling and in the organic matter transformations into forms suitable for the soil food web (Polis & Strong, 1996). In addition, several studies showed their implication in bioremediation processes consisting in the transformation of pollutants (e.g. pesticides and polycyclic aromatic hydrocarbons) into harmless compounds. In this respect, recent literature showed that fungal mycelia can act as dispersal networks of catabolically active bacteria, facilitating bacteria´s access to the pollutants and thereby improve bioremediation performance (Banitz *et al*., 2011). Furthermore, adhesive effects of bacterial metabolites together with fungal hyphae can stabilize smaller soil particles into larger aggregates enhancing soil water holding capacity and preventing further desertification (Melope *et al*., 1987).

It is clear that the properties of different soil types including soil fertility are mainly determined by the soil microbial biodiversity, abundance and activity. The major factors influencing the soil microbial communities are the soil structure (Gelsomino *et a*l.,1999), the soil particle size (Sessitsch *et al*., 2001), the mineral composition (Carson *et al*., 2009), environmental conditions, agricultural practices (Rooney & Clipson, 2009), plant and soil-dwelling insect interactions (Treonis *et al*., 2004; Dawson *et al*., 2004).

**Plant-soil microbe interactions**

The interaction between plants and soil microbes can vary from neutral to beneficial on the one side, and deleterious on the other side when plant-pathogenic organisms are involved (Lugtenberg *et al*., 2002; Singh *et al*., 2004; Mercado-Blanco & Bakker, 2007; Raaijmakers *et al.*, 2009).

These interactions between plants and soil microbes take place in the rhizosphere of the plants which is defined as the soil layer surrounding roots and influenced by the root plant metabolism. Root processes can affect rhizosphere pH, redox potential and chemistry (Marschener, 1998). Plant roots continuously produce and excrete into the rhizosphere compounds which consist in ions, free oxygen and water, mucilage and a broad array of primary and secondary metabolites (Uren, 2000). The main plant metabolites at the soil-root interface are organic acids, sugars, amino acids, lipids, flavonoids, coumarins, proteins, enzymes, aliphatics and aromatics compounds.

Several studies have shown that root exudates represent a mechanism through which a plant shapes the soil microbial populations inhabiting the rhizosphere. In particular, Bröckling *et al.* (2008) showed that the addition of *in vitro*-generated root exudates to soil fungal communities produced an effect qualitatively and quantitatively similar to that one observed when plants are grown in the corresponding soil type.

But in which way the root exudates may affect the microbe communities in the soil? Some of these exudates are suitable substrates for a wide range of microorganisms which consequentially may enhance their biomass and their activity compared to the microbes in the bulk soil. Small organic molecules excreated from the roots (a.g. carbonic acids, amino acids and sugars) can display chemotactic activity or serve as a signal to initiate the symbiosis with rhizobia and mycorrhizal fungi (Akiyama *et al.*, 2005; Badri & Vivanco, 2009). In this cross-talk between microbes and plant roots flavonoid compounds have important roles. Flavonoids excreted from soybean roots were shown to attract simultaneously the beneficial bacterium *Bradyrhizobium japonicum* and the pathogenic fungus *Phytophthora sojae* (Morris *et al.*, 1998). Other flavonoid compounds isolated from white lupin roots may mobilize inorganic phosphorus and decrease soil microbial respiration, citrate mineralization and soil phosphohydrolase (Berg & Smalla, 2009). Recently, the importance of plant secreted proteins in the process of signaling and recognition between compatible

and incompatible plant-microbe interactions has been shown (De la Pena *et al.*, 2008). Furthermore, some exudates can affect the microbial communities in the rhizosphere explicating a toxic activity. These compounds determined the colonization of the plant roots or of the nearby area by microbial populations expressing a specific detoxificant activity (Rettenmaier & Lingens, 1985).

As shown in many studies, the amount and composition of the root exudates is highly influenced by the soil type and the plant species. These factors can dominate, depending on biotic and abiotic conditions (Berg & Smalla, 2009). Other parameters as well as the cultivar and the development stage of the plant may affect the quality and quantity of the root exudation and consequentially the microbial structure in the soil (Smalla *et al.*, 2001). Nevertheless, pathogen-activated plant defenses may induce changes in the root exudation patterns, forcing the diversification of the microbial communities in the rhizosphere by either attracting beneficial microorganisms or actively repressing pathogen proliferation. For instance, Rudrappa *et al.* (2008) showed that the bacterial infection of *Arabidopsis* foliage with the pathogen *Pseudomonas syringae* pv. *tomato* DC3000 (Pst) caused the recruitment in the plant rhizosphere of the biocontrol strain *Bacillus subtilis* FB17 and consequential formation of a biofilm on infected seedlings. The authors demonstrated that roots of Pst infected plants secrete large amounts of malic acid, which is a chemo-attractant for FB17. Pathogen-activated plant defenses can also result in root secretion of antimicrobial compounds. Hairy root culture of *Ocimum basilicum* inoculated with *Pytium ultimum* produce rosmarinic acid, a caffeic acid active against multiple soil-borne microorganisms (Bais *et al.*, 2002). Another interesting discovery was that the root exudation of the plants may be modulated by the rhizosphere microflora itself. For example, the inoculation of the tomato roots with the pathogenic fungus *Fusarium oxysporum* f.sp. *radicis-lycopersici* leads in the rhizosphere to decreased amounts of citric acid and to increased amount of succinic acid compared to the non treated control plants (Kamilova *et al.*, 2006). Last but not least, a growing body of evidences showed that herbivore insects may change the root exudation (carbon flux to the soil) with consequential shifts of the soil microbial communities (Treonis *et al.*, 2004; Denton *et al.*, 1998; Grayston *et al.*, 2001; Dawson *et al.*, 2004). In particular an increased utilization of some sugars, carboxylic and amino acids in presence of belowground insect feeders has been shown (Grayston *et al.*, 2001; Dawson *et al.*, 2004).

Due to the assumed impact of the soil type and of the cultivar on the microbe-plant interactions three different soil types and four different maize cultivars were used in this study.

**Insect herbivore-plant interactions**

Together with the soil microbes, the plants may interact with various herbivorous arthropods, which are the most diverse and abundant group of plant consumers (Zheng & Dicke, 2008). The strength and the direction of these interactions depend mainly on two factors: the plant defences against the phytophagus insect and the plant quality in term of nutritional status.

Plant defenses may be constitutively expressed or induced by insect-mediated damages. Plants may employ against herbivourous insects either physical and chemical direct defenses (e.g. thorns, trichomes, toxins and antifidants) or indirect defences to promote the effectiveness of natural enemies of the insect (Pineda *et al*., 2010). For instance, several evidences showed that upon herbivore attack, the plant may synthesize and release volatile organic compounds (VOCs) attracting natural enemies of both above- and belowground herbivores (van Tol RWHM *et al.*, 2001; Kessler & Baldwin, 2001; D'Alessandro *et al.*, 2006; Soler *et al.*, 2007; Köllner *et al.*, 2008). In particular, European maize (Zea mays) roots release, as a response to root damage caused by *Diabrotica virgifera virgifera* larvae, the volatile sesquiterpene (*E*)-β-caryophyllene, a strong attractant for the entomopathogenic nematode *Heterorhabditis megidis* (Rasmann *et al.*, 2005; Köllner *et al.*, 2008). Plants can respond to insect attack also by the expression of the wound induced resistance (WIR) and of the root herbivore-induced shoot resistance (RISR). These resistances are activated upon above- and belowground herbivore attack, respectively, and they induce systemic defense responses to co-occurring insect attackers. While the WIR is predominantly regulated by bioactive jasmonic molecules (Glauser *et al.*, 2008; Howe & Jander, 2008), the mechanisms mediating the RISR expression are more unclear. However, recent literature reported that WCR root feeding induce aboveground resistance against the generalist insect *Spodoptera littoralis* and also against the necrotrophic fungus *Serosphaeria turcica* (Erb *et al.*, 2009). Abscisic acid biosynthesis as long distance signal and hydraulic changes in maize leaves seem to mediate such responses (Erb *et al.* 2011). The

importance of the cultivar on the plant defenses trigger by herbivores should be mentioned in this context. Several evidences showed that in response to the same herbivore, the plant may activate cultivar-dependent transcriptomic changes (Heidel & Baldwin, 2004; Reymond *et al.*, 2004; De Vos *et al.*, 2005; Brökgaarden *et al.*, 2007). For instance, two cultivars of the white cabbage, *Brassica oleracea* var. *Capitata*, differ considerably in the global gene expression patterns induced by the attack of the caterpillar *Pieris rapae* as well as the level of direct defences against the insect feeding (Brökgaarden *et al.*, 2007). It is clear that the cultivar selection and the metabolic changes triggered upon insect attack (such as VOCs production, WIR and RISR expression) might interfere with the root exudation. Because the root exudates shape the microbial communities associated to the plant (see "Plant-soil microbe interaction"), in Chapter III we investigate for the first time the effect of the WCR larval feeding on the bacterial and fungal populations associated to the roots of four maize cultivars.

The second factor which may influence the plant-herbivore interactions is the nutritional status of the plants. The parameters which affect the quality of the host plant are several and include not only soil nutrient availability, air temperature, water balance, light, atmospheric carbon dioxide, but also plant-associated microbes such as rhizobia, endophytes, and mycorrhizal fungi (Barbosa *et al.,* 1991; Carter *et al.,* 1997). For this reason several investigations have been done to evaluate the effect of microorganisms, especially AMF, on the performance of herbivores. For instance, Goverde *et al.* (2000) showed that the larval survival of the common blue butterfly, *Polyommatus icarus* (Lycaen idae), feeding on the leaves of *Lotus corniculatus* (Fabaceae) plants, was 3.8 times lower on non-mycorrhizal plants than on plants inoculated with single AMF species. These differences in larval performance were explained by differences in leaf chemistry, since mycorrhizal plants had a three times higher leaf P concentration and a higher C/N ratio. Moreover, this work showed a higher lipid concentration of the adult butterflies when the insects feed on mycorrhized plant material indicating a positive effect of AMF on the insect fecundity and longevity (Brown & Chippendale, 1974; Tuskes & Brower, 1978).

**Arbuscular mycorrhizal fungi**

Arbuscular mycorrhiza fungi (AMF) are considered ancient fungi which have coevolved with plants in the last 400 million years, assisting plants in the conquest of dry lands (Parniske, 2008; Schüβler *et al.*, 2009). Based on the SSU (18S) rRNA gene, AMF have been classified as a monophyletic group belong to the *Glomeromycota* phylum divided into four orders: the *Glomerales*, still representing the larges "genus" within the AMF; the *Diversisporales;* and the two ancestral lineages *Archeosporales* and *Paraglomerales* (Schüßler *et al.*, 2001).

AMF form a mutualistic association with the roots of the majority (70-80 %) of terrestrial plants (Smith & Read, 2008). During the symbiosis, the AMF form within the plant cells, tree-shaped fungal structures called arbuscules (Fig. 6). These structures are thought to be the interface of nutrient and signal exchange between the two partners (Parniske *at al*., 2008): the AMF provide, through an extensive hyphal network (up to 100 m/cm$^3$ of soil) (Miller *et al.*, 1995), mineral nutrients to the host plant (e.g. phosphate, nitrogen, zinc and copper); in return, up to 20% of plant-fixed carbon is transferred to the fungus (Smith & Read, 1997; Fitter *et al*., 2006). Radiotracer studies showed that AMF enhanced carbon fixation activity in the leaves, products of which are translocated to the roots (Black *et al*., 2000).

The symbiosis may improve plant survival in harsh environments by enhancing several plant functions (Newsham *et al.*, 1995; Smith & Reed 2008) including drought resistance (Davies *et al.*, 2002), tolerance to heavy metal contaminations (Gildon & Tinker, 1983), protection against pathogens through microbial antagonism and increased plant defensive capacity (Newsham *et al.*, 1995). It is still unclear whether this may be due to an improved nutritional status of the plant and therefore to increased plant fitness or to induced systemic resistance (Parniske, 2008). Furthermore, AMF are prominent through their well-established ability to affect insect-herbivore-plant interactions (Gehring & Bennett, 2009). Several reports showed that AMF can affect the behavior, development and insect performance (Gange *et al*., 1994; Wardle 2002; Davet 2004; Bezemer and van Dam 2005; Hartley & Gange 2009; Koricheva *et al*., 2009), either changing the nutritional status of the plant or triggering plant defense responses (Goverde *et al*., 2000; Nishida *et al.,* 2010). Bennett *et al.* (2007) showed that plant feeders tend to be negatively or positively influenced by the AMF species which the plant is associated with. In particular, the mycorrhizal fungus *Glomus white* do not alter the response of the

narrow-leaved plantain (*Plantago lanceolata*) to the specialist lepidopteran herbivore, *Junonia coenia*; the plant association with the AMF *Archaeospora trappei* leads to tolerance to herbivore in the form of an increased plant growth rate; the association with the fungus *Scutellospora calospora* reduces plant tolerance to the herbivores. It must be noticed that, due to monitoring difficulties, belowground herbivore insects have been seldom examined. However, Gange *et al.* (1994) showed the effect of the AMF, *Glomus mosseae,* on the reduction of black vine weevil (*Otiorhynchus sulcatus* Fabricius) larval growth. Another work showed the effect of AMF on the compensation of the damage caused by root feeders: AMF hyphae extending into the soil may effectively replace some of the root functions (e.g. water and mineral uptake) that are reduced by the root feeding (Gange, 2001). In contrast, Borowicz *et al.* (2010) addressed a negative effect of the AMF on the root damage: wild strawberry plants (*Fragaria virginiana Duchesne*) inoculated with AMF showed significant higher root damage compared to the non-mycorrhized plants.

In addition to the effect on plant-insect interactions, AM fungi can, through the release of hyphal compounds, influence nutrient dynamics in the soil and consequentially the activity and the structure of the soil- and root associated microbial communities (Wamberg *et al.,* 2003; Marschner & Baumann, 2003; reviewed by Jones *et al*., 2004; Offre *et al.,* 2007).

In Chapter IV of this PhD work the effect of the arbuscular mycorrhizal fungus *Glomus intraradices* on the WCR development and fitness was investigated. This set of data was produced by Benedikt Kurz from the Department of Crop Science, Agricultural Entomology, Georg-August University Göttingen. In order to understand if the *Glomus* effect on the herbivore insect was mediated by other microorganisms or not, shifts of the natural microbial communities inhabiting the maize endorhiza (plant roots) and rhizosphere were investigated as well.

*Glomus intraradices* was chosen in our experiments because it is widespread and present in different ecosystems throughout the world, including temperate and tropical locations (Smith & Read, 2008), and it colonizes many plant species. Furthermore, it is one of the most commonly studied AMF and part of several commercial inocula.

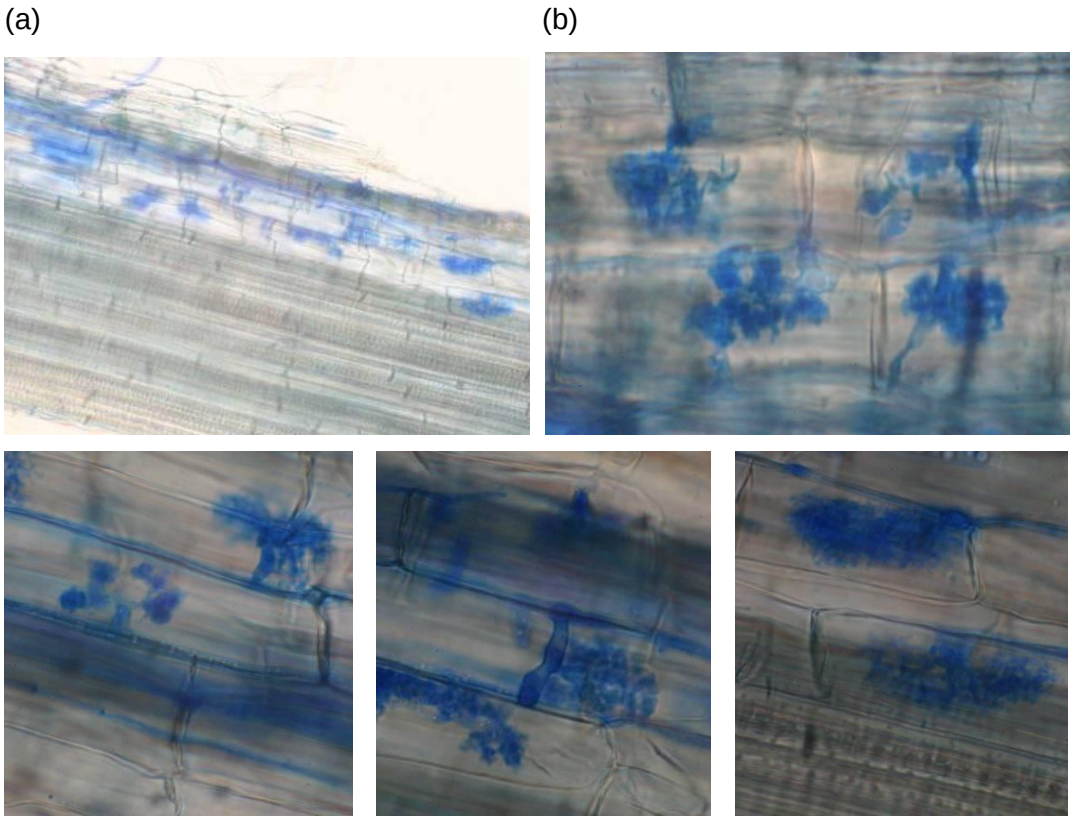


Fig. 8 (a) Maize root segment showing mycorrhizal arbuscules and hyphae in epidermic cells. (b,c,d,e) 40X magnification of mycorrhizal arbuscules in maize root segments. The roots were stained with 1 % cotton blue in lactic acid (Vallino *et al*., 2006).

**Gut microbial composition of WCR larvae**

Microorganisms inhabiting the insect gut can play important roles in the host's nutrition, development, resistance to pathogens, reproduction and efficacy of Bt-insecticides (Brand *et al.* 1975; Brune, 2003; Moran *et al.*, 2005; Broderick *et al.*, 2006). Loss of microorganisms often results in abnormal development and reduced survival of the insect host (Eutick *et al.* 1978, Fukatsu & Hosokawa, 2002).

Despite the importance of microbes in the digestive tract of the insects, little is known about the microbial composition and about their biological role in such environment. With regard to the Western Corn Rootworm several studies revealed the presence of *Wolbachia* sp., intracellular bacteria, maternally transmitted from parent to offspring and responsible for reproductive incompatibilities between infected and uninfected individuals in the gut of WCR (Clark *et al.*, 2001; Roehrdanz & Levine, 2007).

Due to the potential ability of the yeast to degrade several mycotoxins, Molnar *et al.* (2008) studied the yeast diversity in the guts of several pests of maize. They showed that *Metchnikovia* sp. and *Candida* sp. are the most dominant in WCR gut, but they could not exclude the effect of the environment (soil and plant) on the microbial composition of the WCR gut observed.

In Chapter V we investigated the effect of three different soil types on the fungal and bacterial composition in the gut of the WCR larvae. Moreover, to distinguish the microbes which are either parentally transmitted to the offspring or taken up during the root larval feeding from the external environment, we performed a comparative analysis of the microbial communities present in the gut, in the rhizosphere and in surface-sterilized WCR eggs.

**Methods to assess complex microbial community structures of environmental samples and to characterize specific members of those communities**

In the past, the microbial community composition in environmental samples such as soil or plant systems was mainly investigated by phenotypic characterization of isolates (Buchner, 1965; Dasch *et al.,* 1984; Lysenko, 1985). The lack of knowledge of the real conditions under which most of the microorganisms are growing in their natural habitat, and the difficulty to assess cultivation media accurately resembling these conditions, led to the development of cultivation-independent DNA-based methods.

*Total community DNA extraction from environmental samples*

Cultivation-independent methods require an efficient DNA extraction. Yield and purity of the DNA extraction is determined by the method (direct or indirect) choosen for the extraction of the nucleic acids, cell lysis and DNA purification.
The direct DNA extraction method, based on lysis *in situ* of cells, allows high DNA yield but results in increased DNA shearing (Roose-Amsaleg *et al*., 2000). The indirect method is based on the centrifugation recovery of the cell fraction before lysis. Compared to the direct lysis, this method gives 10-fold lower DNA yield, but shows a greater purity of the DNA extracted with a low degree of fragmented DNA (Tien *et al*., 1999). Direct methods can recover more than 60 % of the total theoretical bacterial DNA (More *et al.*, 1994), while indirect methods recover bacterial fraction representing only 25-50 % of the total endogenous bacterial communities (Bakken *et al*., 1995). Therefore, direct lysis procedures are preferred when large quantities of nucleic acids are required for the detection of non-abundant microorganisms, and when the entire diversity of an environmental sample is investigated with minimum bias (Robe *et al*., 2003). Another critical factor influencing the yield and the quality of the DNA extracted is the cell lysis. Disruptive methods for lysing microbial cells include enzymatic digestion, physical disruption or the combination of both approaches. Quite popular among laboratories is the use of beat beating systems. These harsh-lysis methods allow the disruption of solid aggregate often included in the environmental sample. Furthermore, they disrupt Gram-positive bacterial cells and spores, which are more resistant to lysis than

Gram-negative cells (Frostegard *et al*., 1999; Kauffmann *et al*., 2004). On the other end, the beat beating can lead to damage of nucleic acids resulting in loss of probe or primer annealing sites (Smalla & van Esas, 2010). A balance is therefore required between applying beat beating for sufficient time to enable lysis of all cells and prevent DNA shearing (Prosser *et al*., 2010). In the recovering of nucleic acids from the environment, the DNA purification cannot be neglected. Humic acids are a major contaminant of soil samples and can inhibit PCR reactions (Tsai & Olson, 1992; Porteous *et al*., 1994; Zhou *et al*., 1996), restriction enzymes (Porteous *et al*., 1994), and reduce transformation efficiency (Tebbe & Vahjen, 1993). The separations of environmental DNA from humic substances and other contaminants need to be performed before to apply any DNA-based method. The method for purifying DNA should remove efficiently all impurity present in the sample and recover the highest amount of DNA from it.

Several kits for DNA extraction and DNA purification are nowadays commercially available, and all of them recover nucleic acids useful for molecular biology purposes. However, it is a matter of truth that any of these DNA extraction methods recover sufficient DNA to assess "all" microorganisms in the soil. Thus, the improvement of DNA extraction technology from soil or other environmental samples is still needed (Smalla & van Elsas, 2010).

FastDNA SPIN Kit for soil (Q-Biogene, Carlsbad, CA, USA) and GENECLEAN SPIN Kit (Q-Biogene, Heidelberg, Germany) showed in our lab a high DNA extraction efficiency and minimal loss of template during purification procedure. Therefore, those kits were used in this PhD work to examine the microbiota in natural environments such as soil, rhizosphere, plant roots and gut of insects. A direct DNA extraction method was used for all types of samples above listed, except for the rhizosphere samples where an indirect DNA extraction approach was applied.

*Marker genes to study microbial communities by PCR-based methods*

The total community DNA recovered from environmental samples can be used to amplify phylogenetically informative genes. 16S rRNA gene is the most commonly used bacterial molecular marker in microbial ecology due to its essential function, ubiquity, and evolutionary properties (Ward *et al.*, 1990; Head *et al*., 1998). In each bacterium the 16S rRNA gene copy number ranged from 1 to 15, with an average of

4.2 copies per genome (Case *et al*., 2007). The multiple copies of this gene can differ in sequence, leading to the identification of multiple ribotypes for a single organism. Case *et al*. (2007) showed that the intragenomic heterogeneity influenced 16S rRNA gene tree topology, phylogenetic resolution and operation taxonomic unit (OUT) estimates at the species level or below.

For a better resolution at the species level of bacteria, protein-encoding genes such as *rpoB* can be used. Case *et al*. (2007) investigated *rpoB* properties as a marker for microbial ecology studies. Advantages and disadvantages of *rpoB* are here summarized: (i) as a protein-encoding gene, *rpoB* allows the phylogenetic analysis at the amino acid and nucleotide level; (ii) *rpoB* is universally present in all prokaryotes; (iii) it is an housekeeping gene, therefore it is less susceptible to gene transfer; (iv) it has a large size containing phylogenetic information; (v) it contains slowly and quickly evolving regions for the design of specific probes and primers. The main drawbacks of using *rpoB* for microbial ecology studies are: (i) no resolution between closely related organisms, e.g. species and subspecies levels; (ii) difficulties to design universal primer for *rpoB* due to the saturation of all third codon position over a long evolutionary timescale.

Compared to *rpoB* or others single-copy genes encoding proteins, the 16S rRNA has the advantage to be present in higher concentration in environmental samples. This allows the detection of a bigger fraction of microorganisms occupying specific ecological niches (see paragraph above). Thus, the 16S rRNA gene is still used as main marker for the bacterial communities in ecological investigations. However, the detection of microorganisms using protein-encoding genes with improved phylogenetic resolution at the subspecies level, is an existing perspective.

In order to characterize the fungal diversity in natural environments, the molecular markers that can be used are mainly two: the SSU (18S) rRNA gene (White *et al*., 1990; Smit *et al*., 1999; Borneman &d Hartin, 2000; Vainio & Hantula, 2000) and the internal transcribed spacer (ITS) regions (White *et al*., 1990; Gardes & Bruns, 1993; Larena *et al*., 1999). The advantage to use the 18S rRNA gene as molecular marker is mainly related to the big gene size (ca. 1650 bp), carrying a lot of information. Due to the rather high conservation of the non-coding rRNA gene within the fungi, the 18S rRNA gene allows taxonomic discriminations only at the genus or family level (Hugenholtz & Pace, 1996). However, in the context of symbiotic arbuscular mycorrhizal fungi (AMF) there is a sufficient variation in 18S rRNA gene sequences of different species to allow discrimination between isolates to species and

sometimes below species level (Vanderkoornhuyse & Leyval, 1998). Thus, this molecular marker is more commonly used to study this group of fungi. Compared with the non-coding rRNA gene, the ITS regions have higher intra-specific variability that results in a higher systematic resolution between closely related species (Anderson *et al*., 2003). The main limit of this marker is the short size of the ITS regions (ca. 500 bp).

PCR amplifications of all marker genes above introduces can be used directly for downstream molecular biological experiments such as molecular fingerprints, clone library, sequencing, pyrosequencing, restriction enzyme digestion, Real-Time PCR, and so on.

*Molecular fingerprinting methods and DGGE*

PCR products can be analysed by using whole-community fingerprinting methods such as denaturing gradient gel electrophoresis (DGGE), single-strand conformational polymorphism analysis (SSCP), terminal restriction fragment-length polymorphism (T-RFLP) or automated ribosomal intergenic spacer analysis (ARISA). Principles, specificity, resolution and throughput of these methods are reviewed by Oros-Sichler *et al.* (2007).

Denaturing gradient gel electrophoresis (DGGE) is perhaps the most commonly used among the culture-independent fingerprinting techniques. The DGGE method was pioneered by Gerard Muyzer *et al.* (1993) and it allows the electrophoretic separation of PCR amplicons whose sequences differ as little as 0.1% (e.g. 1 bp in 1000). The principle of this technique relies on the use of a denaturing gradient polyacrylamide gel which confers the double stranded amplicons into single stranded DNA through melting domains which will decrease their mobility. Thus, different sequences will result in different origins of melting domains and consequentially in different final positions in the gel. A “GC-clamp” attached to the 5’- end of one of the primers to prevent complete denaturation of the PCR products during the electrophoresis (Fig. 7).

DGGE technique allows a rapid, simultaneous and reproducible analysis of multiple environmental samples (Muyzer & Smalla, 1998; Kowalchuk *et al*., 2006). When combined with cloning and sequencing of specific bands, information on the phylogenetic affiliation of particular community members can be gathered (Smalla &

van Elsas, 2010). The main drawback of the DGGE method is that only strains of higher relative abundance in the total community DNA (> 1% of the target group) can be detected (Muyzer *et al*., 1993; Stephen *et al*., 1999). To improve the resolution of the DGGE analysis taxon-specific primers can be used. Several PCR primers have been designed and successfully employed to amplify 16S rRNA gene fragments of the four major bacterial phyla (*Alphaproteobacteria*, *Betaproteobacteria*, *Pseudomonas* and *Actinobaceria*) from total community DNA (Heuer *et al*., 1997; Gomes *et al*., 2005; Costa *et al*., 2006; Weinert *et al*., 2009). Other primers targeting the partial 18S rRNA gene of the fungal phyla *Ascomycota*, *Basidiomycota*, *Chytridiomycota* and *Zygomycota* are nowadays available (Smit *et al*., 1999; Borneman & Hartin, 2000). No primers providing total coverage of the phylum *Glomeromycota* containing all known AMF (Schlüsser *et al*., 2001) were developed for DGGE analysis until yet. Kowalchuk and collaborators (2002) described a 18S-DGGE approach to discriminate AMF species belonging exclusively to the *Glomerales* genus. They showed that *Glomus* species shared a short range of electrophoretic mobility, which might result in difficulties to discriminate differentiating bands. Furthermore, it has been shown that different species could not be distinguish from each other, while some other can produce more than a single DGGE band, most likely due to the heterogeneity between different rRNA operons within a single AMF spore (Clapp *et al*., 1999). In order to increase the reliability of the DGGE method for AMF, alternative chromosomal regions need to be targeted. Recently, Krueger *et al.* (2009) developed new primers suitable for specifically amplifying all AMF lineages from environmental samples. These primers target the SSU-ITS-LSU fragments that allows phyogenetic analyses of AMF with species level resolution. Thus, the refinement of these primers for DGGE analysis could be matter of high interest for AMF fingerprinting.

In this PhD work 16S- and ITS-DGGE were used to investigate the shifts of the microbial communities due to WCR larval feeding, and to assess bacterial and fungal community structures in the soil, rhizosphere, endorhiza of maize, eggs and gut of WCR larvae. ITS-DGGE was chosen because it showed for single strains a higher discrimination power compared to 18S-DGGE (Fig. 8). To study the AMF populations the alternative but more time-consuming PCR-RFLP method was used.

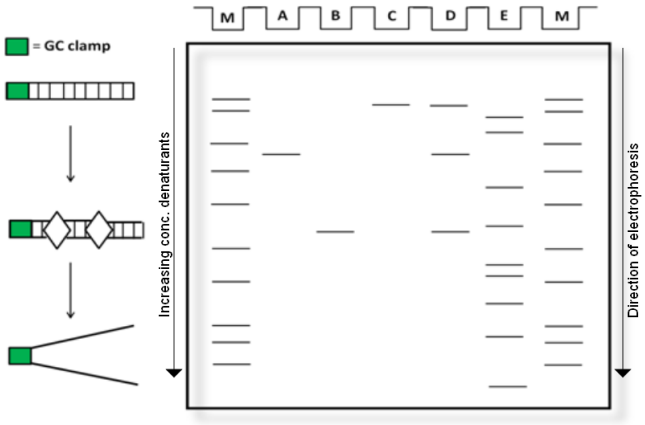


Fig. 7. Principle of DGGE method. M: marker; A: organism 1; B: organism 2; C: organism 3; D: mix of organisms 1, 2 and 3; E: unknown sample. Reproduction of a image developed by Vanhoutte *et al*. and available at the web site http://bccm.belspo.be/newsletter/17-05/bccm02.htm.

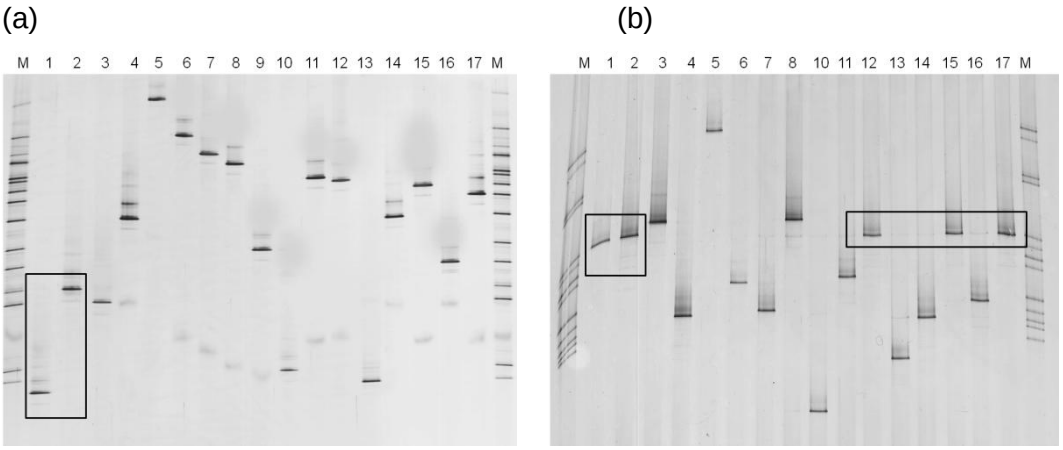


Fig. 8 (a) ITS-DGGE and (b) 18S-DGGE of single strains. The figure shows the higher resolution power of the ITS regions compared to the 18S fragments. M: marker; lane 1: *Verticillium nigrescens*; lane 2: *Paecilomyces marquandii;* lane 3: *Trichoderma* sp.; lane 4: *Penicillium canescens*; lane 5: *Rhizoctonia solani*; lane 6: *Sclerotinia sclerotiorum*; lane 7: *Microdochium bolleyi*; lane 8: *Fusarium redolens*; lane 9: *Verticillium dahliae*; lane 10: *Basidiomycete* sp.; lane 11: *Fusarium solani*; lane 12: *Fusarium* sp.; lane 13: *Sporothrix inflate*; lane 14: *Penicillium canescens*; lane 15: *Nectria haematococca*; lane 16: *Doratomyces sp.*; lane 17: *Fusarium graminearum.*

*PCR-RFLP analysis and sequencing of AMF clone library*

The characterization of the arbuscular mycorrhizal fungi (AMF) is extremely difficult due to several factors: (i) as obligate biotrophs, AMF can be cultured only in presence of a host plant; (ii) microscopic analysis does not allow to distinguish species belonging to a single genus due to the extremely limited variety of discernible structures that AMF forms *in planta*; plus several lineages do not stain with standard procedures (Redecker *et al.*, 2000); (iii) spores of the same species contain a multiple and polymorphic genome (Hijri and Sanders, 2005).

In the last decade, to study AMF populations in root samples, molecular approaches have been developed. Almost all identification systems for AMF are based on the ribosomal DNA, which allows to distinguish taxa at many different level (Redecker *et al*., 2003). The restriction fragment length polymorphism (RFLP) of cloned amplicons of the SSU (18S) gene fragments from total community DNA was shown to be sensitive, reproducible, and highly robust (Vallino *et al.*, 2006). However, this approach amplifies most, but not all *Glomeromycota*. Only members of the *Glomerales* family can be detected, while members more rare of the *Archeosporales* and *Paraglomerales* are excluded.

To increase the spectrum of detectable AMF in root samples Lee *et al.* (2008) developed an alternative approach based on a specific AMF nested-PCR encompassing all known AMF families.

Both RFLP type analysis and specific AMF nested-PCR were tested during my PhD work not only for root material, for which the methods were developed, but also for soil samples.

AMF nested-PCR was less laborious than PCR-RFLP type analysis and has higher species level resolution (populations belonging to the *Archeosporales* and *Paraglomerales* could be detected). But unfortunately, when applied to soil samples mainly fungi belonging to the phylum *Ascomycota* were amplified, indicating that the primers were not specific.

As the *Glomerales* represent the biggest group of AMF known, and the PCR-RFLP described by Vallino *et al*. (2006) can be applied on total community DNA from root and soil samples, it was used for the investigation in this thesis.

Although the DGGE and RFLP methods are of great help for the study of the microbial communities in environmental samples they do not provide quantitative data.

*Quantitative PCR (qRT-PCR)*

Quantitative real-time PCR technologies allow quantification of the copy number of a target DNA present in environmental samples by comparing the observed amplified signal intensity with a standard curve (Fig. 9a). The standard curve is usually constructed using serial dilutions at 10- or 5-fold of a standard DNA template. The signal intensity of amplified DNA products during the PCR amplification is recorded using a selected fluorescent-reporting system, and then normalized. Common fluorescence reporting chemistries include *Taq*Man probes, molecular beacons and DNA intercalating dyes such as SYBER Green (Giulietti *et al*., 2001). By selecting an arbitrary threshold, usually set at a level that is 10 times the standard deviation of the baseline signal observed between cycles 3 and 5, the corresponding threshold cycle (Ct) at each reference template concentration can be defined (Prosser *et al*., 2010).

An important parameter that needs to be considered in order to obtain accurate and reproducible results is the efficiency of the reaction, which should be as close as possible to 100% (e.g., two-fold increase of amplicon at each cycle). The qRT-PCR efficiency can be calculated by the following equation: $E = 10^{(-1/slope)} - 1$. This corresponds to a slope of -3.1 to -3.6 in the $C_t$ vs log-template amount standard curve.

For SYBR Green based amplicon detection, it is important to run a melting curve following the real time amplification. This is due to the fact that SYBR Green will detect any double stranded DNA including primer dimers, contaminating DNA, and PCR product from misannealed primer. Because each dsDNA has a melting point (Tm) at which temperature 50% of the DNA is single stranded, and the temperature depends on the length of the DNA, sequence order or G/C content, the dissociation curve of a single target should produce only one pick. Contaminating DNA or primer dimers would show up as an additional peak separate from the desired amplicon peak. A typical plot of the derivative of the dissociation curve is shown in Figure 9b.

Real-time PCR allow the quantification of up to four different targets simultaneously down to a concentration theoretically close to 1-2 copies of DNA template contained in environmental samples (Giulietti *et al.,* 2001).

The qRT-PCR reaction based on SYBER Green 1 was used in this work to evaluate the root mycorrhization level of *Glomus intraradices* according to Alkan *et al.* (2006).

The primers target the ITS1 and 18S rRNA regions and produce amplicons with 101 bp length.

(a) (b)

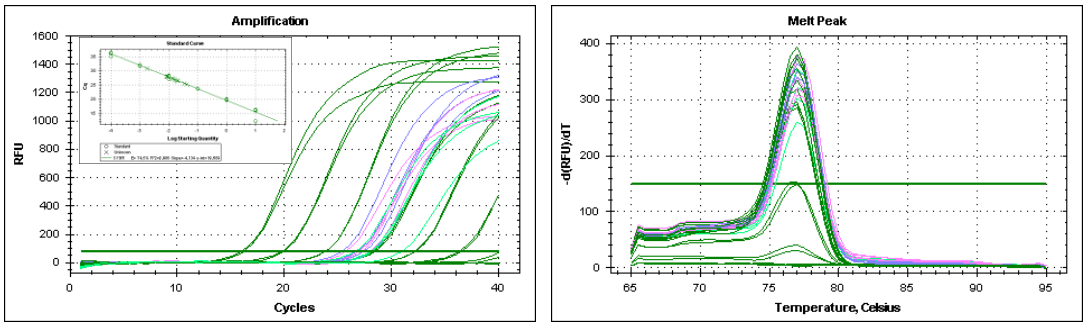


Fig.9. (a) Fluorescent intensity of specific *Glomus intraradices* sequences (in violet) in maize roots and of serial dilutions of standard samples (in green) obtained by quantitative Real Time PCR. The inset illustrate the reaction between the $C_t$ value and the standard gene copy number. (b) The derivative melting curve of standard and unknown samples from Fig. 9a. The melting curve shows only one pick around 76 °C, indicating the specificity of the qRT-PCR reaction.

## Objectives

The objectives of the present study were:

1. To investigate the effects of the root larval feeding of the WCR on the rhizospheric microbial communities;

2. To study the complex interactions among WCR, *Glomus intraradices* (*G.i*.) and microbial communities in the rhizosphere and endorhiza of maize plants;

3. To assess the effect of the soil type on the fungal and bacterial communities inhabiting the digestive tract of WCR larvae;

4. To investigate the dominant microorganisms associated with the gut and eggs of the WCR, and their transovarial transmission.

## Thesis outline

**Chapter 1** gives an overview about the WCR and the multiple interactions among herbivorous insects, plants, soil and rhizospheric or endophytic microorganisms. Furthermore, molecular methods to assess complex microbial community structures of environmental samples and to characterize specific members of those communities are reported.

**Chapter 2** presents cultivation-independent methods to study plant endophytic fungal communities. 18S- and ITS-DGGE methods are proposed to investigate the total fungal communities, while PCR-RFLP analysis or specific nested PCR followed by cloning and sequencing were presented for the study of arbuscular mycorrhizal fungi. A detailed description of these methods, their potential and limitations are reported.

**Chapter 3** aims to investigate the effects of WCR larvae on the fungal and bacterial communities in the rhizosphere of maize. These effects were assessed in four

maize genotypes grown in three different soil types. Microbial communities were investigated by means of ITS- and 16S-DGGE analyses. Cloning and sequencing of specific DGGE bands were performed to identify specific microbial populations responding to WCR larval feeding.

**Chapter 4** presents the complex interactions among WCR, *Glomus intraradices* (*G.i*.) and microbial communities in the rhizosphere and endorhiza of maize plants. Plant inoculated or not with *G.i.* were exposed to WCR larval feeding for 20 days. Treatment effects were assessed with respect to the larvae and to the arbuscular mycorrhizal, bacterial and fungal communities in the rhizosphere and endorhiza of maize. In order to study the microbial communities microscopic analyses and molecular methods such as quantitative Real Time PCR, restriction fragment length polymorphism, cloning and sequencing, and DGGE analyses were used.

**Chapter 5** reports a study aiming to investigate the effects of the soil type on the fungal and bacterial communities inhabiting the digestive tract of WCR. The effects were assessed for one maize genotype in three soil types by ITS- and 16S-DGGE technique. Furthermore, this study provides data on the most dominant gut- and egg-associated microorganisms by DGGE fingerprints and band sequencing. Their transovarial transmission was investigated by comparative DGGE fingerprints, sequencing and phylogenetic analysis of microbial communities in gut and egg samples.

**Chapter 6** summarizes the overall studies and the main findings presented in this PhD thesis.

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# Chapter II

## Study of fungal endophytes in plant roots versus rhizosphere and soil fungal communities.

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**Study of fungal endophytes in plant roots versus rhizosphere and soil fungal communities.**

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## 2.1 Theory

### 2.1.1 Abstract

The plant roots represent a dynamic interface between plants and their environment. In this context, the root inhabiting communities, the endophytes, have a fundamental role in the persistence of the plants in the field. Several studies have shown in fact that the colonization of plant host by fungal root endophytes may lead to higher disease resistance, enhance the growth of the host plant, and increase the tolerance to biotic and abiotic stress. The main problems related to the study of endophytic fungi are the difficulties of isolating them *in vitro* and defining their taxonomy based on morphological markers. The aim of this chapter is to present cultivation-independent methods to study plant endophytic fungal communities. The 18S-DGGE analysis was applied to study the effect of T4-lysozyme, produced by transgenic potato lines, on endophytes. The ITS-DGGE analysis was used to study the endophytic population in maize roots. For studying arbuscular mycorrhizal fungi,

two different strategies were applied to assess the endophytic fungal communities in maize root in comparison with the communities of the soil.

### 2.1.2. Background

Endophytic fungi can be defined as fungi which can be isolated from the tissue of surface-sterilised symptomless root material or which can be detected in the total community DNA extracted from these roots (Götz *et al.*, 2006). Fungal endophytes can colonise plants in a local or systemic manner, and their growth can be inter- or intracellular (Boyle *et al.*, 2001; Schulz and Boyle, 2005). Still their function is unclear, although several studies have shown positive effects of fungal endophytes on the fitness of the host plants (Römmert *et al.*, 2002). The higher performance is particularly notable under stressful conditions, such as high temperature or nutrient and water deficiency. Due to the production of antitumor agents, such as taxol, the endophytic fungi can be considered a potential source for new natural bioactive agents (Wang *et al*., 2000).

In the past, culture-dependent methods and microscopic approaches have been used to investigate the endophytic fungal communities in different types of plant (Arnold *et al*., 2001; Wilberforce *et al.*, 2003). Those methods are quite laborious, time consuming and not suitable for comparing large numbers of samples. In addition, fungi at quiescent stage, or with special growth requirements, are often not retrieved.

In the last decade culture-independent methods, based on the analysis of nucleic acid extracted from plant tissues, have been developed and allow also the study of endophytic fungi which cannot be cultivated *in vitro*. The analysis of total community DNA extracted from plants by means of PCR-based methods and sequencing of the specific gene fragments used as molecular markers led to the discovery of thousands of new sequences. Furthermore, these methods have allowed the study of the composition of fungal communities in different environmental habitats.

The molecular markers used for the phylogenetic study of fungi are mainly represented by the SSU (18S) rRNA gene fragments and the ITS regions. The advantage to use the 18S rRNA gene fragments as molecular marker is related to the size of the fragment (ca. 1650 bp) which carries a lot of information. Due to the rather high conservation of the rRNA genes within the fungi, some SSU rRNA gene fragment may not contain the necessary variation to allow discrimination between

closely-related taxa (Gomes *et al*., 2003). In contrast, the ITS regions have higher intra-specific variability that results in a higher discrimination power. The limitation of ITS marker is mainly represented by the 500 bp size.

Both these molecular markers have successfully been used in fungal community studies based on DGGE fingerprinting. The DGGE method was pioneered by Gerard Muyzer *et al*. (1993) and it enables the electrophoretic separation of PCR amplicons of equal length in a sequence-specific manner. The principle of this technique relies on the use of a denaturing gradient polyacrylamide gel and on the use of "GC-clamp" attached to the 5' end of one of the primers to prevent complete denaturation of the products during the electrophoresis. The advantages of the DGGE technique are the rapid, simultaneous and reproducible analysis of multiple samples represented by amplicons from complex environments, such as soil or plant systems. In addition, it is possible to determine the sequences of bands of interest by excision of the band from the gel, re-amplification and sequencing. The main limitation of the method is the detection of minor populations, as only strains of higher relative abundance in the total community DNA (up to 1% of the target) can be detected. But there are other limiting factors related to this technique that need to be considered. For example, the bands representing different fungal species often share the same electrophoretic mobility. Alternatively, a single isolate or population is characterised by a multiple banding pattern caused by sequence heterogeneities in the fragments amplified, which can lead to an overestimation of the number of the populations observed. In Figure 1, typical 18S- and ITS-DGGE profiles from soil samples can be observed. In the experimental procedures chapter, we provide the protocols in detail for the study of the total endophytic fungal communities in maize plant roots through 18S- and ITS-DGGE fingerprinting techniques.

Within the endophytic fungi, the arbuscular mycorrhizal fungi (AMF) represent a really important group. Due to their symbiotic nature and the high genetic heterogeneity, the study of AMF communities needs, compared to other taxa, alternative molecular strategies.

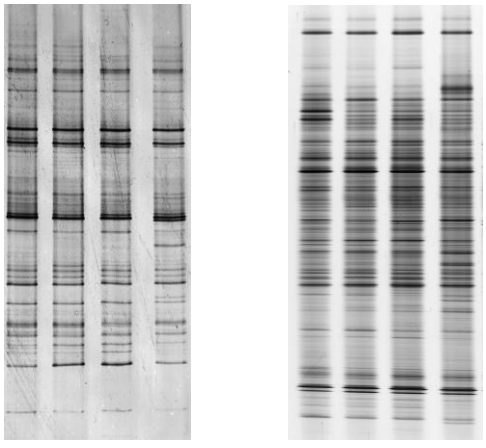
According to the SSU rRNA gene sequences, the AMF belong to the phylum of *Glomeromycota* which include four statistically highly supported main orders: *Glomerales*, *Diversisporales*, *Archeosporales* and *Paraglomerales* (Schüßler *et al*., 2001). They typically penetrate the root cells and produce tree-like structures termed "arbuscules". AMF form mutualistic symbiotic associations with roots of ca. 80% of all terrestrial plant species, and they have a significant impact on the plant

biodiversity, productivity and ecosystem stability (van der Heijden *et al.,* 1998). The benefits due to the mycorrhization of the plant may differ depending on the interacting partners. In general, AMF hypae are well known to increase the soil area explored by the roots and to enhance the uptake of mineral nutrient from the soil; AMF can improve host-plant disease resistance (de la Pena *et al*., 2006) and enhance resistance to water stress and heavy metal contaminations.

In the experimental procedure chapter we present two strategies for studying the structure of the AMF communities in plant roots in relationship with the fungal communities in bulk soil or in the plant rhizosphere. The first strategy is based on a PCR-RFLP technique applied according to Vallino *et al*. (2006) and the second one is based on a nested-PCR amplification developed by Lee *et al*. in 2008.

The PCR-RFLP strategy is group-specific for AMF belonging to the order of the *Glomerales* and is based on the PCR amplification of the SSU gene fragments from total DNA, cloning, restriction and sequencing. The main advantage of PCR-RFLP is the flexibility of the method as it can be applied to bulk or rhizosphere samples, so that comparison can be made between them. The disadvantage of the procedure is that it is time consuming and restricted to the *Glomerales* excluding populations belonging to the *Archeosporales* and *Paraglomerales.*

The second strategy is based on the use of primers designed to detect all fungi belonging to the taxa of the AMF. Compared to the PCR-RFLP strategy, the nested-PCR approach is less laborious and has a higher species level resolution. The main disadvantage is its low specificity when applied to soil samples.



a.18S-DGGE b. ITS-DGGE

Figure 1. Typical 18S-DGGE and ITS-DGGE profile from four replicates of the same soil samples.

### 2.1.3. Study of the influence of the T4-lysozyme on the endophytic fungal community in potato plants by 18S DGGE fingerprinting

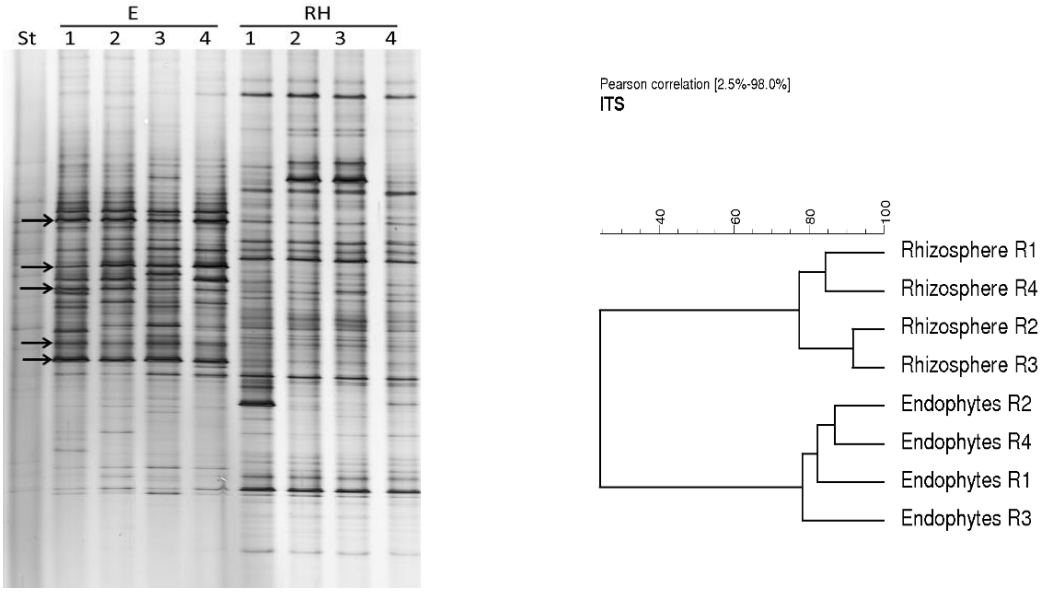
Endophytic fungi in surface-sterilised roots of potato plants were analysed by means of traditional isolation methods and a DNA-based, cultivation-independent analysis to test the hypothesis that endophytic fungi are affected by T4-lysozyme secreted into the apoplast (Götz *et al.*, 2006). Transgenic T4-lysoyme producing plant line (DL 11) and parental line Désirée were grown in field trials in Groß Lüsewitz, Germany (Federal Center for Breeding Research, BAZ). The tubers were planted in a randomised-block design with six replicates per clone or line. For the analysis of the endophytic fungi, root samples of the parental potato line Désirée and transgenic line DL 11 were taken at growth stages 91-97. The plants were carefully removed from each plot and the total DNA from roots was extracted after surface-sterilisation. 18S rRNA gene fragments amplified from total community DNA were analysed by DGGE and by cloning and sequencing. The 18S rRNA gene fragments were also amplified from the genomic DNA of abundant endophytic fungi that were isolated from root segments. A standard, composed of PCR-amplified 18S rRNA gene fragments of different isolates, was used as the marker for the 18S rRNA gene DGGE fingerprints. The DGGE fingerprints showed for both the transgenic and the parental line a high number of bands indicating a high colonisation rate with a high number of fungal species. Comparison of the patterns showed differences between the endophytic populations of the parental line Désirée and the transgenic T4 line DL 11. Remarkably, the electrophoretic mobility of 18S rRNA gene fragment of most isolates could be assigned to dominant bands in the community patterns. However, an identical electrophoretic mobility does not necessarily mean identical sequences (Gomes *et al.*, 2003). Due to the difficulties to successfully re-amplify 18S rRNA genes from excised bands (possibly due to the size of the PCR product), we decided to use a cloning and sequencing approach. Sequencing of 18S rRNA gene fragments from root DNA and isolates revealed that the sequences of dominant fungal endophytes were identical to those of dominant clones. Interestingly, the isolates and the clones that were most frequently obtained were affiliated to *Verticillium dahliae* and *Colletotrichium* sp. However, cloning and sequencing of 18S rRNA gene fragments amplified from total DNA also revealed that three clones were chimeric sequences. Interestingly, one of these chimeric sequences had an identical electrophoretic mobility as the dominant band in the DGGE community pattern that was only detected for the Désirée samples.

In the study by Götz *et al.* (2006) differences in the composition and relative abundance of endophytic fungi were revealed with both cultivation-dependent and independent methods indicating an effect of the T4-lysozyme expression on endophytic fungi. Moreover, the analysis of 18S rRNA gene fragments that was used for both methods, helped to link both approaches.

### 2.1.4. Assessment of the endophytic fungal community structure in maize root by ITS-DGGE fingerprinting

Maize plants were grown in the greenhouse in pots containing Schwarzerde soil. The maize cultivar used in our experiment is the commercial line KWS 13 (Einbeck, Germany). The soil type was collected nearby Göttingen (Germany) in an area used for agriculture. The maize growing conditions were the following: 40% relative humidity, 24°C mean temperature and 16 hours of additional illumination with sodium lamps (400W, HS2000, Hortilux Schréder, Monster, The Netherlands). After 4 weeks, the plants were harvested, the roots were surface-sterilised and the rhizosphere was isolated for the total microbial DNA extraction. The total DNA extracted from the soil was also included in our experiment for comparisons. The procedures for the rhizosphere isolation and the DNA extraction from soil were done according to Weinert *et al*. (2009).

ITS-DGGE was applied to the rhizosphere- and root samples in order to investigate the composition of the endophytic communities in maize roots in comparison with fungal communities present in the rhizosphere. The ITS-DGGE profile in Figure 2A revealed a highly complex endophytic community in maize roots. The DGGE analysis enabled us to identify fungal populations that were detected only in the roots and not in the soil. Many fungal populations detected in the rhizosphere were not found in the fingerprints of the endophytic communities. Cluster analysis of the DGGE gel clearly revealed that fungal communities present in the rhizosphere share only 22.4% similarity with the endophytic population whereas the replicates of DGGE fingerprints of endophytic fungi shared more than 78.2% similarity. Both the presence of bands specific for the endophytic fungi in the DGGE fingerprinting and the dendrogram (Figure 2B) showed a distinct community composition of the dominant fungal endophytes versus the rhizosphere fungal communities.




A B

Figure 2A) ITS-DGGE fingerprinting obtained from root DNA (E) and rhizosphere DNA (RH) samples. Four independent replicates (R) per treatment are reported. B) Dendrogram obtained by GELCOMPAR analysis of the DGGE gel.

### 2.1.5 Detection of arbuscular mycorrhizal fungi: two different strategies

Our first strategy to investigate and compare the AMF communities in root and in soil samples was based on the PCR-RFLP analysis. The soil type, the maize cultivar and the design of the experiment were the same described in our experimental procedures. The total DNA from roots and from soil was amplified with the primers AM1/NS31, targeting the 18S gene fragments. The amplicons were cloned into pGEM Easy Vector and transformed into *Escherichia coli* JM109. Around 160 clones from soil and 200 clones from plant roots were digested with the restriction enzymes *Hinf1* and *Hin1II,* and ten clones representative of each RFLP type found were sequenced. The RFLP types were defined according to Vallino *et al*. (2006). The results obtained from the soil analysis show the presence of several RFLP types belonging to AMF species with a potential role in the fertility of the soil and in the plant nutrition. The analysis of amplicons from the root DNA showed a co-colonisation of different AMF of the maize plant (Figure 3). RFLP types 8, 2, 10 and 11 were detected in the soil and in the root samples but in a different frequency. The RFLP type 8, corresponding to *Glomus mosseae* species, was the most abundant RFLP type present in the soil whereas the RFLP type 11, corresponding to *Glomus*

*intraradices* species, was dominant only in the roots. The results of the comparison between soil and roots show that AMF present in the soil in a really low concentration can become dominant in the host plant.

The second strategy applied to optimise the working time and increase the resolution level of the RFLP technique, was based on a nested PCR approach carried out with primer specific for AMF (Lee *et al*., 2008), cloning and sequencing. This approach was applied to study the same soil and root samples used for the RFLP analysis. The sequencing of the root amplicons confirmed the results obtained with the RFLP method, but surprisingly, several sequences obtained from the soil matched with *Ascomycota* spp. fungi instead with AMF. For this reason, the RFLP method is still recommended for soil samples.

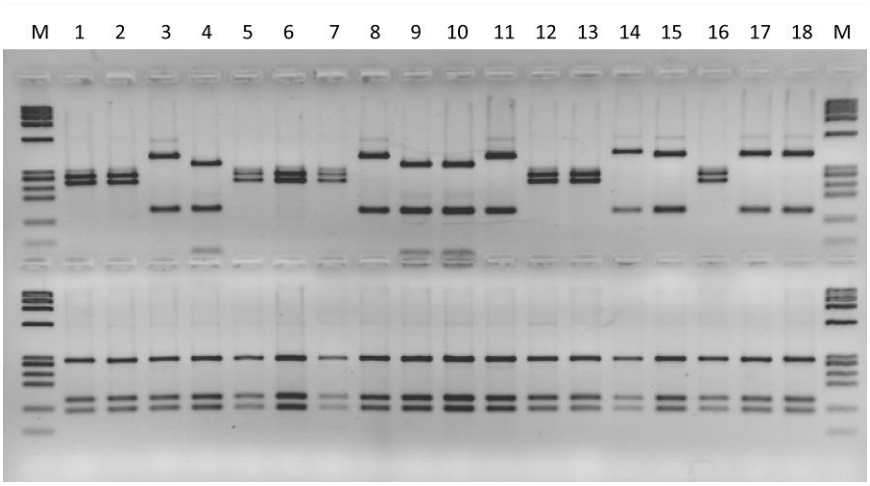



Figure 3. RFLP profiles of clones isolated from maize roots. Each lane shows the RFLP profile of a single clone when digested separately with the enzyme Hinf1, in the upper part of the gel, and with the enzyme Hin1II, in the lower part of the gel. Lines 1, 2, 5, 6, 7, 12, 13: RFLP type 8; Lines 3, 8, 14, 15, 17, 18: RFLP type 11; Lines 4, 9, 10: RFLP type 10. M: molecular weight marker IX (Boehringer Mannheim GmbH, Germany).

## 2.2. Experimental procedures

### 2.2.1. Equipment and materials

A. Equipment

| Equipment | Type | Producer |
|---|---|---|
| Basic stir plate | MR-3001K | Heidolph |
| Centrifuge | 5415C | Eppendorf |
| DGGE machine and accessories: glass plate, spacers, sandwich clamps, combs, alignment card, casting stand, rubber strip, buffer tank, central core | DCode™ System | Bio-Rad |
| Electrophoretic chambers and accessories | Power Pac™ Basic | Bio-Rad |
| FastPrep bead beating system | FastPrep FP 120 | Bio-101 |
| Gel Documentation System (UV transillumination table + camera) | UV System INTAS ® | Mitsubishi Electric Corporation |
| Gradient maker | GM-100 | C.B.S. Scientific |
| Laminar flow | HB2472 | Heraeus Instruments |
| Magnetic Stirrer | 33998-326 | VWR |
| Peristaltic Pump | Miniplus 2 | Gilson |
| pH meter | 643 | Ingold |
| Pipettes | - | Gilson |
| Power supply | Power Pac™ Basic | Bio-Rad |
| Silver nitrate trays | - | - |
| Sodium hydroxide trays | - | - |
| Thermocycle | Biometra | Biometra |

B. Chemicals and consumables

| Product name | Product number | Supplier |
|---|---|---|
| ***Chemicals*** | | |
| Acetic acid | 1.00063.1011 | Merck |
| Agarose | 840004 | Biozym |
| Agarose | 840004 | Biozym |
| AmpliTaqGold with | 4311806 | Applied |

| | | |
|---|---|---|
| GeneAmp | | Biosystems |
| AmpliTaq DNA Polymerase Stoffel Fragment | 58002069-01 | Applied Biosystems |
| Ammoniumperoxodisulfate | K 31009201 | Merck |
| Bacto™-yeast extract | 212750 | Becton Dickinson |
| Bromophenol blue | 32712 | Riedel de Haen |
| Competent cells JM109 | L2001 | Promega |
| Deoxynucloeside thriphosphate Set | 11 969 064 001 | Roche Diagnostics |
| Dimethylsulfoxide | 41639 | Fluka |
| Ethanol | 1.08543.0250 | Merck |
| Ethidium bromide | 1.11628.0030 | Merck |
| Ethylene Diamine Tetraacetic Acid | 8043.2 | Roth |
| FastDNA SPIN Kit for Soil | 11-6560-200 | MP Biomedicals |
| Formaldehyde 37% | 4979.1 | Roth |
| Formamide | 6749.1 | Roth |
| GENECLEAN Spin Kit | 1101-600 | MP Biomedicals |
| Glycerin | 1.04094.1000 | Merck |
| Glucose | 1.08342.100 | Merck |
| High DNA Mass Ladder | 10496-016 | Invitrogen |
| *Hinf 1* Enzyme | ER0801 | Fermentas |
| *Hin1*II Enzyme | ER1831 | Fermentas |
| Isopropyl-beta-thio galactopyranoside | 2316.3 | Roth |
| LB–Agar (Lennox) | X965.2 | Roth |
| Magnesium chloride hexahydrate | 1.05833.100 | Merck |
| Magnesium sulfate heptahydrate | 1.05882 | Merck |
| MinElute PCR purification Kit | 28006 | Qiagen |
| Molecular weight marker IX | 1449460 | Boehringer Mannheim |
| Primers | without GC clamp | MWG |
| | plus GC clamp | IBA Nucleic Acids Synthesis |
| Potassium chloride | 1.04936.0500 | Merck |
| pGEM-T vector system | A1380 | Promega |
| Rotiophorese gel 30 (37.5:1) | 1.01201.0100 | Roth |
| Serdolit MB-1 | 40701 | Serva Electrophoresis |
| Silver nitrate | 7908.1 | Roth |
| Sodium carbonate | 8563.1 | Roth |
| Sodium chloride | 3957.2 | Roth |
| Sodium hydroxide | 1.06498.1000 | Merck |
| Sodium hypochlorite 12% | 017011001 | EWG (EINECS) |

| | | |
|---|---|---|
| Streptomycin sulphate | 85880 | Fluka |
| Tetramethylethylendiamine | 35930.02 | Serva Electrophoresis |
| Tris Acetate EDTA | 4855.2 | Roth |
| Tryptone Peptone | 211705 (0123-17) | Becton Dickinson |
| Urea | 3941.2 | Roth |
| Xylene cynole | 806801 | MP Biochemicals |
| X-Gal | R0401 | Fermentas |
| ***Consumables*** | | |
| DGGE gel loading tips | 729011 | Biozym |
| GelBond pag film | 54731 | Lonza |
| Petri dishes | 82.1195 | Sarstedt |
| Tips | - | Sarstedt |
| Tubes | - | Eppendorf |

### 2.2.2. Solutions

**EDTA (Ethylene Diamine Tetraacetic Acid), 0.5M pH 8:** dissolve 186.1 g EDTA into 800 mL of distilled water. Add ca. 20 g of NaOH pellets and adjust the pH to 8.0. Add the last few grams slowly to avoid overshooting of the right pH. Filter with 0.5 micron filter and autoclave. Store at room temperature.

**TBE (Tris Borate EDTA) Buffer, 5X:** dissolve 27.5 g boric acid, 54 g Tris base and 20 mL of 0.5 M EDTA pH 8.0 in 800 mL of distilled water. Bring the volume up to 1 liter and store at room temperature.

**TAE (Tris Acetate EDTA) Buffer for DGGE, 50X:** dissolve 242.2 g Tris base, 18.6 g EDTA and 57.1 mL acetic acid in 1L of distilled water. Store at room temperature.

**Deionised formamide:** add 10 g/L Serdolit MB-3 (Serva) to the formamide and stir slowly for about 30 min. Filter the solution to remove the ionic exchange resin through a *Whatman* filter-paper, aliquot in 50 mL falcon tubes and store at -20°C.

**Denaturing gradient acrylamide stock solutions:** the denaturant gradient is produced considering that 100% denaturant solution contains 40% deionised formamide and 7 M urea (Muyzer *et al*., 1993) (see note §1.6.).

- **18% denaturant gradient 7.5% acrylamide stock solutions:** dissolve in 100 mL of Milli-Q water 18.93 g of urea, 5 mL of 50X TAE, 18 mL deionised formamide and 62.5 mL acrylamide/bisacrylamide (radiophorese gel). Adjust the volume up to 250 mL in a volumetric flask and filter sterilise. Aliquot 14.5 mL of the solution in 15 mL polypropylene conical tubes (falcon) and store at -20°C.
- **58% denaturant gradient 9% acrylamide stock solutions:** dissolve in 100 mL of Milli-Q water 60.87 g of urea, 5 mL of 50X TAE, 58 mL deionised formamide and 75 mL acrylamide/bisacrylamide (radiophorese gel). Adjust the volume up to 250 mL in a volumetric flask and filter sterilise. Aliquot 14.5 mL of the solution in falcon tubes and store at -20°C.
- **43% denaturant gradient 7.5% acrylamide stock solutions:** dissolve 45,195 g of urea in 100 mL Milli-Q water, 5 mL of 50X TAE, 43 mL deionised formamide and 62.5 mL acrylamide/bisacrylamide (radiophorese gel). Adjust the volume up to 250 mL in a volumetric flask and filter sterilise. Aliquot 14.5 mL of the solution in falcon tubes and store at -20°C.

**Ammoniumperoxodisulfate (APS):** prepare 10% APS solution (w/v) in Milli-Q water and store in aliquots at -20°C.

**Loading buffer 6X for DGGE:** dissolve 25 mg bromophenol blue, 25 mg xylene cyanole and 3 mL of glycerol. Add distilled water up to 10 mL. Store at 4° C.

**Marker for DGGE:** the marker for DGGE is composed of ITS PCR products obtained from single fungal isolates with different electrophoretic mobility in the denaturant gradient acrylamide gel.

**Staining solutions**

- **Fixation solution:** for 2L of fixation solution add 10 mL acetic acid and 200 mL ethanol to 1790 mL of Milli-Q water. Stir mix and store at room temperature.
- **Staining solution:** for 100 mL of staining solution solubilise 0.2 g of silver nitrate in 100 mL Milli-Q water. The staining solution must be freshly prepared.
- **Developing solution:** for 100 mL of developing solution add 400 µL of 37% formaldehyde to 100 mL 1.5% sodium hydroxide. The developing solution must be freshly prepared.
- **Stopping solution:** for 2L stopping solution dissolve 7.5 g of sodium carbonate in 1L Milli-Q water. Store at room temperature.

- **Conservation solution:** for 100 mL conservation solution, mix 250 mL ethanol and 100 mL glycerin in 650 mL of Milli-Q water. Store at room temperature.

**IPTG (Isopropyl-beta-thio galactopyranoside) solution, 0.1M:** dissolve 1.2 g of IPTG in 50 mL of distilled water. Filter-sterilise with a 0.22 µm syringe filter, aliquot in 1.5 mL tubes and store at 4 °C.

**X-Gal (5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside) stock solution, 20 mg/mL:** dissolve 5 g of 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside in 10 mL N, N´-dimethyl-formamide. Cover with aluminium foil and store at -20°C.

**Ampicillin, 50 µg/mL:** dissolve 0.5 g of ampicillin in 10 mL of distilled water. Filter-sterilise through a 0.2 µm syringe filter, aliquot in 1.5 mL tubes and store at 4 °C.

**$Mg^{2+}$ stock solution, 2M:** add 101.5 g magnesium chloride hexahydrate ($MgCl_2$ $6H_2O$), 123.3 g magnesium sulfate heptahydrate ($MgSO_4$ $7H_2O$) to 500 mL distilled water. Filter-sterilise through a 0.2 µm filter unit. Filter-sterilising units should be pre-rinsed with distilled water before use to remove any toxic material.

**NaCl stock, 1M:** dissolve 58.44 g of sodium chloride in 1L of distilled water. Autoclave and store at room temperature.

**KCl stock, 1M:** dissolve 74.55 g of potassium chloride in 1L of distilled water. Autoclave and store at room temperature.

**Glucose stock, 2M:** dissolve 180.16 g of glucose in 500 mL distilled water. Filter-sterilise through a 0.2 µm filter unit and store in aliquots at –20°C.

**SOC medium**: add 2.0 g tryptone peptone, 0.5 g bacto-yeast extract, 1 mL 1M NaCl and 0.25 mL 1M KCl to 97 mL distilled water. Stir to dissolve. Autoclave and cool to room temperature. Add 1 mL 2M $Mg^{2+}$ stock and 1 mL 2M glucose stock solution. The pH should be 7.0.

### 2.2.3. Steps of the Procedure

**Root sterilisation**

Start the root sterilisation from fresh material by carefully prewashing under running tap water. The sterilisation procedure used is described by Götz *et al*. (2006) and is done as follows: 1 min in ethanol (70%), 4.5 min in 5% sodium hypochlorite and three washing steps of 5 min with sterile water. Checking the efficiency of the method is recommended (see note “b” §1.6.).

**DNA extraction from maize roots and cleaning**

- Cut the maize roots into 1-cm segments and mix to randomise the selection of different root areas.
- For each sample, extract the total DNA from 0.4 g of root material using the FastDNA SPIN Kit for Soil (Q-Biogene, Carlsbad, CA, USA) according to the manufacturer´s protocol, with an additional initial step described here: place the root material into bead tubes containing a mixture of ceramic and silica particles (included in the kit) and freeze by immersion in liquid nitrogen.
- Subsequently, process the material twice in a FastPrep bead beating system (Bio-101, Vista, CA, USA) for 1 min at speed 5.5 m $s^{-1}$ to achieve a harsh lysis of the plant cell walls.
- Purify the extracted DNA with the GENECLEAN Spin Kit (Q-Biogene, Heidelberg, Germany) according to the manufacturer´s instructions.

**PCR amplification of the SSU (18S) rRNA gene fragment for DGGE fingerprinting**

Amplification of the 18S rRNA gene fragment (ca. 1650 bp length) for DGGE fingerprinting, is carried out using the primers NS1 and FR1-GC. Primer sequences, together with references are shown in Table 1.

- The PCR is performed in a Tgradient thermal cycler (Biometra, Göttingen, Germany) and the 25 µL reaction mixture contains: 1 µL template DNA (ca. 20 ng), 1 X Stoffel buffer (10 mM KCl, 10 mM Tris-HCl, pH 8.3), 0.2 mM dNTP (Roche Diagnostics GmbH, Mannheim, Germany), 3.75 mM $MgCl_2$, 2% DMSO, 0.2 µM of each primer and 2 U taq DNA polymerase (Stoffel fragment, Perkin Elmer Cetus).
- The PCR conditions are: initial denaturation step at 94°C for 8 min followed by 35 cycles of denaturation at 94°C for 30 s, annealing temperature at 48°C for 45 s, and extension at 72°C for 3 min, subsequently followed by 10 min extension step at 72°C.

Table 1. Molecular markers and relative primers to identify endophytic fungal communities in maize roots

| Marker | Primer pair | Primer | Sequence (5´→3´) | References |
|---|---|---|---|---|
| 18S rRNA | ***Direct PCR*** | | | |
| | NS1/ FR1-GC | NS1 | GTAGTCATATGCTTGTCTC | Vainio and Hantula, (2000) |
| | | FR1-GC | GCclamp[a]AICCATTCAATCGGTAIT | Vainio and Hantula, (2000) |
| ITS region | ***Nested PCR*** | | | |
| | ITS1F/ ITS4 | ITS1F | CTTGGTCATTTAGAGGAAGTAA | Gardes and Bruns, (1993) |
| | | ITS4 | TCCTCCGCTTATGATATGC | White *et al*. (1990) |
| | ITS2/ITS1F-GC | ITS2 | GCTGCGTTCTTCATCGATGC | White *et al*. (1990) |
| | | ITS1F-GC | GCclamp[b]CTTGGTCATTTAGAGGAAGTAA | Anderson *et al*. (2003) |

[a] GC clamp sequence of the primer FR1-GC: CCC CCG CCG CGC GCG GCG GCG GGC GGG GCG GGG GCA CGG GCC G

[b] GC clamp sequence of the primer ITS1F-GC: CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG G

**PCR amplification of the Internal Transcribed Spacer (ITS) regions for DGGE fingerprinting**

The ITS fragments of the endophytic fungal communities of maize roots are amplified using a nested PCR approach. The primer set used in the first PCR reaction are ITS1F and ITS 4, while ITS 2 and ITS1F-GC primers are used in the second polymerase chain reaction. Primer sequences, together with references are shown in Table 1.

- Perform the PCR in a 25 µL volume in the Tgradient thermal cycler (Biometra, Göttingen, Germany). The reaction mixture of the first PCR contains approx. 20 ng of template DNA, 1X AmpliTaqGold buffer, 0.2 mM deoxynucleoside triphosphates, 3.75 mM $MgCl_2$, 2% (mg/mL) dimethylsulfoxide, 2 U of Taq DNA polymerase (AmpliTaqGold with GeneAmp, Applied Biosystems, USA) and 0.2 µM concentration of each primer.
- The PCR conditions are: 95°C for 5 min, followed by 30 cycles of 30 s at 95°C, 30 s at 55°C, 1 min at 72°C and a final extension at 72°C for 10 min.
- Use 1 µL of the undiluted PCR product of the first PCR reaction as the template for the second amplification. Perform the second PCR under the same conditions as the first PCR, except with 25 cycles.
- Visualise 5 µL of amplification products gel by electrophoresis in 1.2 % agarose gel, ethidium bromide staining and UV light illumination.

**DGGE (Denaturing Gradient Gel Electrophoresis): 18S and ITS fingerprinting**

The denaturant gradient concentration of the DGGE solutions, the gel casting procedure and the running conditions reported in this book chapter are strictly referred to the Bio-Rad DCode system (note “c” §1.6.).

For the 18S-DGGE a 18-43% denaturant gradient gel (Vainio and Hantula, 2000) is required. The electrophoresis is performed at 180 V constant voltage at 58°C for 18 h. For ITS-DGGE analysis, instead, a denaturant gradient of 18-58% (Anderson *et al*., 2003) should be used and the conditions of the electrophoresis are 75 V constant voltage at 60°C for 18 h.

Analyse the DGGE profiles with the software package GELCOMPARE 4.0 (Applied Math, Kortrijk, Belgium). Subtract the background using a rolling disk method with an intensity of 10 (relative units) and normalise the lanes. Build a UPGMA dendrogram, based on the Pearson correlation indices for the cluster analysis of the DGGE profiles.

The preparation of DGGE gels involves several steps described below in detail.

**Assembly of the gel-chambers**

- Place the biggest glass plate on a plane table. Carefully clean the surface of the glass plate with 97% ethanol.
- Lay the GelBond film (Lonza, Basel, Switzerland) with the hydrophobic side in direct contact with the bigger glass-plate and make sure that the film is perfectly aligned with the short side at the bottom of the glass. Fix the film to the glass with the help of a ruler.
- Position the two spacers to the outermost edges of the largest glass plate and place the small glass on the top.
- Put the glass plates and the spacers together with the sandwich clamps in the casting stand in which a rubber strip is placed at the bottom to prevent leakage. Make sure that the bottom of the plates and the spacers are in the correct position and close the clamps trying to create the same pressure on both sides to prevent the “smiling” of the bands. Use the “alignment card” for this purpose. Do not over-tighten clamps to avoid that they will crack after few uses.
- Insert the comb in the glass plate sandwich.

**Preparation of the denaturing gradient acrylamide solutions**

Add 25 µL of 10% APS and 45 µL of TEMED to the “low” and “high” concentration denaturant solutions and mix gently by inverting them simultaneously a couple of times. Keep the solutions on ice in order to prevent premature acrylamide polymerisation. The preparation of the denaturant solutions is described in §1.4. Solutions.

**Casting of the denaturing gradient gel and polymerisation**

- Place the gradient marker on a stir plate with a small stir bar in the chamber containing the outlet port.
- Connect the gradient maker to the peristaltic pump and make sure that the pump is off and the gradient maker-channel is closed. Put a syringe needle to the tubing of the peristaltic pump and enter it in the middle of the comb, located in between the glass- plate sandwich.
- Pour the solution with the highest concentration of denaturant into the chamber of the gradient maker adjacent to the outlet port. Briefly open and close the valve in order to remove the air between the two chambers. Turn on the stir plate at speed 300 round per min. Pour the solution with lowest denaturant concentration in the empty chamber.

- Simultaneously turn on the peristaltic pump and open the valve between the two chambers. For optimal gradient gels a flow of 5 mL/min is recommended. Ensure that the solutions are not leaking out from the glass plate sandwich, and allow the gel to pour until air bubbles reach the syringe needle.
- After gel casting, remove the needle and flush the gradient marker and tubing with water to discard any remaining of polyacrylamide solution.
- Let the gel polymerise for at least 1 hour.

**Pre-run**

- Assemble one or two gel sandwiches in the core. If only one gel is used, a glass-plate sandwich without spacers must replace the second gel.
- Place the core into the buffer tank filled with TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0). Refresh 50% of the buffer at each new run. Check buffer level, set up the temperature and start the pump.
- When the buffer reaches the run temperature, turn off the system and remove the comb from the gel, which is now ready to be loaded with samples.

**Loading of the samples and electrophoresis**

- Equalise the volumes of PCR products to load the same DNA concentration of the samples.
- Add loading buffer to the samples (1:1) and load them with microcapillary gel loading tips. Note that not more than 20 µL of sample can be loaded without having overflow of the wells.
- Load the standard to the outermost lanes for determination of the band positions and to normalise the gel in the gel analysis procedure.
- Close the system and start the electrophoresis after checking that the buffer is set correctly and that the pomp is working properly.

**Staining procedure, drying and scanning**

- Transfer the gel to a tray for silver nitrate ($AgNO_3$) and pour 100 mL of fixation solution for 2 x 3 min or for 1 x 10 min, or overnight.
- Discard the fixation solution and pour 100 mL of 0.2% silver nitrate staining solution (freshly prepared) on the gel for 15 min.
- Discard the silver nitrate solution in a specific waste (see note d §1.6) and wash the gel at least twice for 1 min with Milli-Q water. Change the silver nitrate trays with one for sodium hydroxide (NaOH).

- Add 100 mL of developing solution (freshly prepared) to the tray with the gel. It is recommended to stop the developing process as soon as the first pale bands become visible.
- Discard the developing solution and add 100 mL of stopping solution for ca. 10 min depending on the darkness of the gel. Keep in mind that the gel still develops during this step. Bands of interest can be excised from the gel and re-amplified for sequence analysis (note “e” §1.6).
- Discard the stopping solution and pour 100 mL of conservation solution on the gel for at least 7 min. Cover up the gel with a cellophane film. Make sure that the film is wet.
- Place the gel in a rigid support and distend the cellophane film on its surface, carefully remove all the air bubbles in between. Fix the film on the gel using frames and clamps. Air-dry the gel at room temperature for 2 days. Note: the gel will become darker after drying.
- Transform the gel image in a digital picture using any of the scanning systems available.

**Detection of endophytic arbuscular mycorrhizal fungi (AMF) by RFLP analysis**

Endophytic AMF in maize roots are studied by a PCR-RFLP method (Vallino *et al.,* 2006) divided in the following step: 18S-PCR amplification, creation of a clone library, clone´s restriction, sequencing and analysis.

**PCR amplification of the SSU(18S) rRNA gene fragment**

The PCR is performed using the universal eukaryotic primer NS31 and the *Glomerales* group specific primer, AM1. Primer sequences, together with references are shown in Table 2.

- The PCR reaction is prepared in a final volume of 25 µL and contains: 1X AmpliTaqGold buffer, 0.2 mM dNTPs, 3.75 mM $MgCl_2$, 2 U of Taq DNA polymerase (AmpliTaqGold with GeneAmp, Applied Biosystems, USA) and 10 pmol of each primer.

- The PCR conditions are as follows: 95°C for 5 min, then 35 cycles at 94°C for 1 min, 62°C for 1 min, 72°C for 2 min, then 72°C for 10 min.

Table 2. Molecular markers and relative primers to identify endophytic AMF population in maize roots

| Marker | Primer pair | Primer | Primer sequence (5´→3´) | References |
|---|---|---|---|---|
| 18S rRNA | ***Direct PCR*** | | | |
| | NS31/ AM1 | NS31 | TTGGAGGGCAAGTCTGGT GCC | Simon *et al.* (1992) |
| | | AM1 | GTTTCCCGTAAGGCGCCGAA | Helgason *et al*. (1998) |
| | ***Nested PCR*** | | | |
| | NS1/ NS4 | NS1 | GTAGTCATATGCTTGTCTC | Van Tuinen *et al*. (1998) |
| | | NS4 | TTCCGTCAATTCCTTTAAG | Van Tuinen *et al*. (1998) |
| | AML1/ AML2 | AML1 | AACTTTCGATGGTAGGATAG A | Lee *et al*. (2008) |
| | | AML2 | CCAAACACTTTGGTTTCC | Lee *et al*. (2008) |

**Clone library and restriction**

- Ligate the amplicons, 550 bp length, in the pGEM-T vector system (Promega) and transform into *Escherichia coli* (JM109 Competent Cells, Promega) according to the manufacturer´s instructions.
- Screen the positive transformants with the primer pair NS31/AM1 and the following PCR conditions optimised for clone targets: 95°C for 10 min, 30 cycles at 94°C for 35 sec, 63°C for 35 sec, 72°C for 45 sec, and final step at 72°C for 10 min.
- Test the positive clones from each sample for RFLP by independent digestion with the enzymes *HinfI* and *Hin1II* (Fermentas), according to the manufacturer´s instructions, and analyse by 3% agarose gel electrophoresis.
- For an appropriate identification of the band size, use the molecular weight marker IX (Boehringer Mannheim GmbH, Germany) as the standard.

**Sequencing and analysis**

Clones representing each RFLP type should be chosen for sequencing. Re-amplify the selected clones with the primers SP6 and T7, purify with the “MinElute PCR

purification Kit" (Qiagen) and sequence. Analyse the DNA sequences by BLAST-n and CLUSTAL W programme at NCBI site for multiple sequence alignments.

## *Glomeromycota*-specific nested PCR

### *Nested PCR*

The 18 rRNA gene fragments of endophytic fungal communities in maize roots can be studied by a nested PCR amplification. The first PCR is performed with the universal eukaryotic primers NS1 and NS4 and the second PCR is performed with AML1 and AML2 primers targeting the taxa of the *Glomeromycota*. Primer sequences, together with the references are shown in Table 2.

- Prepare the PCR reaction mixture in a volume of 25 µL with 1 µL template DNA (ca. 20 ng), Stoffel buffer (10 mM KCl, 10 mM Tris-HCl, pH 8.3), 0.1 mM dNTPs (Roche Diagnostics GmbH, Mannheim, Germany), 3.75 mM $MgCl_2$, 10 pmol of each primer, 2 U taq DNA polymerase (Stoffel fragment, Perkin Elmer Cetus).
- Use the following PCR conditions: an initial denaturation at 95°C for 3 min, followed by 30 cycles at 94°C for 30 s, 40°C for 1 min, 72°C for 1 min, followed by a final extension at 72°C for 10 min.
- Dilute the amplicons from the first PCR by 1:50 with milliQ sterile water and used as the template for the second PCR reaction that is performed in the following PCR conditions: 3 min initial denaturation at 95°C, 30 cycles of 1 min primer annealing at 50°C and 1 min extension at 72°C, followed by final extension at 72°C for 10 min.

### *Cloning, and sequencing analysis*

The PCR products from the nested PCR reaction can be cloned into pGEM Easy Vector (Promega) and transformed into *Escherichia coli* JMP9. Positive clones should be randomly selected for sequencing. The sequences are analysed by BLAST-n programme at the NCBI site.

### 2.2.4. Note

a. The preparation of the DGGE solutions requires the use of highly toxic chemicals like formamide and acrylamide. Take appropriate precautions when handling these compounds.

b. Check the efficiency of the surface sterilisation method imprinting the treated roots on biomalt agar 50 g/L Biomalt (Villa Natura Gesundprodukte GmbH, Kirn, Germany) plus 20 g/L Bacto TM Agar (Becton Dickinson and Company, Sparks, MD, USA), pH 5.6 with the antibiotics penicillin G Na 60 mg/L, streptomycin sulphate 80 mg/L and (Oxy)-tetracycline HCl 50 mg/L.

c. The Bio-Rad DGGE system allows the simultaneous run of two gels with 15-25 lanes for each gel. The system is relatively easy to manipulate and can produce really high quality gels. The main disadvantage of the Bio-Rad apparatus is the design: the lid contains the motor, the rotor and the heating element (altogether they are defined the “core” of the machine), and they can be damaged when the lid is removed for moving gels in and out of the buffer tank.

d. The silver nitrate waste solution is disposed as follows: add 1g of NaCl per 100 mL of waste solution, cook it and stir the waste solution until the silver nitrate precipitates, filter the solution (pH 4) using filter paper and neutralise it with 10 N NaOH. Discard the solution in the regular waste and collect the metallic silver for a special disposal.

e. Single bands can be excised from acrylamide gel and re-amplified as described by Gomes *et al*. (2005).

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# Chapter III

## Multitrophic interaction in the rhizosphere of maize: Root feeding of Western Corn Rootworm larvae altered the microbial community composition

**Multitrophic interaction in the rhizosphere of maize: Root feeding of Western Corn Rootworm larvae altered the microbial community composition**

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**Abstract**

Larvae of the Western Corn Rootworm (WCR) feeding on maize roots cause heavy economical losses in the US and in Europe. New or adapted pest management strategies urgently require a better understanding of the multitrophic interaction in the rhizosphere.

This study aimed to investigate the effect of WCR root feeding on the microbial communities colonizing the maize rhizosphere.

In a greenhouse experiment, maize genotypes KWS13, KWS14, KWS15 and MON88017 were grown in three different soil types in presence and in absence of WCR larvae. Bacterial and fungal community structures were analyzed by denaturing gradient gel electrophoresis (DGGE) of the16S rRNA gene and ITS fragments, PCR amplified from the total rhizosphere community DNA. 16S-DGGE bands were excised from the gel, cloned and sequenced in order to identify specific bacteria responding to WCR larval feeding.

16S- and ITS-DGGE analysis showed that WCR larval feeding affected the fungal and bacterial populations inhabiting the maize rhizosphere in a soil type and plant-genotype dependent manner. DGGE band sequencing revealed an increased abundance of *Acinetobacter calcoaceticus* in the rhizosphere of several maize genotypes in all soil types. Our findings suggest that phenolic compounds released upon WCR wounding led to the observed bacterial community changes.

## Introduction

*Diabrotica virgifera virgifera* (Western Corn Rootworm, WCR; *Coleoptera: Chrysomelidae*), is considered one of the most destructive agricultural pests of maize (*Zea mays* L.) in the US (Sappington *et al.*, 2006). Since the beginning of the 1990s the WCR was accidentally and repeatedly introduced into Europe (Ciosi *et al*., 2008) where a cost damage of about 472 million Euro per year is expected (Wesseler & Fall, 2010). Currently 21 European countries reported finding of this pest (Kiss *et al*., 2005; Michaelakis *et al.,* 2010). Major damages are caused by the larvae feeding on the maize roots resulting in disrupted water and nutrient uptake and thus in plant lodging (Godfrey *et al.,* 1993). The expected severe yield losses (Rice 2004; Sappington *et al*., 2006), the increasing distribution (Kiss *et al.*, 2005; Michaelakis *et al.,* 2010) and the fast spreading of WCR (Spencer *et al.* 2005) prompted the EU to establish mandatory eradication and containment measures since 2003 (Decision 2003/766/EC; Decision 2006/564/EC; Recommendation 2006/565/EC). Up to now the annual crop rotation is considered the most effective and environmentally benign rootworm management method in Europe. However, the reported loss of ovipositional fidelity to maize field of American WCR populations which lead to the reduction of the crop rotation efficacy (Onstad *et al,* 2001, Levine *et al.*, 2002; Gray *et al*., 2009) alert European farmers to pest behavioural changes in maize production areas. New or adapted pest management strategies urgently require a deeper knowledge of the ecology of this soil-dwelling pest and its multitrophic interactions in the rhizosphere of their host plant (Meinke *et al.,* 2009).

Beneficial rhizosphere microorganisms promote plant growth and health by nutrient solubilization, nitrogen fixation and plant hormone production (Hayat *et al*., 2010). Some of them are involved in plant disease suppression or in the reduction of herbivorous insect damage (Van Loon *et al*., 1998; Van Oosten *et al*., 2008). This is either through a direct antagonism of soil-borne pathogens or triggering plant-mediated resistance responses (Van Loon *et al*., 1998; Nishida *et al*., 2010). Clearly, shifts of the rhizosphere microbial communities may affect not only key soil processes and soil fertility, but also the functionality of the agro-ecosystem.

Several studies have shown that the microbial composition in the rhizosphere may be influenced by different biotic and abiotic factors such as soil type, climate, cropping history, plant species, plant developmental stage and to a lesser extent cultivar (Berg & Smalla, 2009). Furthermore, it has been shown that root-feeding insects such as leather jacket larvae (*Tipula paludosa*) or cyst nematodes (*Heterodera trifolii*) may lead to shifts

in the microbial communities colonizing the soil, the root and the plant rhizosphere, most likely by changes of root exudation patterns (Treonis *et al*., 2004; Grayston *et al*., 2001; Dawson *et al*., 2004). Root exudates being suitable substrates for a wide range of microorganisms, were shown to play a fundamental role in shaping the microbial populations in the plant rhizosphere (Brimecombe *et al*., 2001; Bais *et al*., 2006; Broeckling *et al.,* 2008).

Despite the importance of the rhizosphere microorganisms, little is known about the multitrophic interactions between the plant, microbial communities in the rhizosphere and WCR larvae. To our knowledge, only Prischmann *et al.* (2008) provided information on the interaction of WCR and the maize rhizosphere bacterium *Serratia* by means of a cultivation-dependent method.

In this study we aimed at unravelling the multitrophic interactions between WCR and the microorganisms inhabiting the maize rhizosphere. Because different soil types and different maize plant genotypes might support different rhizosphere microbial communities, a greenhouse experiment was performed using three different soil types (Haplic Chernozem, Haplic Luvisol and Eutric Vertisol) and four maize plant genotypes including the traditional breeding lines KWS13, KWS14 and KWS15, and the transgenic maize MON88017. Different maize genotypes were also chosen because Broekgaarden *et al*. (2007) observed that the plant, in response to the same herbivorous insect, may activate cultivar-dependent transcriptomic changes, which might affect the rhizosphere microbial communities. We hypothesize that in response to WCR root feeding changes in the exudation patterns might result in shifts of the microbial communities in the rhizosphere of maize according to the soil type and maize genotype combination. The effects of larval feeding on bacterial and fungal communities in the rhizosphere were investigated by denaturing gradient gel electrophoresis (DGGE) fingerprinting of 16S rRNA gene and ITS fragments amplified from total rhizosphere community DNA.

## Materials and methods

### *Experimental design*

A greenhouse experiment was performed under quarantine conditions. Seeds of each maize plant genotypes were sown in plastic trays (34 cm x 26 cm) containing three different soil types (Haplic Chernozem, Haplic Luvisol and Eutric Vertisol) and the seedlings were transferred one week later into pots (Ø 13 cm). A gauze (voile, 100 % polyester, Alfatex Göttingen, Germany) was glued to the bottom of the pots to avoid the escaping of larvae. For each plant genotype four independent replicates per soil type were prepared. After three weeks of growing (plant developmental stage V3), circa 60 eggs of WCR were injected close to the stem at 5 cm depth. After 20 days of larval feeding the plants were harvested and the rhizosphere isolated from the maize roots for total community DNA extraction and molecular analysis.

### *Soil types*

Three different agricultural soil types, Haplic Chernozem, Haplic Luvisol and Eutric Vertisol, were collected nearby Göttingen (Germany) in June 2008 (Supplemental information, Table S1). 400 kg of each soil type were taken from four different spots per field, five meters apart from each other, along a transect. The soil was taken to a depth of 25 cm. In order to avoid any alteration of the microbial content, the soils were immediately transported to the laboratory and homogenized by a soil crusher machine (Unifix 300, Möschle, Ortenberg, Germany) and sieved through 10 mm mesh to remove stones and plant residues. The majority of the soil was used for the greenhouse experiment, while little volumes were collected in four falcon tubes (50 mL) per soil type and used as replicates to investigate the soil microbial composition.

### *Maize genotypes and growth conditions*

The maize genotypes used in this study were three Northern European maize breeding lines provided by the seed company KWS (Einbeck, Germany: KWS13, KWS14, KWS15) and the transgenic maize MON88017 (Monsanto, St. Louis, USA). The genetically modified maize was developed to express two proteins: the insecticidal Cry3Bb1 protein from *Bacillus thuringiensis* subsp*. kumamotoensis*, and the CP4 EPSPS protein from *Agrobacterium* sp. conferring glyphosate tolerance.

According to the Canadian Food Inspection Service, the Cry3Bb1 protein is locally expressed in root tissues with concentrations of 100-370 µg $g^{-1}$ dry weight root tissue (EFSA-GMO-CZ-2005-27, 2009).

The maize growing conditions adopted in the greenhouse were as follows: 40 % relative humidity, 24 °C mean temperature and 16 h of additional illumination with sodium lamps (400W, HS2000, Hortilux Schréder, Monster, Netherlands). The pots of plants grown in the same soil were placed in the same tray that was moved twice a week in the greenhouse to randomize the growing conditions. The fertilizer Hakaphos blau (Compo, Münster, Germany; 2.5 %) was applied by watering once a week to plants older than 14 days.

***WCR egg inoculum***

WCR eggs were provided by USDA-ARS (Northern Grain Insect Research Laboratory, Brookings, USA) and stored at 8 °C until their use. In order to stimulate the larval development, the eggs were incubated at 26 °C, 60 % relative humidity in dark conditions for 12 days and checked for visible larvae presence using a dissecting microscope. Afterwards the eggs were washed in a sieve (Ø 250 µm) and suspended in 0.15 % agar solution. A hatch test was prepared to assess the hatch time and the hatch rate as follows: 0.5 mL of egg suspension were applied on a sterile humid filter paper and incubated at the same conditions as described for larval development. The eggs were counted and checked daily for hatching. The mean values estimated for the hatch time and hatch rate were two days and 72 %, respectively.

***Rhizosphere sampling and microbial cells extraction***

Six-week old maize plants were removed from the soil and shaken vigorously. The soil tightly adhering to the roots was considered as rhizosphere and collected using a Stomacher blender (Stomacher 400, Seward, England) as described by Costa *et al.* (2006). The microbial pellets were harvested by centrifugation at 10,000 *g* at 4 °C for 30 min and homogenized with a spatula.

***Total community DNA extraction***

The TC-DNA was extracted from 0.5 g of soil and from 0.5 g of rhizosphere pellet. The cells were lysed mechanically twice with the FastPrep FP120 bead beating system (Q-Biogene, Carlsbad, CA, USA) for 30 s at high speed. Thereafter the DNA

was extracted with the FastDNA SPIN Kit for Soil (Q-Biogene, Carlsbad, CA, USA) according to the instructions of the manufacturer. The extracted DNA was purified with the GENECLEAN SPIN Kit (Q-Biogene, Heidelberg, Germany) according to the manufacturer´s protocol. The TC-DNA was checked on 0.8 % agarose gel and DNA concentrations were estimated visually using the quantitative marker High DNA Mass Ladder (Invitrogen). Genomic DNA samples were differently diluted in MilliQ sterilized water to obtain ca. 20 ng/µL DNA for use as a PCR template.

***PCR amplification of the Internal Transcribed Spacer (ITS) regions and 16S rRNA gene fragments***

The ITS fragments of the fungal communities were directly amplified from TC-DNA extracted from soil and rhizosphere samples obtained from plants grown with or without WCR larvae. The ITS amplification was performed using a nested PCR approach with the primer pair ITS1F/ITS4 and ITS2/ITS1F-GC according to Weinert *et al.* (2009). The same TC-DNA samples extracted from soil and plant rhizosphere were used to amplify the 16S rRNA gene fragments using the primer pair F984GC/R1378 (Heuer *et al*., 1997). Reaction mixture and PCR conditions applied were described by Costa *et al.* (2006).

***Denaturing Gradient Gel Electrophoresis (DGGE)***

The DGGE analyses of the fungal and bacterial communities were carried out in the PhorU2 machine (Ingeny, Goes, The Netherlands). ITS- and 16S-DGGE gels were prepared as described by Weinert *et al*. (2009). Gels were silver stained and air dried according to Heuer *et al.* (2001). Gel images were digitally captured using an Epson 1680 Pro scanner (Seiko-Epson, Japan) with high resolution setting.

***DGGE data analysis and statistical testing***

DGGE profiles were analyzed with the software package GELCOMPAR II 4.5 (Applied Math, Ghent, Belgium) as described by Gomes *et al.* (2003). Cluster analysis (UPGMA) based on the Pearson correlation indices was performed to evaluate the percentage of similarity shared among samples. Pair-wise statistical analysis (Permutation test) was applied on the values of the similarity matrix according to Kropf *et al.* (2004). The differences between groups (*D* value) and significant values (*P* value < 0.05) were always reported.

***Identification of specific 16S-DGGE bands***

In order to identify the main population responding to WCR feeding, bands 1 (Fig. 4), occurring in the 16S-DGGE fingerprints of the rhizosphere samples of most of the maize genotypes grown in the three soil types in presence of WCR larvae, were excised from the acrylamide gels and transferred in 1.5 mL tubes. The replicates of band 1 were excised and combined per plant genotype and soil type. Gel slices were crushed with the top of a sterile tip and the contained DNA was suspended into sterile TE buffer, pH 8, by overnight incubation at 4 °C. After centrifugation at 11,000 x *g* for 60 s, the supernatant containing the band DNA was transferred to a new tube and 1 µL of it was used as template for a new PCR reaction. The PCR was performed using the same conditions described for the bacterial community amplification, except for the use of a forward primer without GC-clamp (F984). PCR products were ligated in the pGEM-T vector system (Promega) and transformed into *Escherichia coli* (JM109 Competent Cells, Promega) according to the manufacturer´s instructions. The clones were re-amplified with the primer pair T7/SP6 to select the transformants carrying the insert with the right size. The T7/SP6 amplicons of the positive clones were re-amplified with the primers F984-GC/R1378 to identify on DGGE gel the clones carrying the differentiating band. Per each plant genotype and soil type combination three to four clones per DGGE band were sequenced. 16S-rRNA gene sequences were analyzed using BLAST-n program at the NCBI site.

*Nucleotide sequence accession numbers*: nucleotide sequences determined in this study were deposited in the GenBank database under accession numbers JN836602-JN836633.

## Results

### *Microbial communities in three different soil types*

In order to verify the hypothesis that Haplic Chernozem, Haplic Luvisol and Eutric Vertisol supported different microbial communities, fungal and bacterial populations in these soils were investigated and compared by means of ITS- and 16S-DGGE fingerprints, respectively. Both ITS- and 16S-DGGE revealed complex patterns with ca. 40 bands for each soil type and showed differences in the relative abundance of several microbial populations among soils (data not shown). UPGMA dendrograms of fungal and bacterial communities showed that the different soil types clustered apart from each other (Fig. 1a and b). Permutation testing showed high statistically supported differences ($P$ < 0.04) of the microbial communities among the three different soils. The high dissimilarity ($D$ >16) of both fungal and bacterial populations inhabiting the three soils indicated a soil type specific microbial community structure (Supplemental information, Table S2).

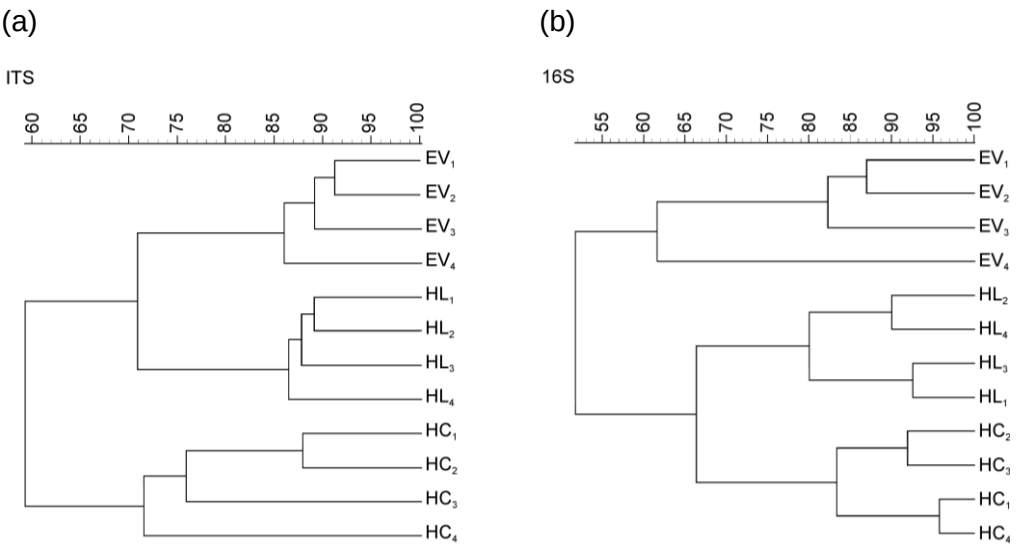


Fig.1. Fungal (a) and bacterial (b) UPGMA dendrograms generated by cluster analysis of DGGE fingerprints of three different soil types and showing separate clusters for each soil type. HC: Haplic Chernozem; HL: Haplic Luvisol; EV: Eutric Vertisol. The independent replicates are labeled from 1 to 4. The dendrograms were constructed using the Pearson correlation coefficient. The scale shows similarity values.

***Rhizosphere effect of four maize genotypes in three soil types***

In order to elucidate the influence of the four maize genotypes used in our experiment (KWS13, KWS14, KWS15 and MON88017) on the soil microbial structure, the fungal and bacterial DGGE profiles of each soil type were compared with the microbial fingerprints of the rhizosphere samples of all four maize genotypes grown in the corresponding soil. ITS-DGGE patterns of the fungal communities in the bulk soil Haplic Chernozem and in the rhizosphere of KWS13, KWS14, KWS15 and MON88017 grown in the same soil type are exemplarily shown in Fig. 2a. ITS-DGGE fingerprints showed a similar number of bands between bulk soil and rhizosphere samples of all four maize genotypes in all soil types. However, differences of the fungal communities in soil and rhizosphere samples, measured as absence/presence or band intensity, were always observed (see arrow in Fig. 2a). The cluster analysis of all DGGE gels showed that the fungal communities of bulk soil samples clustered always separately from the rhizosphere samples (e.g. Fig. 2b).

The fungal composition of soil and rhizosphere patterns of each maize line in all three soil types were statistically different ($P$ = 0.03), with $D$ values ranging between 3 and 17.2 (Supplemental information, Tab. S3).

The same set of samples was analyzed by 16S-DGGE fingerprinting to investigate the rhizosphere effect of four different maize genotypes grown in three soil types on the bacterial populations. Similar to the fungal communities the bacterial fingerprints showed a similar number of bands in bulk soil and rhizosphere samples of all four maize lines in all soil types. Differences measured as absence/presence or band intensity between soil and rhizosphere samples were always observed (data not shown). The comparison between soil and rhizosphere samples revealed for all the maize lines significant rhizosphere effects ($P$ = 0.03) in all three soil types investigated, with $D$ values ranging between 10 and 58. In comparison to the fungal populations a higher dissimilarity in the bacterial community composition between soil and rhizosphere samples was observed (Supplemental information, Tab. S3).

(a) (b)

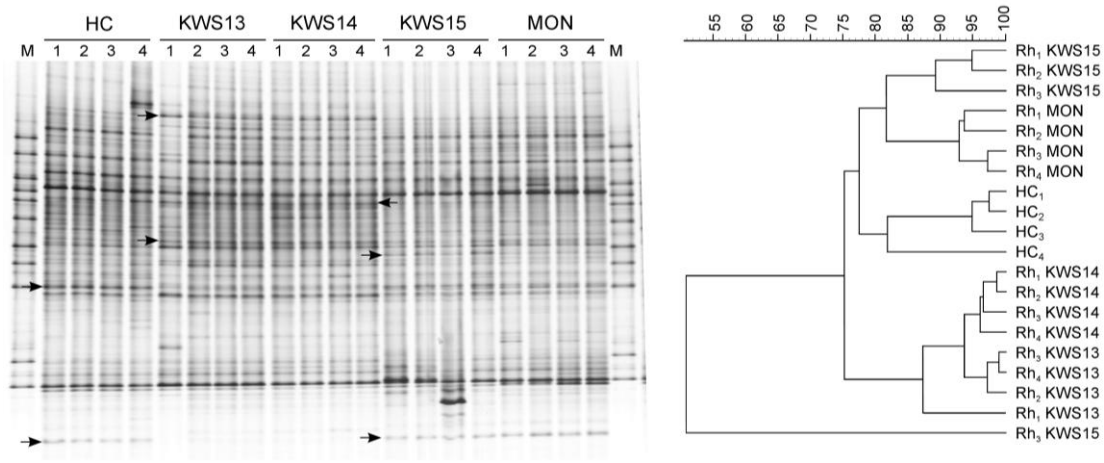


Fig.2. (a) ITS-DGGE fingerprinting of the fungal communities in the soil Haplic Chernozem (HC) and in the rhizosphere of four different maize genotypes (KWS13, KWS14, KWS15 and MON88017) grown in the same soil type. The profile represent ITS regions, PCR- amplified from TC-DNA extracted from soil- and rhizosphere samples. The independent replicates are indicated with numbers from 1 to 4. M: fungal marker prepared with the ITS sequences of *Verticillium nigrescens*, *Basidiomycete* sp., *Trichoderma* sp., *Doratomyces* sp., *Verticillium dahliae*, *Penicillium canescens*, *Fusarium graminearum*, *Nectria haematococca*, *Fusarium solani*, *Fusarium redolens*, and *Sclerotinia sclerotiorum*. Arrows indicate maize genotype effects. (b) Corresponding UPGMA dendrogram constructed using the Pearson correlation coefficient. The scale shows similarity values. Rh: rhizosphere samples.

***Maize genotype effect on the microbial communities in the rhizosphere***

In order to test the hypothesis that different maize genotypes affect the rhizosphere microbial communities, a pair-wise comparison of DGGE profiles of the fungal and bacterial populations in the rhizosphere of KWS13, KWS14, KWS15 and MON88017 grown in the same soil type was performed and differences were tested for significance.

The pair-wise comparison of the rhizosphere fungal fingerprints obtained from different maize lines showed genotype-dependent differences in the relative abundance of several fungal populations in all three soils (e.g. Fig. 2a). Although a

clear clustering between the plant genotypes was not always observed, likely due to the variability among replicates, UPGMA-dendrograms of the fungal communities revealed, independently from the soil type, always two groups including KWS13/KWS14 from one side and KWS15/MON88017 from the other side (e.g. Fig. 2b). The statistical analysis showed significant differences ($P$ = 0.03) of the rhizosphere fungal populations between all maize genotypes in all three soil types, except between KWS13 and KWS14 in Haplic Chernozem and between KWS15 and MON88017 in Haplic Luvisol (Table 1). Small differences of the rhizosphere fungal communities between KWS13 and KWS14 ($2 < D$ values $< 6.2$) and between KWS15 and MON88017 ($1.2 < D$ values $< 9.3$) were observed in all three soil types.

Pair-wise comparison of rhizosphere bacterial fingerprints obtained from different maize genotypes revealed different bacterial community structures among maize lines in all three soil types (data not shown). UPGMA-cluster analysis showed that the bacterial communities in the rhizosphere of each maize line clustered apart from each other in all soil types, except for KWS13 and KWS14 in Haplic Chernozem and for KWS14 and KWS15 in Haplic Luvisol, which formed a mixed cluster due to high variability within KWS13 and KWS14 replicates (data not shown). Bacterial community patterns obtained from the rhizosphere of MON88017 clustered separately from those of the other genotypes in both Haplic Chernozem and Luvisol. In Eutric Vertisol, KWS15 and MON88017 formed one cluster sharing low similarity (36 %). Statistical testing revealed significant differences ($P$ = 0.03) of the bacterial community structure in the rhizosphere between all maize genotypes, except for KWS14/KWS15 in Haplic Luvisol (Table 1). Thus, the bacterial communities in the maize rhizosphere, as well as the fungal communities, were influenced by the maize genotype in a soil type specific manner.

Table 1. Percentage dissimilarity (*D*) and significant values (*P*) of rhizosphere fungal or bacterial fingerprints between different maize genotypes (KWS13, KWS14, KWS15 and MON88017) grown in the soil types Haplic Chernozem, Haplic Luvisol and Eutric Vertisol.

| | **Haplic Chernozem** | | **Haplic Luvisol** | | **Eutric Vertisol** | |
|---|---|---|---|---|---|---|
| | ***D*** | ***P*** | ***D*** | ***P*** | ***D*** | ***P*** |
| ***Fungi*** | | | | | | |
| KWS13/KWS14 | 2.2 | 0.06 | 6.2 | **0.03** | 2 | **0.03** |
| KWS13/KWS15 | 14 | **0.03** | 14.1 | **0.03** | 8.7 | **0.03** |
| KWS13/MON | 21.2 | **0.03** | 16.6 | **0.03** | 8.8 | **0.03** |
| KWS14/KWS15 | 14.8 | **0.03** | 18.7 | **0.03** | 11.1 | **0.03** |
| KWS14/MON88017 | 21.7 | **0.03** | 17.8 | **0.02** | 8.6 | **0.03** |
| KWS15/MON88017 | 9.3 | **0.03** | 1.2 | 0.3 | 5.5 | **0.03** |
| ***Bacteria*** | | | | | | |
| KWS13/KWS14 | 9.3 | **0.03** | 15.4 | **0,03** | 30 | **0.03** |
| KWS13/KWS15 | 18.1 | **0.03** | 27.3 | **0.03** | 50 | **0.03** |
| KWS13/MON88017 | 15 | **0.03** | 26.6 | **0.03** | 65.2 | **0.03** |
| KWS14/KWS15 | 17.5 | **0.03** | 9.5 | 0.06 | 50.6 | **0.03** |
| KWS14/MON88017 | 14.2 | **0.03** | 24.3 | **0.03** | 56.8 | **0.03** |
| KWS15/MON88017 | 27.2 | **0.03** | 16.9 | **0.03** | 12.8 | **0.03** |

Values of $P < 0.05$ indicate significant differences between rhizosphere samples of different maize genotypes grown in the same soil type. Values obtained by Permutation testing using 10.000 simulations. Bold values show significant differences.

### *WCR larval feeding effect on the fungal communities in the rhizosphere of maize*

The effects of WCR larval feeding on the rhizosphere fungal communities was investigated for all maize genotypes grown in three soil types by comparing the ITS-DGGE fingerprints of the treatments with or without larvae.

Only in the fungal fingerprinting of KWS14 grown in Haplic Chernozem a pronounced shift upon larval feeding was observed (see arrow in Fig. 3a). In the same soil type minor variations of the fungal communities due to larval presence

and activity, were observed in the rhizosphere of KWS13, while no shifts were visible in the rhizosphere of KWS15 and MON88017 between samples with (L+) and without (L-) larvae (data not shown). UPGMA dendrograms showed that the fungal communities in the rhizosphere of KWS14 (L+) and (L-) grouped separately (Fig. 3b). Although the patterns of KWS13 (L+) and (L-) shared a high similarity (82.4 %), separate clusters for treatments with and without larvae were still found (data not shown). In contrast, the fungal communities in the rhizosphere of KWS15 (L+) and (L-) grouped together as well as the rhizosphere fungal populations of MON88017 (L+) and (L-). Permutation testing revealed significant differences of the fungal communities between treatments with or without larvae only in the rhizosphere of KWS13 and KWS14 ($P$ = 0.03), indicating a significant effect of the larval feeding on the relative abundance of fungi inhabiting the rhizosphere of these maize lines. Only in the rhizosphere of KWS14 these shifts were highly pronounced ($D$ value = 22.8). No significant effect of the larval feeding was observed on the fungal communities in the rhizosphere of KWS15 and MON88017 (Table 2).

In Haplic Luvisoil and Eutric Vertisol, ITS-DGGE profiles displayed little variations in the relative abundance of the fungal populations in the rhizosphere of KWS13 and KWS14 in response to larval feeding. The fungal communities in the rhizosphere of MON88017 showed shifts in response to larval feeding only in Eutric Vertisol. No shifts in presence of larvae were observed in the fingerprinting of the fungal populations in the rhizosphere of KWS15 in both soils. UPGMA dendrograms showed clearly separated clusters of (L+) and (L-) samples in the rhizosphere of KWS13 and KWS14 in both soils and in the rhizosphere of MON88017 in Eutric Vertisol (data here not shown). Permutation testing revealed between (L+) and (L-) samples, highly supported differences ($P$ = 0.03) of the fungal communities inhabiting the rhizosphere of KWS13, KWS14 in Haplic Luvisoil and Eutric Vertisol.

Unexpectedly, a significant effect of larval feeding on the fungal population was observed in the rhizosphere of MON88017 grown in Eutric Vertisol (Tab. 2).

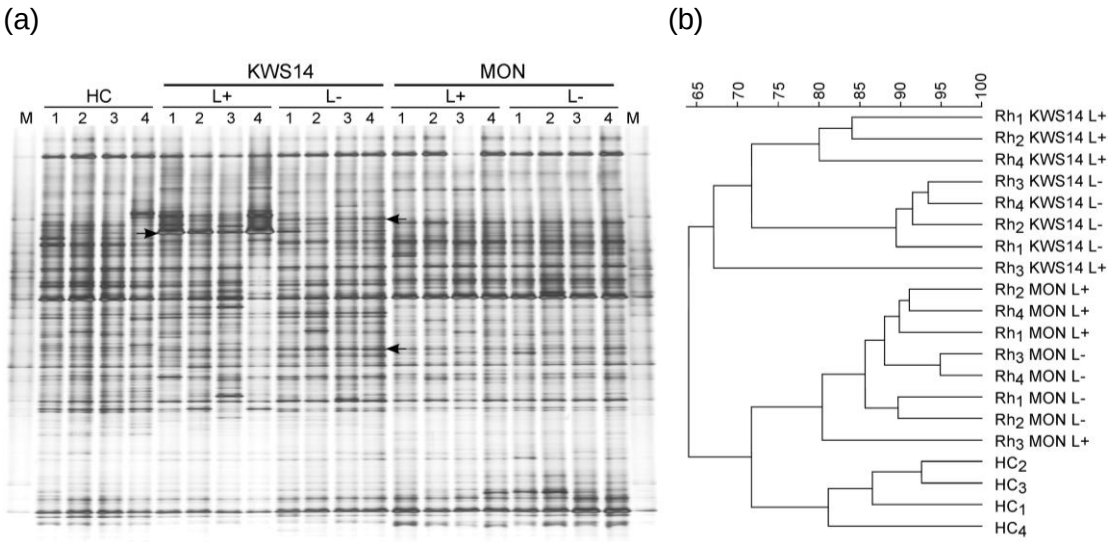


Fig. 3. (a) ITS-DGGE profile of the fungal communities in the soil type Haplic Chernozem (HC) and in the rhizosphere samples of the maize genotypes KWS14 and MON88017 grown in the same soil type in presence (L+) or absence (L-) of WCR larvae. The profile represents ITS regions, PCR amplified from TC-DNA extracted from soil- and rhizosphere samples. Independent replicates are indicated with numbers from 1 to 4. M: Fungal marker. Arrows indicate WCR larval effects on the rhizosphere fungal communities. (b) Corresponding UPGMA dendrogram generated by cluster analysis of Pearson's similarity indices. The scale shows similarity values.

***WCR larval feeding effect on the bacterial communities in the rhizosphere of maize***

The effects of WCR larval feeding on the bacterial populations in the maize rhizosphere were tested by DGGE analysis of 16S rRNA gene fragments amplified from rhizosphere TC-DNA of four different maize genotypes (KWS13, KWS14, KWS15, MON88017) grown in three soil types (Haplic Chernozem, Haplic Luvisol and Eutric Vertisol) in presence and absence of larval feeding.

In Haplic Chernozem pronounced shifts due to WCR larval feeding on the bacterial populations colonizing the maize rhizosphere were observed for all maize genotypes investigated, except for MON88017 (Fig. 4). The analysis UPGMA showed that the bacterial communities in the rhizosphere of all the KWS lines formed separate clusters (L+) and (L-), although one or two replicates per maize lines clustered as an

out-group due to the variability within replicates (data not shown). A mixed cluster was observed for the bacterial rhizosphere populations of MON88017 grown with and without larvae. Permutation testing revealed significant differences of the rhizosphere bacterial communities between (L+) and (L-) samples of KWS13, KWS14, and KWS15 ($P$ = 0.03) in Haplic Chernozem, indicating a significant effect of the larval feeding on the bacteria inhabiting the rhizosphere of those maize lines. No effects of the larval feeding were observed on the bacterial communities in the rhizosphere of the transgenic maize MON88017 in Haplic Chernozem.

In Haplic Luvisol and Eutric Vertisol pronounced shifts in the bacterial community patterns were observed upon root larval feeding as well (gel not shown, but see Tab. 2). UPGMA dendrograms displayed separate clusters between (L+) and (L-) samples of KWS13, KWS14, KWS15 and MON88017, although one or two replicates clustered as an out-group due to the variability within replicates. Permutation testing revealed in response to larval feeding significant differences in the bacterial populations inhabiting the rhizosphere of all maize lines investigated in Haplic Luvisol and Eutric Vertisol (Table 2).

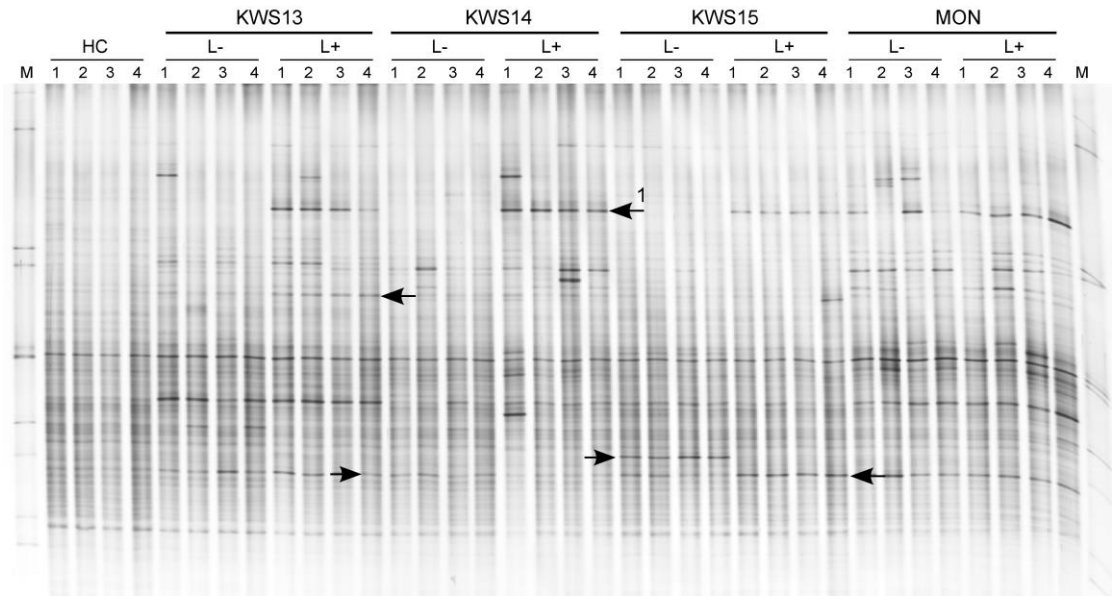


Fig. 4. 16S-DGGE profile showing the bacterial community structure in the bulk soil Haplic Chernozem (HC) and in the rhizosphere of the maize genotypes KWS13, KWS14, KWS15 and MON88017 (MON) grown in the same soil type, in presence (L+) or absence (L-) of WCR larval feeding. The profile represents 16S-rRNA gene fragments amplified from TC-DNA extracted from soil- and rhizosphere samples. Independent replicates are indicated with numbers from 1 to 4. M: Bacterial marker (Heuer *et al.*, 2001). Arrows indicate WCR larval effects on the rhizosphere bacterial communities.

Table 2. Percentage dissimilarity (*D*) and significance values (*P*) of rhizosphere fungal or bacterial fingerprints between maize genotypes in presence and in absence of WCR larval feeding (Larvae +/-), in the soil types Haplic Chernozem, Haplic Luvisol, and Eutric Vertisol.

| | **Haplic Chernozem** | | **Haplic Luvisol** | | **Eutric Vertisol** | |
|---|---|---|---|---|---|---|
| | **Larvae+/-** | | **Larvae+/-** | | **Larvae+/-** | |
| | ***D*** | ***P*** | ***D*** | ***P*** | ***D*** | ***P*** |
| ***Fungi*** | | | | | | |
| KWS 13 | 5.8 | **0.03** | 11.1 | **0.03** | 7 | **0.03** |
| KWS 14 | 22.8 | **0.03** | 8.9 | **0.03** | 3.3 | **0.03** |
| KWS 15 | 0.9 | 0.3 | 3.7 | 0.06 | 3.8 | 0.17 |
| MON88017 | 2 | 0.1 | 2 | 0.2 | 5.9 | **0.03** |
| ***Bacteria*** | | | | | | |
| KWS 13 | 15.8 | **0.03** | 15.6 | **0.03** | 15.7 | **0.03** |
| KWS 14 | 31.3 | **0.03** | 25.5 | **0.03** | 48.4 | **0.03** |
| KWS 15 | 23.6 | **0.03** | 11.9 | **0.03** | 25.4 | **0.03** |
| MON88017 | 6.4 | 0.06 | 4.1 | **0.03** | 19.2 | **0.03** |

*P* values <0.05 indicate significant differences between rhizosphere samples of the same maize genotype grown with and without larval feeding in the same soil type. Values obtained by Permutation testing using 10.000 simulations. Values in bold show significant values.

***Identification of bacteria responding to WCR larval feeding***

16S-DGGE of the bacterial communities inhabiting the rhizosphere of the four maize genotypes investigated, grown with and without larvae in Haplic Chernozem, showed a dominant band (Band 1, Fig. 4) with identical electrophoretic mobility in the fingerprints of all rhizosphere samples of KWS cultivars grown with larvae and of MON88017 grown with and without larvae. Cloning, sequencing and blast analysis of this band revealed for most of the clones a high sequence similarity to *Acinetobacter calcoaceticus* (99-100 % identity, sequence accession no. JN836603-JN836608 and JN836610-JN836621). Only two clones showed 99 % similarity to *Sphingomonas* sp. (accession no. JN836602) and 99 % similarity to *Massilia* sp. (accession no. JN836609). A band with the same electrophoretic mobility of band 1 in Haplic Chernozem was observed in the bacterial fingerprints of KWS13 and KWS14 in Haplic Luvisol (Supplemental information, Fig. S1), and of KWS13, KWS15 and MON88017 in Eutric Vertisol (data not shown). The sequencing of this band from the 16S-DGGE fingerprints of the bacteria in the rhizosphere of KWS13 from both soils Haplic Luvisol and Eutric Vertisol revealed again the highest similarity to *Acinetobacter calcoaeticus* (99-100 % identity, sequence accession no. JN836622-JN836629).

Bacterial community fingerprints of rhizosphere samples from KWS13, KWS14, KWS15 and MON88017 grown in Haplic Luvisol revealed a faint band with a slightly lower electrophoretic mobility of Band 1 (Band 2, Supplemental information, Fig. S1). Band 2 occurred in all rhizosphere replicates of KWS15 and in some replicates of KWS13 and KWS14 in presence of larvae. Band 2 was identified by cloning, sequencing and blast analysis as *Enterobacter ludwigii* (100 % identity, sequence accession no. JN836630-JN836633).

## Discussion

This is the first study on the effects of the WCR maize root feeding on the bacterial and fungal communities inhabiting the maize rhizosphere. To understand the influence of the soil type and of the plant genotype on microbial population dynamics upon larval attack, four maize genotypes KWS13, KWS14, KWS15 and MON88017, were grown in the soil types Haplic Chernozem, Haplic Luvisol and Eutric Vertisol.

Kurtz (2010) investigated the effect of the same maize genotypes KWS13, KWS14, KWS15 and MON88017 grown in the soil types mentioned above on the plant growth and on the larval development of WCR 3$^{rd}$ instars. Plant dry weight was significantly lower for all cultivars in Haplic Chernozem compared to plants grown in Haplic Luvisol and Eutric Vertisol. The larval development was not influenced by the soil type but by the maize genotype. As expected, larval survival was drastically reduced in all three soils for MON88017. In Haplic Luvisol and Eutric Vertisol the cultivars KWS13 and KWS15 supported larval development better than KWS14 and MON88017, while in Haplic Chernozem no significant plant genotype-dependent differences on larval development were observed.

The microbial community analysis of Haplic Chernozem, Haplic Luvisol and Eutric Vertisol revealed that the three different soils harbored distinct bacterial and fungal communities. A significant rhizosphere effect of all maize genotypes was observed for both bacterial and fungal communities in each soil type. The influence of the maize rhizosphere was more pronounced on bacterial communities ($10 < D$ values $< 58.4$) than on fungal communities ($3 < D$ values $< 17.2$). This result indicated either that the fungi were less affected by maize root exudates than the bacteria or that the resolution power of the ITS region is lower than the 16S rRNA gene fragment. The effect of the soil type and of the rhizosphere on the microbial community structure was already reported in several studies (Kandeler *et al.*, 2002; Gomes *et al*., 2001, 2003; Baumgarte *et al.*, 2005). However, maize genotype effects on the composition of bacterial and fungal communities in the rhizosphere shown in this study were not observed by others (Baumgarte *et al.*, 2005; Miethling *et al.*, 2010).

ITS-DGGE fingerprinting and statistical analysis revealed that rhizosphere fungal communities were significantly affected by larval feeding in all soil types and according to the plant genotype. The strongest shift upon larval feeding was observed in the rhizosphere of KWS14 grown in Haplic Chernozem. Bacterial

communities in the maize rhizosphere were more responsive than the fungal communities to larval feeding: Pronounced shifts of bacteria were observed in the rhizosphere of all tested maize genotypes in all soil types with just one exception for MON88017 in Haplic Chernozem (Table 2).

Interestingly, the 16S-DGGE profiles of the bacterial communities displayed Band 1 (Fig. 4) in the rhizosphere samples of all WCR-treated plants grown in Haplic Chernozem. A band with the same electrophoretic mobility was observed in the other two soils upon WCR attack, but not for all plant genotypes. In the bacterial fingerprint of the rhizosphere samples of MON88017 in Haplic Chernozem Band 1 occurred even in absence of larvae. Sequencing of Band 1 from the rhizosphere bacterial fingerprints of all plant genotypes here investigated grown in presence of larvae and of MON88017 also in absence of larvae revealed the bacterium *Acinetobacter calcoaceticus* (100 % 16S rRNA sequence identity). This bacterium was recently described as a phenol degrading microorganism (Zhang *et al*., 2011). Poerschmann *et al.* (2008) showed that roots of MON88017 have a higher total lignin content compared to the iso-line. Lignin is a phenolic compound and the secretion of phenolic compounds such as t-cinnamic acid by barley plant roots was recently introduced as a novel belowground plant defence mechanism (Lanoue *et al.*, 2010). Thus, we assume that the presence of *Acinetobacter calcoaceticus* in the rhizosphere of all maize genotypes in presence of larvae might be due to the exudation of phenolic compounds, triggered by larval feeding. The presence of *Acinetobacter calcoaceticus* in the rhizosphere of MON88017 might be due to the higher lignin content of the root tissues which might support such phenol degrading microorganisms in the rhizosphere of the transgenic plant. Recently it has been reported that WCR larvae are resistant against higher levels of 2,4-dihydroxy-7-methoxy-1,4-bezoxacin-3-one (DIMBOA), a compound specifically enriched in the nutritional superior crown roots (Robert *et al*., 2012) and previously regarded as contributing to the resistance of some maize cultivars against larval feeding (Davis *et al*., 2000). It would be worthwhile to investigate whether *A. calcoaceticus* is also present in higher amount in the crown root part of the maize root system and whether this microorganism contributes to ability of WCR larvae to cope with the different nutritional qualities within the maize root system.

In presence of larvae, a second bacterial population identified as *Enterobacter ludwigii* increased in abundance in the rhizosphere of several maize genotypes in Haplic Luvisol. This *Gammaproteobacterium* was originally isolated from the

rhizosphere of tomato plants and was shown to display *in vitro* and *in planta* strong antagonistic activity towards a range of fungal and oomycete pathogens (Kavroulakis *et al.*, 2010). The potential antagonistic activity of *E. ludwigii* on plant-WCR larval feeding interactions might be a matter of further investigations.

As mentioned before, the larval survival was drastically reduced for MON88017 in all three soils (Kurtz, 2010) but surprisingly, the microbial communities in the rhizosphere of the transgenic maize line MON88017 were also influenced by WCR larval presence. We conclude that either the initial larval feeding and/or the larval body decomposition triggered these changes in the microbial community structure most likely by changes of root exudation patterns. These changes may most likely be mediated by plant defenses to herbivorous insects. For instance, upon WCR larval damage, roots of European maize (*Zea mays* L.) were reported to release the volatile compound sesquiterpene (E)-ß-caryophyllene. This compound is a strong attractant for the natural WCR enemy, *Heterorhabditis megidis*, an entomopathogenic nematode (Rasmann *et al.,* 2005; Köllner *et al.,* 2008). Plants also respond to belowground herbivore attack by the expression of the root herbivore-induced shoot resistance (RISR), which resulting in a systemic response against further herbivore attackers Recently, there has been a first report of an aboveground resistance against the nectrophic fungus *Serosphaeria turcica* (Erb *et al.*, 2009) triggered by WCR root feeding. The finding of our study suggest that RISR might be causing changes of the rhizosphere microbial communities. So far, the influence of the rhizosphere community on plant-belowground herbivore interactions has been investigated only in few studies addressing the effects of soil-borne microorganisms on aboveground herbivores (Wurst*,* 2010; Pineda *et al.,* 2010). Beneficial effects of microbial communities for plants have been shown via promoting plant growth or inducing defenses against herbivore feeding. In most cases the changes in root exudates triggered by microorganisms were regarded as a defense mechanism against soil plant pathogens (Lanoue *et al*., 2010). In this study we found evidence that the feeding activity of WCR larvae influenced the composition of the rhizosphere microbial communities most likely caused by the secretion of phenolic compounds due to wounding. We regard the plant response to WCR feeding as the overriding factor determining the shifts in the microbial community response. Whether the changes in the bacterial and fungal communities in response to WCR feeding influence also the feeding behavior of WCR larvae or

contribute to reduced damage on the roots, acting as a plant induced defense mechanism, remains to be investigated.

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**Supplemental information**

Table S1. Geographic locations of the soil sampling areas, and use, soil texture and physico-chemical parameters

| | **HaplicChernozem (silt)** | **Haplic Luvisol (silt loam)** | **Eutric Vertisol (silt loam)** |
|---|---|---|---|
| **Coordinates** | 51°30`29.44 N<br>9°55`38.26 E | 51°29`52.88 N<br>9°55`38.26 E | 51°28`26.99 N<br>9°59`55.13 E |
| **Land use** | winter wheat | grass land | winter wheat |
| **Elevation in m** | 265 | 153 | 165 |
| **Sand content (%)** | 3. 7 | 20.7 | 11.3 |
| **Silt content (%)** | 83.8 | 68.1 | 67.8 |
| **Clay content (%)** | 12.6 | 11.2 | 20.9 |
| **pH ($H_2O$)** | 7.6 | 7.6 | 7.4 |
| **C/N ratio** | 16.3 | 15.8 | 13.2 |

The soil texture and the physico-chemical parameters were determined by the Institute of Soil Science (Georg-August-University, Göttingen, Germany).

Table S2. Percentage dissimilarity (*D*) and significant values (*P*) of fungal and bacterial communities fingerprints between the soil types Haplic Chernozem, Haplic Luvisol, and Eutric Vertisol.

| | **Fungi** | | **Bacteria** | |
|---|---|---|---|---|
| **Soil type** | ***D*** | ***P*** | ***D*** | ***P*** |
| HC/HL | 24.4 | **0.02** | 18.9 | **0.02** |
| HC/EV | 20.4 | **0.01** | 32.7 | **0.02** |
| HL/EV | 16.7 | **0.01** | 21.9 | **0.04** |

Haplic Chernozem (HC), Haplic Luvisol (HL), and Eutric Vertisol (EV). *P* values were obtained by Permutation testing with 10.000 numbers of simulations. Values of *P* <0.05 indicate significant differences between soils. Values in bold show significant differences in the microbial DGGE fingerprints between soils.

Table S3. Percentage dissimilarity (*D*) and significant values (*P*) of fungal and bacterial fingerprints in the soil and in the rhizosphere of different maize genotypes grown in Haplic Chernozem, Haplic Luvisol and Eutric Vertisol.

| | **Haplic Chernozem** | | **Haplic Luvisol** | | **Eutric Vertisol** | |
|---|---|---|---|---|---|---|
| | *D* | *P* | *D* | *P* | *D* | *P* |
| ***Fungi:Soil/Rh*** | | | | | | |
| KWS 13 | 14.2 | **0.03** | 17.2 | **0.03** | 11.6 | **0.03** |
| KWS 14 | 16.1 | **0.03** | 17.1 | **0.03** | 16 | **0.03** |
| KWS 15 | 9.8 | **0.03** | 3 | **0.03** | 8.6 | **0.03** |
| MON88017 | 14 | **0.03** | 5.5 | **0.03** | 12.5 | **0.03** |
| ***Bacteria: Soil/Rh*** | | | | | | |
| KWS 13 | 16.6 | **0.03** | 44.7 | **0.03** | 37 | **0.03** |
| KWS 14 | 10 | **0.03** | 38.3 | **0.03** | 41.5 | **0.03** |
| KWS 15 | 20.4 | **0.03** | 28 | **0.03** | 43.8 | **0.03** |
| MON88017 | 30 | **0.03** | 30 | **0.03** | 58.4 | **0.03** |

*P* values were obtained by Permutation testing with 10.000 numbers of simulations. *P* values <0.5 indicates a significantly rhizosphere effect.

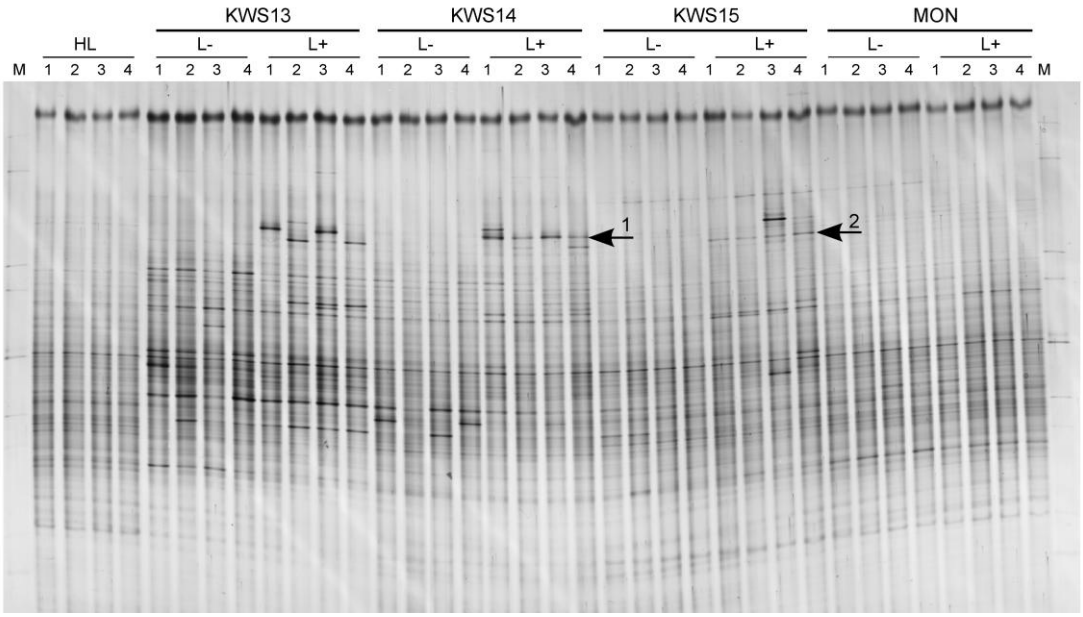


Fig. S1. 16S-DGGE profile showing the bacterial community structure in the bulk soil Haplic Luvisol (HL) and in the rhizosphere of the KWS13, KWS14, KWS15 and MON88017 (MON) grown in the same soil type, in presence (L+) or in absence (L-) of WCR larval feeding. M: Bacterial marker (Heuer *et al*., 2001). Independent replicates are indicated with numbers from 1 to 4. Arrows pointing to band 1 and 2 have been identified as *Acinetobacter calcoaceticus* and *Enterobacter ludwigii*, respectively.

# Chapter IV

## Multitrophic interactions among Western Corn Rootworm, *Glomus intraradices* and microbial communities in the root zone of maize

**Multitrophic interactions among Western Corn Rootworm, *Glomus intraradices* and microbial communities in the root zone of maize**

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**Keywords:** rhizosphere, endorhiza, *Diabrotica virgifera virgifera*, *Glomus intraradices*, arbuscular mycorrhizal fungi, microbial community analysis.

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**Abstract**

In view of new control strategies against the maize pest Western Corn Rootworm (WCR), the complex interactions among WCR, *Glomus intraradices* (*G.i*.) and microbial communities in the rhizosphere and endorhiza of maize have been investigated.

In a greenhouse experiment maize plants were pre-grown for six weeks in pots containing the soil Hapic Chernozem inoculated or not with *G.i*. Thereafter 200 non-diapausing WCR eggs were added or not to each pot. 20 days later, larval number/survival, developmental stage and root feeding of WCR were measured. qRT-PCR was used to quantify *G.i.* in the roots. Root colonization levels by arbuscular mycorrhizal fungi (AMF)s were estimated by microscopic analysis. Dominant AMF species in soil and endorhiza were analysed by cloning of 18S rRNA gene fragments of AMFs amplified from total community (TC) DNA, restriction fragment length polymorphism and sequencing. Bacterial and fungal communities in the rhizosphere and endorhiza were investigated by denaturing gradient gel electrophoresis of 16S rRNA gene and ITS fragments, PCR amplified from TC-DNA extracted from rhizosphere and root material.

*G.i*. reduced significantly the WCR larval development and strongly affected the endophytic populations of AMFs, and to a lesser extent bacterial communities. Thus, we assumed that *G.i.* could contribute to the control of WCR larvae either directly or indirectly through shifts of the endophytic microbial communities via plant-mediated mechanisms. Furthermore, we first reported the effect of WCR larval feeding on the maize endophytic bacterial populations.

## Introduction

The Western Corn Rootworm (WCR), *Diabrotica virgifera virgifera* LeConte, is an invasive maize pest in North America and in Europe. WCR larvae feed on maize root tissues causing bent stalks (goose necking) and lodging. Economic losses are mainly due to difficulties in mechanical harvesting of injured maize plants. Successful and long-term resistance management strategies of the pest need to be developed. An improved knowledge of the ecology of this soil-dwelling insect and its multitrophic interactions in the rhizosphere and endorhiza are important prerequisites to achieve this goal.

The rhizosphere and endorhiza are dynamic environments in which plant, fungi, bacteria, viruses, nematodes and herbivore insects interact with each other influencing the agro-ecosystem functionality, and thus the sustainability of the crop production (Weller & Thomashow, 1994; Berg and Smalla, 2009). Beneficial rhizospheric microorganisms promote plant growth and health by nutrient solubilization, nitrogen fixation and plant hormone production (Hayat *et al*., 2010). Microbial endophytes influence plant fitness as well affecting plant-microbe-arthropod interactions (Finkes *et al*., 2006; Rudgers *et al*., 2007). Within the endophytes, the arbuscular mycorrhizal fungi (AMF)s are well known to improve plant survival in harsh environments by enhancing several plant functions (Newsham *et al.*, 1995; Smith & Reed 2008) including drought resistance (Davies *et al.*, 2002), tolerance to heavy metal contaminations (Gildon & Tinker, 1983), protection against pathogens through microbial antagonism and increased plant defensive capacity (Newsham *et al.*, 1995). Furthermore, AMFs affect the interaction between plants and herbivorous insects (review by Gehring & Bennett, 2009). Several reports showed that certain AMF species influence the behavior, the development and the performance of aboveground insects (Gange *et al*., 1994; Wardle 2002; Davet 2004; Bezemer & van Dam 2005; Hartley & Gange 2009; Koricheva *et al.,* 2009). These effects are assumed to occur either due to changes of the nutritional status of the plant or by triggering plant defense responses (Goverde *et al*., 2000; Nishida *et al*., 2010). To date the effects of AMFs on belowground herbivorous insects have been only rarely examined (Gange *et al.*, 1994).

It has been shown that AMFs may influence directly or indirectly the activity and the community structure of the rhizospheric- and root-associated microorganisms either

through the release of hyphal compounds or through changes in the plant root exudation patterns (Wamberg *et al*., 2003; Marschner & Baumann, 2003; reviewed by Jones *et al*., 2004; Offre *et al.,* 2007).

The microbial community assembly can be affected also by belowground insect attackers (Denton *et al*. 1998; Grayston *et al*. 2001; Dawson *et al.* 2004; Currie *et al*., 2006; Dematheis *et al.*, submitted), most likely via plant-mediated mechanisms. Upon insect attack, changes in the plant transcriptome, in the production of volatiles or root exudates have been often detected (Köllner *et al.,* 2008; Dicke *et al*., 2009). Larval feeding effects on the bacterial and fungal community composition in the maize rhizosphere were recently investigated and soil-type and cultivar dependent shifts in the microbial populations were observed (Dematheis *et al*. submitted). However, effects of WCR larval feeding on the indigenous microbial communities inhabiting the maize endorhiza remained unexplored. In addition, no studies on the effect of *G.i*. on WCR larval fitness and on both rhizospheric and endophytic microbes of maize have been reported yet.

The present study aimed to investigate the multitrophic interaction among WCR, *G.i*. and the microbial communities in the maize root zone (rhizosphere and endorhiza). We specifically addressed the following questions: (1) Does *G.i.* mycorrhization of maize roots affect the WCR larval fitness measured as larval number/survival, developmental stage and root feeding? (2) Does *G.i.* inoculation affect the composition of microbial populations in the rhizosphere and endorhiza of maize? (3) Does the feeding of WCR larvae alter the microbial communities in the endorhiza and rhizosphere of maize, and is this effect influenced by *G.i.*-soil inoculation?

In the present study AMF, total fungal and bacterial communities were investigated. AMF communities naturally occurring in the soil and colonizing the maize endorhiza were studied by PCR-RFLP analysis and sequencing of AMF-specific 18S rRNA gene fragments amplified from total community (TC) DNA. The total fungal and bacterial communities in both rhizosphere and endorhiza of maize were analyzed by means of denaturing gradient gel electrophoresis (DGGE) of ITS and 16S rRNA gene fragments amplified from TC-DNA.

## Materials and methods

### *Experimental setup*

A greenhouse experiment was performed under quarantine conditions. Maize plants, cultivar KWS13, were pre-grown in pots containing the soil Haplic Chernozem inoculated or not with *Glomus intraradices* (*G.i.*) (treatments G and C). After six weeks of plant growth (plant growth stage V7) approx. 200 non-diapausing WCR eggs were applied close to the plant stems or not resulting in the following treatments: C (control), W (WCR), G (*G.i.*), GW (*G.i.* + WCR). Four independent replicates (one replicate = one plant) per treatment were established. An extra set of four plant replicates per treatment C and G was harvested after six weeks of plant growth in order to verify, by quantitative real-time PCR, *G.i.*-root colonization before WCR egg inoculation.

Nine weeks after sowing (plant growth stage VT) the larvae were collected from the treatments W and GW to evaluate the total number of larvae per plant and the development of the larval instars (L1, L2 and L3). In parallel, the plants were harvested and the fresh weight of the roots was recorded. Per plant, circa 1 m of root pieces randomly taken, were used for the microscopic analysis of the root colonization levels by AMFs. The remaining roots were surface sterilized after the rhizosphere isolation. Total community (TC) DNA was extracted from soil, rhizosphere and surface sterilized roots in order to determine (a) the 18S/ITS rRNA gene copy numbers of *Glomus intraradices* in the roots by quantitative real-time PCR; (b) the AMF community structure in soil and roots by PCR-RFLP of 18S rRNA gene fragments and (c) the bacterial and fungal community structures in the rhizosphere and endorhiza by DGGE analysis of PCR-amplified ITS and 16S rRNA gene fragments.

### *Soil type and sampling method*

The soil used in this study is Haplic Chernozem, collected in 2008 nearby Göttingen (geographic coordinates: 51°30´29.44 N and 9°55´38.26 E). 400 kg were taken from four different spots, five meters apart from each other, along a transect to a depth of 25 cm. In order to avoid any alteration of the microbial content, the soil samples were immediately transported to the laboratory and homogenized by a soil crusher machine (Unifix 300, Möschle, Ortenberg, Germany) and sieved through a 10 mm

mesh to remove stones and plant residues. Fresh soil was used for the experiment described here.

***Glomus intraradices inoculum and application***

The arbuscular mycorrhizal *Glomus intraradices* (Glomeromycota Phylum) was provided by Dr. Henning von Alten (Isolate n° 501, Institute of Plant Disease and Plant Protection, University of Hannover, Germany) as expanded clay material contains a high level of *G.i.* spores. The inoculum was mixed as 5 % of the total volume of soil estimated for the whole experiment (Dehne & Backhaus, 1986).

***WCR egg inoculum and application***

Non-diapausing WCR eggs were provided by USDA-ARS (Northern Grain Insect Research Laboratory, Brookings, USA) and stored at 8 °C until their use. In order to stimulate the larval development, the eggs were incubated at 26 °C, 60 % relative humidity in dark conditions for 12 days and checked for visible larvae presence using a dissecting microscope. Afterwards the eggs were washed in a sieve (Ø 250 µm) and the collected eggs were suspended in 0.15 % agar solution. 0.5 mL of egg suspension were applied on a sterile humid filter paper and incubated at the same conditions as described for larval development and checked daily to assess the hatch time (HT) and the hatch rate (HR). HT and HR mean values were two days and 72 %, respectively. Approx. 200 eggs with those HR and HT values were applied into the soil, at 5 cm depth close to the stem of the plants for the establishment of the treatments W and GW.

***Maize cultivar and growing conditions***

The maize cultivar used in this study was KWS13, a Northern European maize breeding line developed by the seed company KWS (Einbeck, Germany). Maize seeds were sterilized according to Benziri *et al*. (1994) and pre-germinated at room temperature in Petri dishes containing sterile wet filter paper. The seedlings were planted singly into pots (13 cm diameter) containing Haplic Chernozem. The maize growing conditions were the following: 40 % relative humidity, 24 °C mean temperature and 16 h of additional illumination with sodium lamps (400W, HS2000, Hortilux Schréder, Monster, Netherlands). Plants were placed into the same tray that was moved twice a week in the greenhouse to randomize the growing conditions.

After the first 14 days of growth, each plant was fertilized with 20 ml 0.2% Wuxal top N (Manna, Düsseldorf, Germany) by watering.

***WCR larval extraction from the soil, larval development, root feeding evaluation and data analysis***

Larvae were extracted from the soil of plants inoculated with 200 WCR diapausing eggs (treatments W and GW) using a high gradient Kempson extraction system (Kempson *et al*. 1968). The larvae extracted from each plant were counted and classified into larval stages (L1, L2 and L3) by measuring head capsule width as described by Hammack *et al.* (2003). The WCR root feeding was evaluated based on the root fresh weight of four plant replicates for each treatment.

The root weight values and total numbers of larvae per plant were analyzed with one-way ANOVA combined with Tukey`s HSD test to evaluate statistical differences among treatments. The analysis of the composition of larval stages was performed using a Tukey`s HSD test under a generalized linear model via a logistic function for binomial data. The program used was R add-on package multicomp.

***Rhizosphere isolation***

At growth stage VT maize plants were taken out from the soil and shaken vigorously. The soil tightly adhering to the roots was considered as rhizosphere and collected using a Stomacher blender (Stomacher 400, Seward, England) as described by Costa *et al.* (2006). The microbial pellet was obtained from the cell suspensions by centrifugation at 10,000 *g* at 4 °C for 30 min. The microbial pellet of each root was homogenized with a spatula and 0.5 g were used for the TC-DNA extraction.

***Root sterilization***

Fresh root material was prewashed under running tap water and surface sterilized as described by Götz *et al*. (2006). Afterwards, each root was cut into 1 cm-segments and mixed to randomize the selection of different root areas. 0.4 g of root pieces per plant were used for the TC-DNA extraction.

***Total community (TC) DNA extraction from rhizosphere and root samples***

The TC-DNA was extracted from 0.5 g of rhizosphere pellet and from 0.4 g of surface sterilized root pieces using the FastDNA SPIN Kit for Soil (Q-Biogene, Carlsbad, CA, USA) according to the manufacturer´s protocol. The treatment of the root material required the following additional initial step: root fragments were placed into bead tubes containing a mixture of ceramic and silica particles (included in the kit), frozen by immersion into liquid nitrogen and subsequently processed twice for 1 min at speed 5.5 m $s^{-1}$ in a FastPrep bead beating system (Bio-101, Vista, CA, USA). All TC-DNA samples were purified with the GENECLEAN Spin Kit (Q-Biogene, Heidelberg, Germany) according to the manufacturer´s protocol. DNA concentrations were estimated visually by 0.8 % agarose gel electrophoresis using the quantitative marker High DNA Mass Ladder (Invitrogen). TC-DNA from both rhizosphere and root samples were diluted in MilliQ sterilized water to obtain ca. 20 ng/ µL to use as a PCR template.

***Root mycorrhization levels and data analysis***

Roots of plants at the 9$^{th}$ week of growth were cleared in 10 % KOH at 60 °C for 30 min and then stained with 0.1 % cotton blue in lactic acid. One hundred root pieces about 1-cm long, randomly sampled from the whole root system of each plant were inspected under the optical microscope to quantify the AMF colonization according to Trouvelot *et al.* (1986). Parameters as frequency of root colonization (F %), colonization intensity of the root cortex (M %) and arbuscule abundance in the root cortex (A %) were calculated with the program Mycocalc at the web site: http://www.dijon.inra.fr/mychintec/Mycocalc-prg/download.htlm.

Mycorrhization parameters were submitted to the angular transformation of percentage values prior performing the statistical analyses. The transformed values were statistically analysed by one-way ANOVA and Turkey's HSD test using the mycorrhization parameters as a factor with four levels (one for treatment) and four plant replicates for each treatment. Differences were considered significant when *P* value was < 0.05.

***Preparation of the standard for the quantitative real-time PCR (qRT-PCR) of Glomus intraradices***

TC-DNA of roots colonized by *G.i.* was amplified in qRT-PCR with the primers VC-F and VC-R (Alkan *et al*. 2006). Amplicons, 110 bp length, were ligated in the pGEM-T

vector system (Promega) and transformed into *Escherichia coli* (JM109 Competent Cells, Promega) according to the manufacturer´s instructions. Positive transformants were re-amplified in a Tgradient thermal cycler (Biometra, Göttingen, Germany) with the primers SP6 and T7, purified with the "MinElute PCR purification Kit" (Qiagen) and sequenced. The BLAST analysis of DNA sequences at NCBI site showed 100% identity with *G.i.* (accession no. JN83667-JN836670). The PCR products from single clones amplified with SP6 and T7 were quantified with the *NanoDrop* Spectrophotometer ND-1000 (Peqlab, Erlangen, Germany) and serial dilutions $10^{-4}$ to $10^{-10}$ were used as a standard for the detection and quantification of *G.i.* into the root samples.

***Detection and quantification of Glomus intraradices by quantitative real-time PCR (qRT-PCR)***

The abundance of *Glomus intraradices* was determined in the maize roots of all treatments by means of qRT-PCR using the primer pair VC-F/ VC-R targeting in a specific manner the ITS1+18SrRNA gene fragments of the mycorrhizal fungus (Alkan *et al*. 2006). The qRT-PCR was carried out in the CFX96 Real Time PCR System (Biorad, Hercules, California). The reaction mixture and cycling program were performed as described by Alkan *et al.* (2006) with few modifications: 25-µL aliquot of reaction mixture contained 1 µL DNA template and 2X SYBR Green qRT-PCR Master Mix (Fermentas, St. Leon-Rot, Germany).

The qRT-PCR was calibrated with the clonal ITS1+18SrRNA fragment of *G.i.* used in this study. From the standard calibration curves, the amount of *G.i.* in 1 g of plant root was calculated.

***Restriction fragment length polymorphism analyses (RFLP) and sequencing***

To investigate the AM fungal communities the partial 18S rRNA gene fragments (550 bp) were amplified from TC-DNA extracted from a composite soil sample and four root samples from each treatment. The PCR was performed with the primer pair NS31/AM1 according to Vallino *et al.* (2006) with the following modifications: no BSA was added to the PCR reaction mixture and 2 U of Taq DNA polymerase (AmpliTaqGold with GeneAmp, Applied Biosystems, USA) and 10 pmol of each primer were used. Moreover, the PCR extension temperature was increased to 62 °C. PCR modifications were made to optimize the AM fungal amplification in the soil. Due to a multiple pattern obtained from the soil sample, 550 bp length amplicons

were cut out from the agarose gel and purified by "QIAEXII gel extraction kit" (Quiagen GmbH, Hilden, Germany).

Amplicons of 550 bp length from soil and roots were ligated in the pGEM-T vector system (Promega) and transformed into *Escherichia coli* (JM109 Competent Cells, Promega) according to the manufacturer´s instructions. Positive transformants were amplified with the primer pair NS31/AM1 to select the clones carrying the insert with the right size. The PCR conditions were optimized for the cloned target sequence as follows: 95 °C for 10 min, 30 cycles at 94 °C for 35 s, 63 °C for 35 s, 72 °C for 45 s, and final step at 72 °C for 10 min. Positive clones (180 clones obtained from a soil composite sample and 140 to 155 clones obtained from root samples per each treatment) were tested for RFLP type by independent digestion with the enzymes *HinfI* and *Hin1II* (Fermentas), as recommended by the manufacturer and analysed on 3% agarose gel electrophoresis. For an appropriate identification of the size of restricted fragments, the Molecular weight marker IX (Boehringer Mannheim GmbH, Germany) was used as a standard. Each clone was identified as RFLP type according to Vallino *et al.* (2006). Representative clones per each RFLP type were re-amplified with the primers SP6 and T7, purified with the "MinElute PCR purification Kit" (Quiagen GmbH, Hilden, Germany) and sequenced. The DNA sequences were analysed by BLAST-n program at the NCBI site for multiple sequence alignment.

***PCR amplification of the Internal Transcribed Spacer (ITS) regions and 16S rRNA gene fragments for DGGE fingerprinting***

ITS fragments of the fungal communities in the endorhiza and rhizosphere of maize were amplified from TC-DNA extracted from plants of the treatments C, W, G, GW. The ITS amplification was performed using a nested PCR approach with the primer pair ITS1F/ITS 4 and ITS 2/ITS1F-GC according to Weinert *et al*. (2009).

The 16S rRNA gene fragments of complex bacterial population contained in the same set of samples were amplified by direct PCR performed with the primer pair F984GC/R1378 (Heuer *et al*., 1997). PCR conditions applied were as described by Costa *et al.* (2006).

***Denaturing Gradient Gel Electrophoresis (DGGE) and data analysis***

The DGGE analyses of the fungal and bacterial communities were carried out in the PhorU2 machine (Ingeny, Goes, The Netherlands). ITS- and 16S-DGGE gels were prepared as described by Weinert *et al*. (2009). Gels were silver stained and air dried according to Heuer *et al.* (2001). Digitalized DGGE gel images were analysed with the software package GELCOMPAR II program, version 4.5 (Applied Math, Kortrijk, Belgium) as described by Rademaker *et al.* (1999). Background was subtracted and lanes were normalized as described by Gomes *et al.* (2003). Cluster analysis based on the Pearson correlation coefficient (UPGMA) was performed to evaluate the percentage of similarities among samples.

Pair-wise statistical analysis (Permutation test) was applied on the values of the similarity matrix according to Kropf *et al.* (2004) to evaluate if the differences observed were statistically supported. *P* values and *D* values were always reported.

***Identification of specific endophytic fungi by ITS-DGGE band sequencing***

Four ITS-DGGE bands which occur exclusively in the roots of plants treated with *G.i.* (treatments G and GW) were excised from the acrylamide gel. DNA was eluted during overnight incubation of the gel slices at 4° C in sterile TE buffer, pH 8. After centrifugation at 11,000 x *g* for 60 s, the supernatant was transferred to a new tube and 1 µL of it was used as a template in the second PCR amplification described for ITS-DGGE analysis, except for the use of primers without GC clamp (ITS1F/ITS2). PCR products were ligated into the pGEM-T vector system (Promega) and transformed into *Escherichia coli* (JM109 Competent Cells, Promega) according to the manufacturer´s instructions. Positive clones were re-amplified with the primers ITS1F-GC/ITS2 and the electrophoretic mobility of the cloned fragments was checked by DGGE gel. To identify different ribotypes co-migrating on acrylamide gel, four to five clones per excised DGGE band were sequenced. The DNA sequences were analysed with BLAST-n program at NCBI site for multiple sequence alignments with sequences available in the database.

*Nucleotide sequence accession numbers:* nucleotide sequences determined in this study were deposited in the GenBank database under the accession numbers JN836634-JN836670.

## Results

### *Glomus intraradices detection and quantification in maize roots*

Total community DNA extracted from maize roots of the extra-set of plants grown for six weeks (growth stage V7) in Haplic Chernozem with and without *G.i.* inoculant (C, G) was analyzed by qRT-PCR method to assess *G.i.* abundance in the endorhiza of maize plants before WCR egg inoculation. The qRT-PCR revealed a specific *G.i.*-signal exclusively in the roots of plants grown in the soil inoculated with *G.i.* (G) with a mean of 9.5 $x10^5$ copy numbers of 18S/ITS fragments per g root (s.d. 0.5).

After nine weeks (growth stage VT) of growth, the maize roots of each treatment were analyzed by qRT-PCR as well, in order to study the treatment effect on *G.i.* root colonization. A specific qRT-PCR signal was detected only in the roots of plants grown in soil inoculated with *G.i.* in presence and in absence of WCR larvae (treatments G and GW). The *G.i.* mean value was about 1.8 $x10^6$ (s.d. 0.2) and 2 x $10^6$ (s.d. 0.3) copies of 18S/ITS fragments per g root in the treatments G and GW, respectively. No significant differences were observed between these treatments ($P$ = 0.8) indicating that WCR larval feeding did not influence the abundance of *G.i.* in the roots. Differences in the *G.i.* abundance were instead observed between plants at the growth stages V7 and VT ($P < 0.05$), indicating that the mycorrhization increased during the nine weeks of plant growth.

### *Does Glomus intraradices inoculation affect the root biomass, the WCR root feeding, the larval number and development?*

In order to evaluate the effect of *Glomus intraradices* (*G.i.*) on the root biomass and on the WCR root feeding, the root fresh weight of plants (growth stage VT) from the treatments C, W, G and GW were determined. Significant differences of the root fresh weight between the treatments with and without larvae ($P < 0.01$) indicated a clear larval effect on the root biomass with approx. 20 % reduction of the root tissues for the treatments W and GW. No significant differences of root biomass were observed between the treatments with and without *G.i.*-soil inoculation (C/G and W/GW), indicating that *G.i.* mycorrhization did not improve the belowground plant development and did not affect the root larval feeding.

The numbers of WCR larvae determined for the treatments W and GW did not significantly differ from each other indicating that *G.i*. mycorrhization did not affect

the viability of the WCR eggs or the larval survival. However, the analysis of the larval instars composition in the treatments W and GW revealed a significant reduction of the WCR larval development in presence of the *G.i.* (Fig. 1) with the relative number of 3rd larval instars being significantly lower in the GW than in the W treatment ($P = 2\ e^{-16}$).

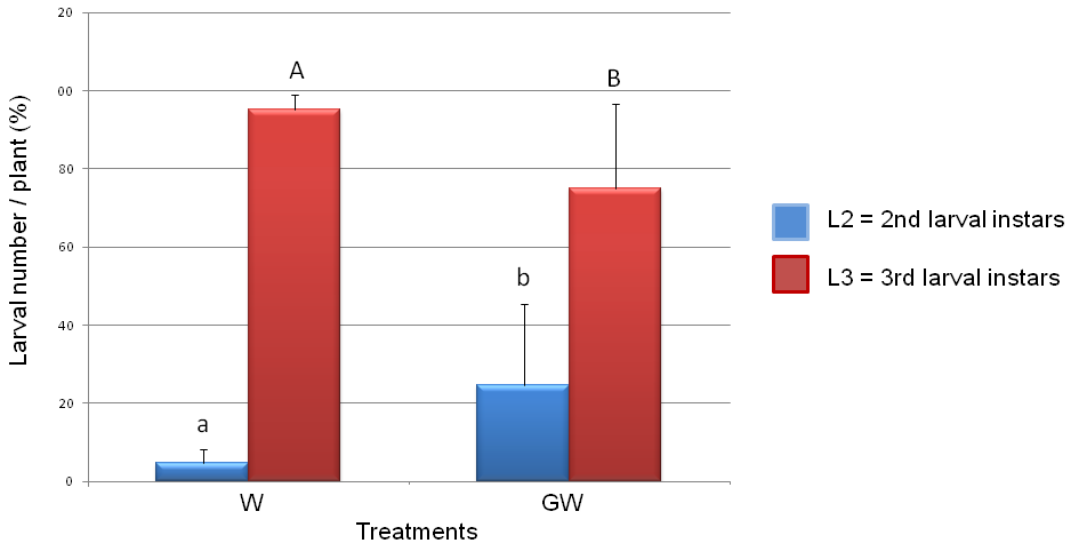


Fig. 1. Effect *of Glomus intraradices* on larval development. The number of 3rd larval instars (L3) was significantly lower in the *Glomus*-treated plants (treatment GW) than in untreated control plants (treatment W). The error bars represent standard deviations. Lowercase letters above columns indicate significance of difference between the number of L2 larval instars, while uppercase letters indicate significance difference between L3 larval numbers ($P=2e^{-16}$).

***Microscopic analysis and AMF root colonization level***

To assess the level of root colonization by arbuscular mycorrhizal fungi four plant replicates per treatments were analyzed by microscopy. Mycorrhization parameters such as frequency (F %), colonization intensity (M %) and abundance of arbuscules in the root cortex (A %) were significantly higher in the roots from the treatments where *G.i.* was applied ($P$ <0.01). No significant differences between plants from the treatment C and W and between G and GW were observed (Fig. 2).

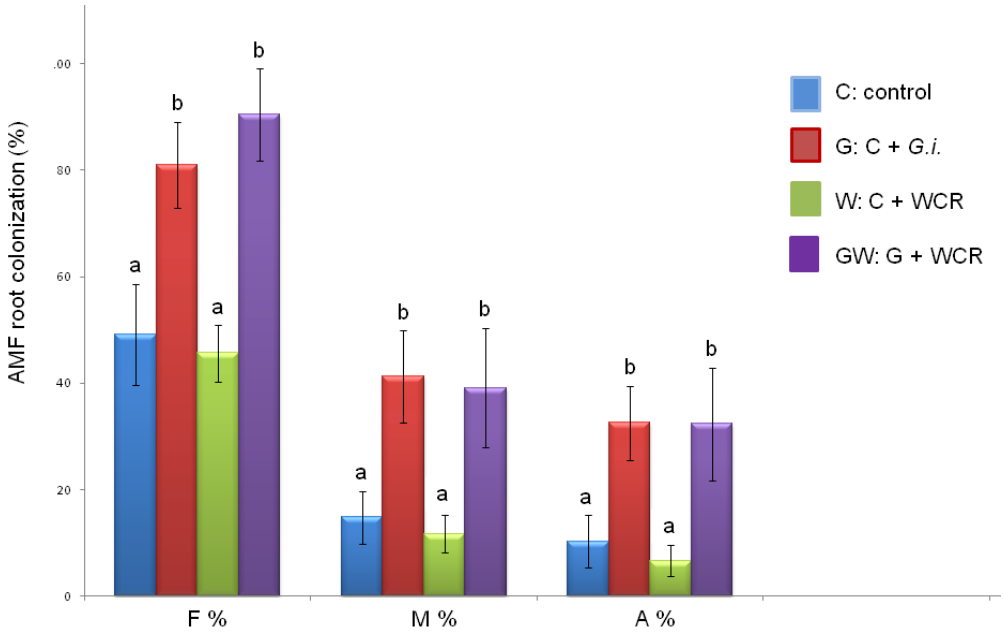


Fig. 2. Colonization of the root system of maize plants by *Glomus intraradices* shown for the treatments (C, G, W and GW). F %: frequency of mycorrhization defined as ratio between colonized root fragments and total number of root fragments; M %: intensity or amount of cortical cells occupied by AMF structures; A %: arbuscule richness in the root system. The error bars represent standard deviations. Letters above columns indicate significance of difference according to ANOVA <0.05.

***PCR-RFLP and AMF composition in soil and root samples***

To assess (i) the AMF community structure in the soil, (ii) the AMF populations naturally occurring in the maize roots and (iii) the effect of both *G.i.*-soil inoculation and WCR larval feeding on the endophytic AMF communities a PCR-RFLP analysis was performed on the TC-DNA extracted from one composite soil sample and from four root samples for each treatment (C, W, G and GW). The PCR-RFLP analysis of 180 cloned 18S rRNA gene fragments obtained from the soil sample revealed five different RFLP patterns including RFLP types 1, 2, 3, 6, 8 and several (ca. 32 %) unclassified RFLP types. Among the unclassified RFLP profiles, one occurred more often and was here generally described as RFLP X. The dominant AMF in the soil belonged to the RFLP types 8 and 1. The percentage of clones carrying 18S rRNA gene fragments of AMFs on the total number of clones investigated by means of

RFLP method are reported in Table 1. The RFLP analyses of 140 to 155 cloned 18S rRNA gene fragments obtained from root samples per treatment revealed that the AMF colonizing the maize roots from the treatments C and W belong to the RFLP types 2, 3, 6 and 8. In these roots, the RFLP types 8 and 3 were dominant. Differently, in the roots of plants from the treatments G and GW the RFLP analysis showed a significant reduction of the AMF evenness almost exclusively to the RFLP type 11. Cloned 18S rRNA gene fragments representative of each RFLP type were sequenced and virtually digested with the enzyme *Hinf1* and *Hin1II* in order to obtain clear information about the restriction fragment lengths characterizing each RFLP type. Database searches of 18S rRNA gene sequences representative of each RFLP type allowed the identification of different AMF species from the genus *Scutellospora* (RFLP type 6) and *Glomus* (RFLP types 1, 2, 3, 8, 11 and X). RFLP types found in Haplic Chernozem and plant roots, the source of isolation; the corresponding accession number, the species with highest identity sequence found in the GenBank, and the exact coordinates and restriction fragment lengths are reported in Table 2.

Table 1. RFLP types and their relative abundance in Haplic Chernozem and in root samples from the treatments C, W, G and GW grown in the same soil type.

| | **Relative abundance of RFLP types in soil and maize roots** | | | | |
|---|---|---|---|---|---|
| **RFLP type** | **Soil** | **Treatment C** | **Treatment W** | **Treatment G** | **Treatment GW** |
| **RFLP 1** | 14,4 | 0 | 0 | 0 | 0 |
| **RFLP 2** | 10 | 5,8 | 3,5 | 0 | 0 |
| **RFLP 3** | 2,2 | 18,7 | 25,3 | 0 | 0 |
| **RFLP 6** | 1,1 | 2,5 | 1,4 | 0 | 0 |
| **RFLP 8** | 40 | 62 | 60 | 7 | 5,7 |
| **RFLP 11** | 0 | 0 | 0 | 93 | 94,3 |
| **RFLP X** | 6,7 | 0 | 0 | 0 | 0 |
| **Unclassified RFLP profiles** | 25,5 | 11 | 9,8 | 0 | 0 |

The relative abundance of the RFLP types found in soil and roots was calculated as percentage of clones carrying the insert of a certain RFLP type on the total number of clones digested with *HinfI* and *Hin 1II* per soil or plant treatment.

Table 2: RFLP types found in Haplic Chernozem and plant roots, the source of isolation; the corresponding accession number, the species with highest identity sequence found in the GenBank, and the exact coordinates and restriction fragment lengths (bp) obtained with the enzyme *Hinf1* and *Hin1II* by virtual digestion at BioLabs web site.

| | | | | ***Hinf 1*** | | **Hin1II** | |
|---|---|---|---|---|---|---|---|
| **RFLP type** | **Source** | **Access. n°** | **Identity sequence (ID)** | **Coordinates** | **Length (bp)** | **Coordinates** | **Length (bp)** |
| **RFLP1** | Soil | JN836649 | *G. etunicatum* (99% ID) | 268-552 | 285 | 1-297 | 297 |
| | | | | 1-267 | 267 | 388-552 | 165 |
| | | | | | | 298-387 | 90 |
| **RFLP2** | Soil | JN836650 | *Uncultured Glomus* (99%ID) | 280-523 | 244 | 258-548 | 291 |
| | Root C | JN836641 | | 1-189 | 189 | 1-257 | 257 |
| | Root W | JN836645 | | 190-279 | 90 | | |
| | | | | 524-548 | 25 | | |
| **RFLP3** | Soil | JN836651 | *Uncultured Glomus* (98%ID) | 280-523 | 244 | 258-548 | 291 |
| | Root C | JN836642 | | 1-189 | 289 | 1-164 | 164 |
| | Root W | JN836646 | | 190-279 | 90 | 165-257 | 93 |
| | | | | 524-548 | 25 | | |
| **RFLP 6** | Soil | JN836652 | *Scutellospora calospora* (99% ID) | 1-301 | 301 | 260-547 | 288 |
| | Root C | JN836643 | | 302-522 | 221 | 1-169 | 169 |
| | Root W | JN836647 | | 523-547 | 25 | 170-259 | 90 |
| **RFLP8** | Soil | JN836653 | *G. mosseae* (100 % ID) | 267-550 | 284 | 1-295 | 295 |
| | Root C | JN836644 | | 23-266 | 244 | 296-438 | 143 |
| | Root W | JN836648 | | 1-22 | 22 | 439-550 | 112 |
| | Root G | JN836636-37 | | | | | |
| | Root GW | JN836640 | | | | | |
| **RFLPX** | Soil | JN836654 | *G. aurantium* (99% ID) | 283-550 | 268 | 260-550 | 291 |
| | | | | 1-141 | 141 | 1-169 | 169 |
| | | | | 142-282 | 141 | 170-259 | 90 |
| **RFLP 11** | Root G | JN836634-35 | *G. intraradices* (99% ID) | 142-524 | 383 | 259-549 | 291 |
| | Root GW | JN836638-39 | | 1-141 | 141 | 117-258 | 142 |
| | | | | 525-549 | 25 | 1-116 | 116 |

***ITS-DGGE of the fungal communities in the endorhiza and rhizosphere of maize and DGGE bands identification***

Comparative analysis of ITS-DGGE profiles showed highly similar fungal community structure between the treatments C/W and between the treatments G/GW in the endorhiza of maize. Four dominant differentiating bands appeared exclusively in the endophytic fungal fingerprints of *G.i.*-treated plants (band 1, 2, 3 and 4, Fig. 3). Cluster analysis of ITS-DGGE profiles showed that the treatments G and GW grouped together as well as the treatments C and W, with just one exception (Fig. 4). However, differences ($P$ = 0.03) in the fungal community composition, observed between the treatments with and without *G.i.* (Table 3), indicated a clear effect of the *G.i.*-soil inoculation on the fungal populations in the endorhiza of maize. No WCR larval feeding effect on the composition of the endophytic fungal communities was observed.

The DGGE fingerprints of the fungal communities in the maize rhizosphere showed high similarity among all treatments. A mixed cluster of samples from all treatments was obtained (Fig. 4). Although a significant difference was observed between the treatments G and GW, the corresponding $D$-value was low ($D$= 2.1). Thus we concluded that *G.i.*-soil inoculation did only weakly affect the fungal communities in the rhizosphere as well as the WCR larval feeding.

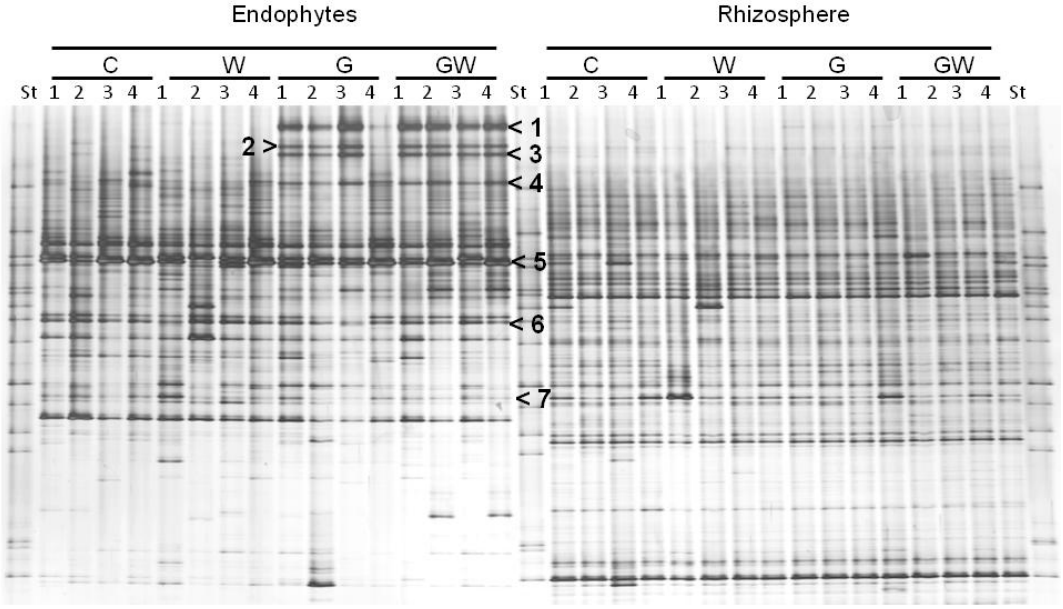


Fig. 3. ITS-DGGE fingerprinting showing the endophytic and rhizospheric fungal communities of maize plants from the treatments C, W, G and GW. C: maize plant grown in Haplic Chernozem, natural source of different mycorrhizal species; W: maize plants characterized by 4 weeks root feeding by WCR larvae; G: maize plants with *G.i.* inoculum added before sowing; GW: maize plants mycorrhized by *G.i.* and characterized by 4 weeks WCR larval feeding on the roots. St: ITS standard. The fingerprinting was generated by separation of ITS fragments amplified from TC-DNA extracted from root and rhizosphere. Arrows: specific bands occurring only in plant inoculated with *G.i.*

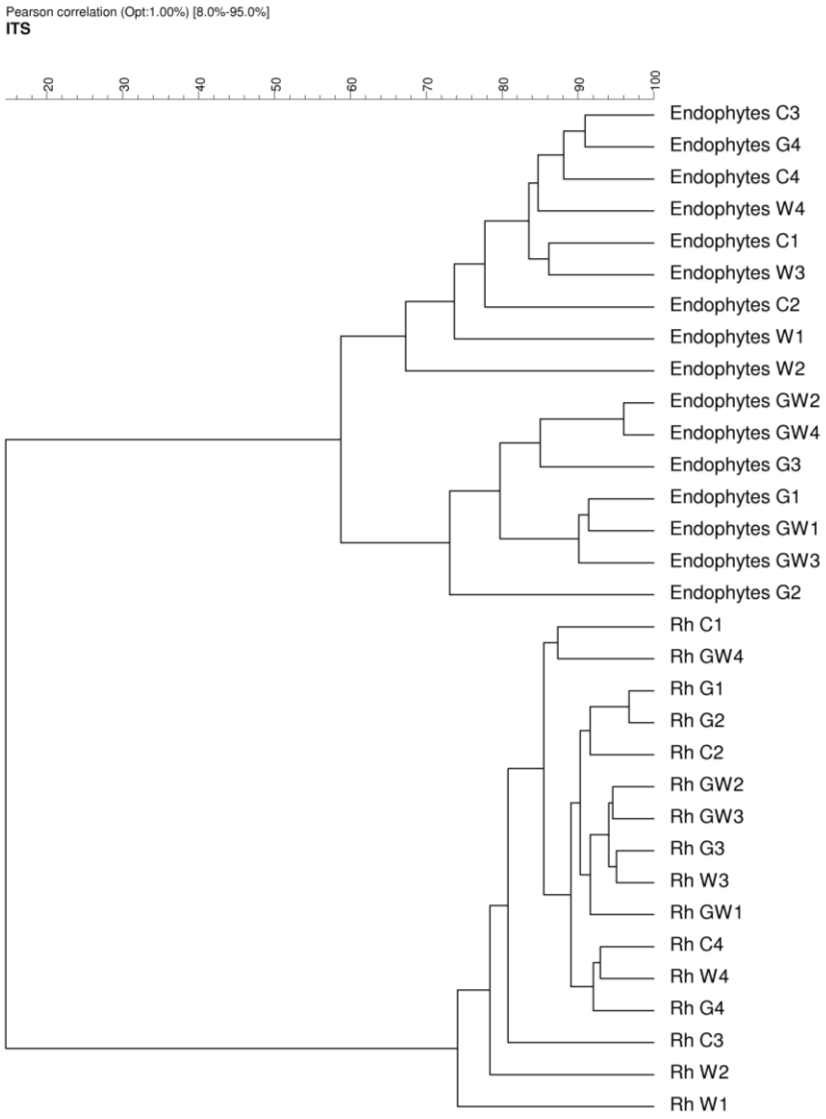


Fig. 4. Dendrogram constructed with the fungal communities fingerprints in the endorhiza and rhizosphere of maize reported in Fig. 3. The differences between the profiles are indicated by percentage of similarity. The dendrogram was based on the Pearson correlation indices and cluster analysis by the unweighted pair group method using arithmetic averages. Microbial patterns of root samples and rhizosphere cluster separately. With one exception, the endophytic communities in the maize roots of the treatments G and GW grouped apart from those of the treatments C and W.

***Identification of specific endophytic fungi by ITS-DGGE band sequencing***

The BLAST analysis of the ITS-sequences obtained by cloning of Bands 1, 2 and 3 (Fig. 3) matched against the same type of *Glomus* sp., although with different percentage of similarity (96-100 % identity) (accession no. JN36655-JN836661). This suggests that a single individual belonging to the species *Glomus* contributed to originate the three bands in the ITS-DGGE fingerprinting of the endophytic fungal communities in the roots of maize. No clones carrying an insert with the electrophoretic mobility of Band 4 were found. Although this study focused on the identification of the four differentiating bands occurring only in *G.i*.-treated plants other bands (Bands 5, 6 and 7 in Fig. 3) were also sequenced. Band 5 was affiliated to *Microdochium bolleyi* with 99 % sequence identity (accession no. JN836662 and JN8366623). Band 6 sequences showed 99 % sequence identity with *Tetracladium* sp. (accession no. JN836664 and JN836665). The sequencing of Band 7 revealed *Periconia macrospinosa* (98 % sequence identity, accession no. JN836666).

***16S-DGGE of the fungal communities in the endorhiza and rhizosphere of maize***

In order to elucidate the interactions among WCR larval feeding and the rhizospheric and root-associated and endophytic bacteria of maize grown in *G.i*.-inoculated and non-inoculated soil, comparative analysis of 16S-DGGE profiles were performed.

The DGGE fingerprints of the bacterial communities in the maize endorhiza showed high variability among replicates. Differences in the relative abundance of two bacterial populations upon WCR larval feeding or of *G.i.*-soil inoculation were observed (bands 1 and 2, Fig. 5). Statistical analysis revealed significant differences in the endophytic bacterial composition between the treatment C and the treatments W G and GW ($P$ = 0.03) indicating a clear effect of both *G.i.*-soil inoculation and WCR larval feeding on the endophytic bacteria of the maize roots. Although a differentiating band (band 2, Fig. 5) in the treatments with G.i.-soil inoculation was displayed, no significant differences were observed between the treatments W/G and W/GW (Table 3).

The DGGE patterns of the bacterial communities in the rhizosphere of maize showed pronounced shifts due to the WCR larval feeding independently by the *G.i*.-soil inoculation, while no shifts were observed in response to *G.i.*-soil inoculation (Fig. 5). No clustering was observed between treatments (Fig. 6). However,

statistical tests revealed significant differences between all of them. The *D* values relatively high (*D* >7.1) confirmed the significance of those data (Table 3).

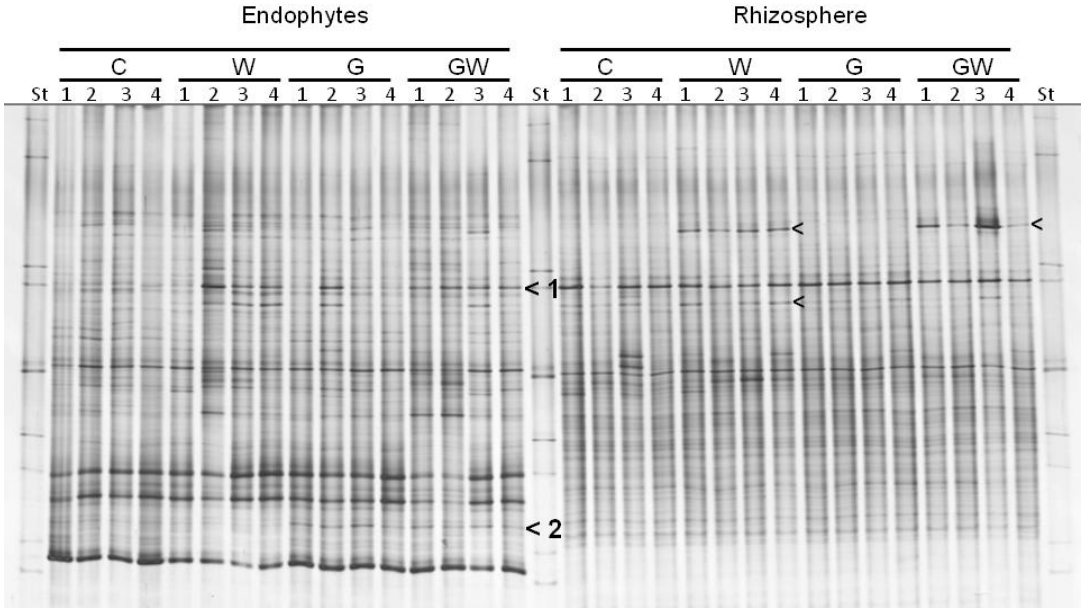


Fig. 5. 16S-DGGE fingerprinting showing the endophytic and rhizospheric bacterial communities of maize plants from the treatments C, W, G and GW.. C: maize plant grown in Haplic Chernozem, natural source of different mycorrhizal species; W: maize plants characterized by 4 weeks root feeding by WCR larvae; G: maize plants with *G.i.* inoculum added before sowing; GW: maize plants mycorrhized by *G.i.* and characterized by 3 weeks WCR larval feeding on the roots. St: ITS standard. The fingerprinting was generated by separation of 16S fragments amplified from TC-DNA extracted from root and rhizosphere. Arrows show treatment dependent bands.

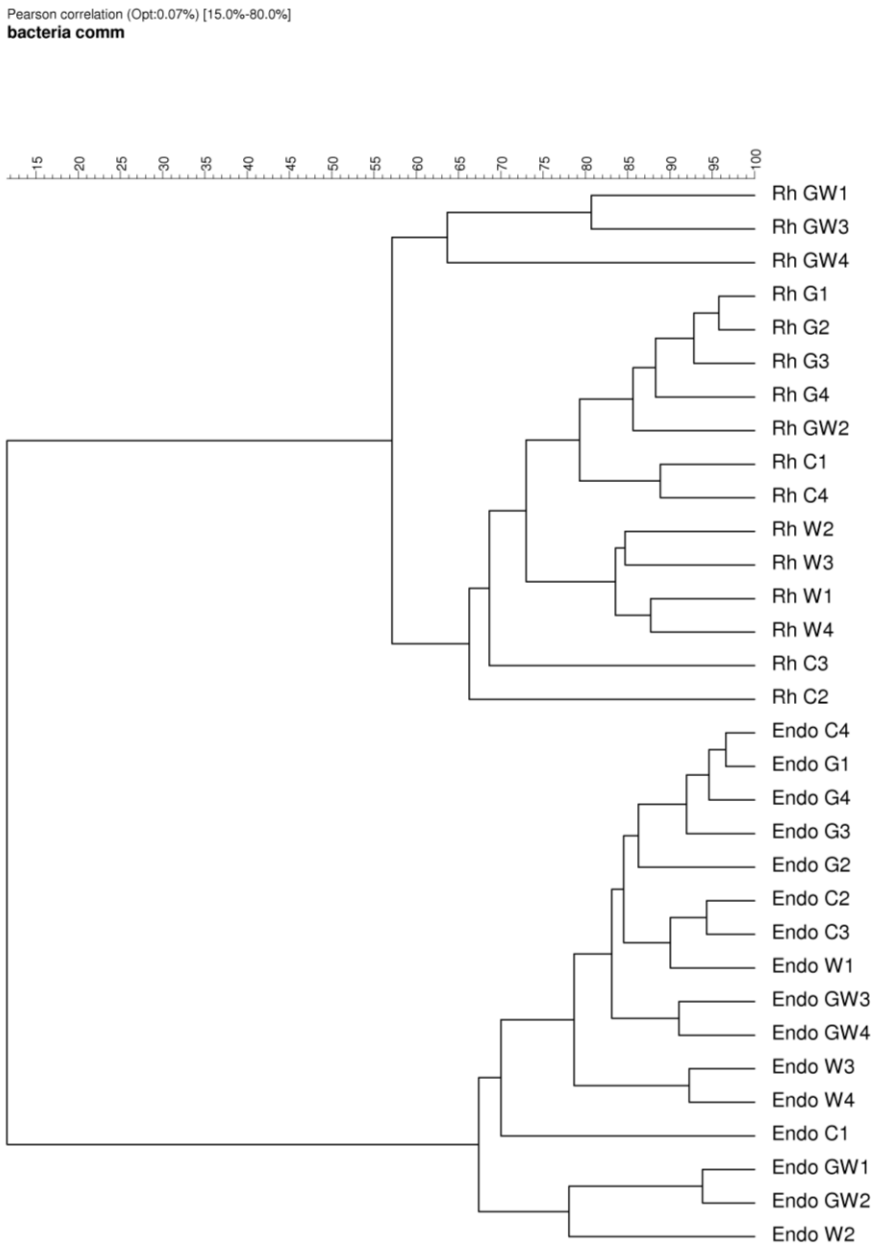


Fig. 6. Dendrogram constructed with the bacterial communities fingerprints in the endorhiza and rhizosphere of maize reported in Fig. 5. The differences between the profiles are indicated by percentage of similarity. The dendrogram was based on the Pearson correlation index and cluster analysis by the unweighted pair group method using arithmetic averages. Microbial patterns of root samples and rhizosphere cluster separately.

Table 3. Significant values (*P* values) and *D* values of pairwise comparisons between treatments (C, G, W and GW) of fungal and bacterial communities fingerprints in the endorhiza and in the rhizosphere of KWS 13 cultivar grown in Haplic Chernozem.

| | **Fungi** | | | | **Bacteria** | | | |
|---|---|---|---|---|---|---|---|---|
| | **Endorhiza** | | **Rhizosphere** | | **Endorhiza** | | **Rhizosphere** | |
| | ***P*** | ***D*** | ***P*** | ***D*** | ***P*** | ***D*** | ***P*** | ***D*** |
| C/W | 0.3 | 0.8 | 0.5 | 0.3 | **0.03** | 8.2 | **0.03** | 7.8 |
| C/G | **0.03** | 8.7 | 0.06 | 2.9 | **0.03** | 5.2 | **0.03** | 7.1 |
| C/GW | **0.03** | 12.4 | 0.17 | 1.2 | **0.03** | 8.4 | **0.03** | 22.1 |
| W/G | **0.03** | 14.7 | 0.3 | 1.3 | 0.06 | 5.2 | **0.03** | 12.4 |
| W/GW | **0.03** | 23 | 0.08 | 1.3 | 0.1 | 5.1 | **0.03** | 13.4 |
| G/GW | 0.2 | 3.6 | **0.03** | 2.1 | 0.06 | 2.4 | **0,03** | 14.4 |

Values of $P < 0.05$ indicate significant differences between rhizosphere samples of different maize genotypes grown in the same soil type. Bold values show significant differences. Simulations: 10.000.

## Discussion

The first question addressed in the present study was whether soil inoculation of the arbuscular mycorrhizal fungus *G.i.* affects the WCR fitness in terms of larval survival, developmental stage and root feeding. A significantly reduced WCR larval development was observed when the maize plants were grown in *G.i*. inoculated soils. Our finding are in agreement with Boucher (2001) that reported a reduction in head capsule diameter of emerging WCR beetles from *G.i.*-treated plants compared to control plants. To our knowledge no other studies on this topic have been published until now. The effect of the mycorrhizal colonization of maize roots might be due either to a direct interaction between WCR and *G.i.* or to a plant-mediated mechanism. Maize secondary metabolites such as hydroxamic acids might, in fact, have a toxic activity towards WCR larvae (Xie *et al*., 1991). Furthermore, root exudates triggered by larval feeding (e.g., protease inhibitors and phenolics) might have limited the assimilation of plant nutrients and thus delayed herbivore growth (Karban & Baldwin 1997). Because the prolonged time in early larval instars rendered them more susceptible to predation by natural enemies, *G.i.*- can be proposed as a biocontrol microorganism for the integrated pest management of WCR larval damages.

The second question addressed in the present study aimed to elucidate the effect of *G.i.* on the indigenous AMFs colonizing the maize roots and on the fungal and bacterial populations living in both rhizosphere and endosphere of maize plants. Microscopic analysis of the plant roots showed that *G.i.*-soil inoculation increased the frequency of the root mycorrhization from about 50 % to 80 % (Fig. 2). PCR-RFLP analysis and sequencing of AMF-18S rRNA gene fragments in *G.i.*-treated and untreated plants revealed that *G.i*.-soil inoculation reduces the AMF richness in the maize roots to almost exclusively the RFLP type 11 identified by sequencing as *G.i.* The dominance of *G.i.* in the roots reflects the preferential establishment of symbiosis between *G.i.* and the maize pant due to the higher abundance of *G.i.* in soil. A selective interaction has been observed also between maize plants and the AMF populations naturally occurring in the soil Haplic Chernozem. PCR-RFLP comparative analysis and sequencing of AMF-18S rRNA gene fragments in the soil and in the roots of plants grown in absence of *G.i*. showed significant differences in the AMF composition between soil and root samples: the soil was dominated by *Glomus mosseae* (RFLP 8) and G*lomus* sp. (RFLP 1), while *Glomus mosseae*

(RFLP 8) and *Glomus* sp. (RFLP 3) were dominant in the endorhiza of plants from the treatments without *G.i.*

The effects of *G.i.* on the fungal and bacterial communities in the endorhiza and in the rhizosphere of maize were assessed by DGGE fingerprinting. *G.i.* strongly affected the fungal community composition in the endorhiza of maize (Fig. 3). ITS sequences of three dominant differentiating bands occurring only in the fingerprints of endophytic fungal communities in *G.i.*-treated plants matched the same type of *Glomus* sp. although with different percentage of sequence identity. This suggested that a single individual belonging to the species *Glomus* contributed to the differentiating bands 1, 2 and 3. Some studies showed that ITS sequences are rarely recovered twice from a single spore (Lanfranco *et al*., 1999; Antoniolli *et al*., 2000), most likely due to the multiple and polymorphic genome of the AMFs (Hijiri and Sanders, 2005). Furthermore, these data might indicate that the ITS region alone has a too low resolution power to differentiate AMFs at the species level.

*G.i.* inoculation affected significantly also the bacterial community composition in the endorhiza of maize, although less pronounced shifts in the DGGE fingerprints were observed (Fig. 5). In the rhizosphere no clear differentiating bands on the DGGE fingerprints of fungal and bacterial communities were observed between the treatments with and without *G.i.*-soil inoculation. However, permutation testing revealed significant effects of *G.i.* on the bacterial communities inhabiting the maize rhizosphere (Table 2). *G.i*. effects on the microbial communities in the rhizosphere and endorhiza of plants were reported in other studies. Filion *et al*. (1999) showed that soluble substances released by the extraradical mycelium of *G.i.* induced differential growth of soil-inhabiting microorganisms. Marschner *et al.* (2003) showed that mycorrhizal colonization by *G.i*. changed the bacterial community structure in the soil and in the root surface of maize.

The final question addressed in this study was if the feeding of WCR larvae altered the microbial community composition in the endorhiza and rhizosphere of maize in *G.i.*-treated and untreated plants. PCR-RFLP method and sequencing revealed that WCR larval feeding did not affect the AMF diversity in the maize endorhiza in both *G.i.*-treated and untreated plants. DGGE fingerprints showed that the total endophytic fungal communities in the roots were not affected by WCR larval feeding. Differently, WCR larvae affected the endophytic bacterial communities of the treatments without *G.i.*: DGGE profiles of the endophytic bacteria from the treatments C and W differed significantly. Between the treatments

G and GW no significant differences were observed indicating that WCR did not affect the bacterial communities in presence of *G.i.* However, the absence of statistically significant differences could have been caused also by the high variability in the DGGE profiles between the replicates. In accord to a previous work (Dematheis *et al*., submitted), WCR larvae did not affect the fungal communities in the maize rhizosphere, while affected significantly the bacterial communities of *G.i.*-treated and untreated plants. WCR influenced mainly the bacterial populations living in the rhizosphere, and to lesser extent, living in the endorhiza. One of the dominant bacterial population occurring, upon larval feeding, in the maize rhizosphere of KWS 13 maize was identified in a previous work as *Acinetobacter calcoaceticus* (Dematheis *et al*., submitted). No information is available on the bacterial populations increased in the endorhiza of maize in presence of WCR larvae. The identification of dominant bacterial populations responding to larval feeding in the maize endorhiza can be further investigated.

**Conclusion**

This work provided new insights into the interaction between WCR, *G.i*. and microorganisms in the rhizosphere and endorhiza of maize.

We first reported inhibitory effects of the WCR grown caused by the *G.i.* root mycorrhization (9.5 x$10^5$ copy numbers of 18S/ITS fragments of *G.i.* per g root). *G.i.* strongly affected the fungal communities in the endorhiza. Less pronounced effects, although significant, were found also on the bacterial communities living in the endorhiza and in the rhizosphere of maize. In contrast, WCR feeding did mainly influence the bacterial communities in the rhizosphere and to a lesser extent in the endorhiza. No WCR effect on the endophytic and rhizospheric fungal communities was observed.

In conclusion, *G.i*. inoculation can be used in integrated pest management as it can delay larval development rendering WCR larvae more susceptible to predation by natural enemies. The mechanisms of this interaction remain unknown. However, our data showed that *G.i.* altered the interactions between the plant and the endophytic fungal and bacterial communities which might affect WCR larval development.

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# Chapter V

## The microbiome in gut and eggs of *Diabrotica virgifera virgifera* LeConte: no effects of the soil type

**The microbiome in gut and eggs of *Diabrotica virgifera virgifera* LeConte:**

**no effects of the soil type**

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**Keywords:** *Diabrotica virgifera virgifera* LeConte; ITS; 16S rRNA gene; DGGE.

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## Abstract

Gaining insight into the microbiome of the Western Corn Rootworm (WCR) is a fundamental prerequisite to develop new pest control strategies. The main objectives of this work were (i) to assess the ability of previously published DGGE method for estimating fungal diversity in gut samples; (ii) to investigate the effect of the soil type on the microbial communities inhabiting the digestive tract of WCR larvae; (iii) to identify the most dominant gut-associated microorganisms; (iv) to investigate their transovarial transmission; and (v) to identify the dominant populations colonizing WCR eggs. Total fungal and bacterial communities, and taxon-specific bacteria were investigated by means of DGGE technique and sequencing of ITS regions and 16S-rRNA gene fragments, PCR-amplified from total community DNA. Phylogenetic analyses were performed to assess the identity between sequences from different environments.This work showed that ITS-DGGE allows the characterization of the WCR gut microflora. Dominant gut-associated fungi and bacteria were shown not to be influenced by the soil type. The fungi *Fusarium* spp. and *Gibberella zeae* were dominant in the gut system and originated most likely from the rhizosphere. Due to their dominance in the gut system we speculate that WCR larvae are vectors of mycotoxin-producing fungi. Within the bacteria, *Wolbachia* sp. was dominant in the WCR gut and transovarially transmitted. Within the *Betaproteobacteria, Herbaspirillum* sp. was dominant in all the intestines and most likely originated from the external environment. Major fungal population identified in the eggs was *Mortierella gamsii;* major bacterial population was *Wolbachia* sp. Within the group specific bacteria *Duganella* sp., endosymbiont of *Mortierella elongata, Pseudomonas* sp., *Lysobacter* sp., *Streptomyces* sp. and *Rhodococcus* sp. were dominant.

## Introduction

The Western Corn Rootworm (WCR), *Diabrotica virgifera virgifera* LeConte (Coleoptera: *Chrysomelidae*) is one of the economically most important pests of maize *(Zea mays* L.) in the US and it is an increasing threat to corn-growing areas in Europe. In the US the WCR causes about $1.3 billion in lost revenue and control costs each year (Rice, 2004), while in Europe more than 470 million Euro cost damages per year are expected (Wesseler & Fall, 2010). Major yield losses are caused by WCR larvae feeding on root tissues resulting in plant lodging.

The high adaptability of this herbivorous insect to prevailing pest management strategies such as annual crop rotation with soybean (Gray *et al*., 2009) or WCR-resistant transgenic plants (Gassmann *et al.*, 2011) alerted maize farmers worldwide. The genetic manipulation of microorganisms intimately associated with the insect gut was already suggested as a novel approach to manage insect pests (Dillon *et al*., 2004; Riehle & Lorena, 2005; Douglas, 2007).

In this work, our attention focused on the microbial biodiversity within the gut systems of WCR larvae and eggs. Microorganisms inhabiting the digestive tracts of insects can play important roles in the nutrition, development, survival, resistance to pathogens and reproduction of the insect host (Eutick *et al*. 1978, Fukatsu & Giordano *et al*., 1997; Brand *et al*., 1975; Hosokawa, 2002; Brune, 2003; Moran *et al*., 2005). Furthermore, the finding that the midgut bacteria are required for *Bacillus thuringiensis* insecticidal activity (Broderick *et al*., 2006) pointed out that these microbes as key players for successful control measures.

Despite the importance of the microbes inhabiting the gut system, little is known about the microbial composition in the WCR gut and about their transovarial transmission. The high complexity of the gut microbiota limited their study. In the past, the gut flora has been mainly investigated by phenotypic characterization of isolates (Buchner, 1965; Dasch *et al.,* 1984; Lysenko, 1985). But, because 99% of the microorganisms existing in nature cannot be cultivated (Amann *et al*., 1995) such studies provided only a partial description of gut-associated microorganisms. Molecular approaches have been developed in the last 20 years to overcome the limitations of culture-based techniques. The molecular fingerprinting based on denaturant gel electrophoresis (DGGE) of 16S rRNA gene fragments was used to identify bacterial species in the gut system of insects (Reeson *et al.*, 2003; Fall *et al*., 2007; Dillon *et al*., 2010). However, no study investigated the bacterial

communities in the gut of the WCR by means of 16S-DGGE. The major research effort has been directed toward the study of bacteria rather than fungi. Due to the potential ability of yeasts to degrade several mycotoxins, Molnár *et al.* (2008) studied the yeast diversity in the gut system of several pests of maize by DGGE technique performed on the D1 domain of the 26S rRNA gene. No literature reported the use of DGGE fingerprinting to study the total fungal communities in the gut of insects.

Different soil types can support different microbial communities. Because the larvae are feeding on maize root tightly attached to soil particles, we hypothesized that different rhizospheric microorganisms can be ingested, modifying the gut microflora.

The main objectives of this work were (i) to assess the ability of previously published DGGE method for estimating the fungal diversity in gut samples; (ii) to investigate the effect of the soil type on the microbial communities inhabiting the digestive tract of the WCR; (iii) to identify the most dominant gut-associated microorganisms; (iv) to investigate their transovarial transmission, and (v) to identify the dominant populations colonizing the WCR eggs.

To achieve these goals a DNA-based approach was used. Fungal community composition was studied by denaturing gradient gel electrophoresis (DGGE) of the 18S rRNA gene fragments and of the internal transcribed spacer (ITS) regions. Both molecular markers were PCR-amplified from total community (TC) DNA extracted from gut or egg samples. Bacterial community composition was investigated by DGGE of the 16S rRNA gene fragments PCR-amplified from TC-DNA as well. Dominant microorganisms in gut and eggs of WCR were identified by cloning and sequencing of specific DGGE bands.

The results of this analysis provide insights into the microorganisms that are associated with gut and eggs of the WCR.

## Materials and methods

### *Experimental setup*

Maize plants were grown in the greenhouse in pots (Ø 13 cm) containing three different soil types: Haplic Chernozem, Haplic Luvisol and Eutric Vertisol. A gauze was glued to the bottom of these pots to prevent the escape of the larvae. Four independent replicates per soil type were prepared. Three weeks after sowing (growth stage V3) 60 eggs of the Western Corn Rootworm (WCR; *Diabrotica v. Virgifera*) were injected in each pot directly into the soil close to the plant stems. The plants were thereafter grown for an additional time of three weeks (growth stage V7). After 21 days of larval feeding on the maize roots, the larvae were collected from the soil (see below) and their guts were immediately removed for the total communities (TC) DNA extraction. In parallel, the plants (growth stage V7) were harvested and the rhizospheres isolated (see below) for the TC-DNA extraction as well.

### *Soil types and sampling method*

Three different soil types, Haplic Chernozem, Eutric Vertisol and Haplic Luvisol, were collected nearby Göttingen (Germany) in 2008. Physico-chemical parameters (e.g. pH, particle size, nitrogen and carbon content) and microbial composition differed among soil types as shown in a previous work (Dematheis *et al.*, submitted). 400 kg of each soil were taken from four different spots, five meters apart from each other, along a transect to a depth of 25 cm. In order to avoid any alteration of the microbial content, the soil samples were immediately transported to the laboratory and homogenized using a soil crusher machine (Unifix 300, Möschle, Ortenberg, Germany) and sieved through a 10 mm mesh. Fresh soil was used for the experiments described here.

### *WCR egg source, stimulation of the larval development and hatch test*

Non-diapausing WCR eggs were provided by USDA-ARS (Northern Grain Insect Research Laboratory, Brookings, USA) and stored at 8 °C until their use. In order to stimulate the larval development, the eggs were incubated at 26 °C, 60 % relative humidity in dark conditions for 12 days and checked for visible larval presence using a dissecting microscope. Afterwards the eggs were washed in a sieve (Ø 250 µm) and the collected eggs were suspended in 0.15 % agar solution. 0.5 mL of egg suspension were applied on a sterile humid filter paper and incubated at the same

conditions as described for larval development and checked daily to assess the hatch time (HT) and the hatch rate (HR). The HT and HR mean values were two days and 72 %, respectively. Approx. 60 eggs with those HR and HT values were applied into the soil, at 5 cm depth close to the plant stems.

***Extraction of WCR larvae from the soil and gut isolation***

After 20 days of feeding, the larvae were extracted from the soil by using a high gradient Kempson extraction system (Kempson *et al*., 1968). The larvae were washed three times with sterile double-distilled $H_2O$ and sedated with ethanol (40 %). Afterwards, the larvae were cut at both ends and the guts were removed aseptically using a tweezer. Single and composite gut samples were prepared. For the composite samples ten guts of larvae grown in the same pot were pooled to obtain approximately 25 mg fresh weight.

***WCR egg surface sterilization and conservation***

The WCR eggs were washed in a sieve (Ø 200 µm) with cold water and transferred to 30 mL of a 5 % $MgSO_4$ solution for about 1 min. The material that sank down was transferred into 65 % $MgSO_4$ solution. Emerging intact eggs were taken and washed with tap water. Subsequently the eggs were transferred into 2 mL reaction tubes containing a sterile washing solution consisting of 0.85 % NaCl and 0.1 % Tween, and vortexed for 30 s. Afterwards the eggs were transferred to a petri dish containing sterile water and placed under UV light for one night. The water with the WCR eggs was placed on sterile filter paper and dried using a water jet vacuum pump. From the sterile filter paper the eggs were transferred to a solution of 0.33 g Nipagin per ml of 70 % ethanol. After 30 min the eggs were washed and stored in 70 % ethanol. The efficiency of the surface sterilization was checked twice plating 50 eggs on PDA media mixed with 600 ppm streptomycin.

***Maize cultivar and growing conditions***

The maize cultivar used in this study was KWS 13, a Northern European maize breeding line developed by the seed company KWS (Einbeck, Germany). The maize growing conditions adopted in our experiments were the following: 40 % relative humidity, 24 °C mean temperature and 16 h of additional illumination with sodium lamps (400W, HS2000, Hortilux Schréder, Monster, Netherlands). Plants grown in the same soil were placed within the same tray that was moved twice a week in the greenhouse to randomize the growing conditions. Fertilizer Hakaphos blau (Compo,

Münster, Germany; 2.5 %) was applied by watering once a week to plants older than 14 days.

***Rhizosphere isolation***

Six-week old maize plants (growth stage V7) were removed from the soil and shaken vigorously. The soil tightly adhering to the roots was considered as rhizosphere and collected using a Stomacher blender (Stomacher 400, Seward, England) following the method described by Costa *et al.* (2006). The microbial pellet was obtained from the cell suspensions by centrifugation at 10 000 *g* at 4° C for 30 min. The microbial pellet of each root was homogenized with a spatula and 0.5 g were used for the TC-DNA extractions.

***Microbial DNA extraction from rhizosphere, gut and egg samples***

The TC-DNA was extracted from 0.5 g of rhizosphere pellet using the FastDNA SPIN Kit for Soil (Q-Biogene, Carlsbad, CA, USA) according to the manufacturer's instructions. TC-DNA was extracted from pools of 10 guts and from four pools of 100 surface sterilized eggs using the same kit used for the rhizosphere DNA extraction. The DNA was extracted following the manufacturer's protocol with some modifications: the material was placed into bead tubes, frozen in liquid nitrogen and subsequently processed for 1 min at speed 5.5 m $s^{-1}$ in a FastPrep bead beating system (Bio-101, Vista, California, USA); the TC-DNA pellet was re-suspended in 100 µL of TRIS-EDTA buffer (pH 7.4) included in the kit. All TC-DNA samples were purified with the GENECLEAN Spin Kit (Q-Biogene, Heidelberg, Germany) according to the manufacturer´s protocol. DNA concentrations were estimated visually by 0.8 % agarose gel electrophoresis using the quantitative marker High DNA Mass Ladder (Invitrogen). TC-DNA from rhizosphere and from eggs were diluted 1:10 for PCR amplifications, while TC-DNA from gut was used undiluted as a PCR template.

***PCR amplification of the SSU (18S) rRNA gene fragment, the Internal Transcribed Spacer (ITS) regions and 16S rRNA gene fragments for DGGE fingerprinting***

The 18S rRNA gene fragments of the fungal communities contained in gut samples were amplified by a semi-nested PCR amplification. The primer pair NS1 and EF3 were used in the first PCR reaction, while NS1 and FR1-GC were used in the second amplification. Reaction mixture and PCR conditions used were described by

Oros-Sichler *et al*. (2006). The ITS fragments of the fungal communities contained in gut and egg samples were amplified using a nested PCR approach with the primer pair ITS1F/ITS 4 and ITS 2/ITS1F-GC according to Weinert *et al*. (2009). The 16S rRNA gene fragments of complex bacterial populations contained in the same set of samples were amplified by direct PCR performed with the primer pair F984GC/R1378 as described by Costa *et al.* (2006). The amplification of the 16S rRNA gene fragments of the bacterial families *Pseudomonas*, *Alphaproteobacteria*, *Betaproteobacteria,* and *Actinobacteria* was carried out using taxon specific primers in a nested PCR amplification according to Costa *et al*. (2006).

***Denaturing Gradient Gel Electrophoresis (DGGE), cluster analysis and statistics***

18S-DGGE gels were carried out in the DCode$^{TM}$ System (Biorad Laboratory, Hercules, CA, USA) as described by Oros-Sichler *et al*. (2006). ITS- and 16S-DGGE gels were carried out in the PhorU2 machine (Ingeny, Goes, The Netherlands) according to Weinert *et al.* (2009). Gels were silver stained and air dried according to Heuer *et al.* (2001). Gel images were digitally captured using an Epson 1680 Pro scanner (Seiko-Epson, Japan) with high resolution setting. Digitalized DGGE gel images were analysed with the software package GELCOMPAR II program, version 4.5 (Applied Math, Kortrijk, Belgium) as described by Rademaker *et al.* (1999). Background was subtracted and lanes were normalized as described by Gomes *et al.* (2003). Cluster analysis based on the Pearson correlation coefficient (UPGMA) was performed to evaluate the percentage of similarities among samples. Pair-wise statistical analysis (Permutation test) was applied on the values of the similarity matrix according to Kropf *et al.* (2004) to evaluate if the differences observed were statistically supported. *P* values < 0.5 indicate significant differences between treatments.

***ITS clone library and screening on DGGE gel***

Products of the first ITS amplification (circa 600 bp) obtained from gut or egg samples were ligated in the pGEM-T vector system (Promega) and transformed into *Escherichia coli* (JM109 Competent Cells, Promega) according to the manufacturer´s instructions. ITS inserts of positive transformants were re-amplified by PCR using the primers pair ITS 1F-GC/ ITS2 and re-analyzed by DGGE to check the electrophoretic mobility. For gut samples five to nine clones per soil type carrying the insert representative for the most dominant fungal population were

selected for sequencing. For each egg sample five clones carrying ITS fragments with different DGGE electrophoretic mobility were sequenced.

***Identification of 16S-DGGE bands by cloning, sequencing and BLAST analysis***

Dominant bands were excised from the 16S acrylamide gel. The gel slices were transferred to a 1.5 mL tube and crushed with the top of a sterile tip. DNA was eluted from the gel slices by incubation overnight at 4 °C in sterile TE buffer at pH 8. After centrifugation at 11,000 x *g* for 60 s, the supernatant was transferred to a new tube and 1 µL of it was used as template for 16S-DGGE analysis. The PCR was performed using the same primer pairs described for the total bacterial communities but without GC clamp (F984/R1378). PCR products were ligated in the pGEM-T vector system (Promega) and transformed into *Escherichia coli* (JM109 Competent Cells, Promega) according to the manufacturer´s instructions. The clones were re-amplified with the primer pair T7/SP6 to select the ones carrying the insert with the correct size. The T7/SP6 amplicons of the positive clones were amplified with the primers F984-GC/R1378 to identify the clones with specific DGGE band. To identify different ribotypes co-migrating on DGGE gel, four to six clones per excised DGGE band were sequenced.

ITS-DNA sequences were analyzed by BLAST-n program at the NCBI site. Differently, the 16S-rRNA gene sequences were analyzed first by CLASSIFIER program at RDP (Ribosomal Database Project) site to identify the sequences at the genus level (selected sequences with higher confidential index) and second, with BLAST-n program at the NCBI site to identify into a specific genus the species higher related to the sequence introduced into the GenBank.

***Phylogenetic analysis***

ITS and 16S rRNA sequences obtained from gut and egg samples were aligned using Clustal W in MEGA 4.0 software. Phylogenetic trees were constructed with MEGA 4.0 using the maximal parsimony algorithm and 500 repetitions for the calculation of the bootstrap values.

***Nucleotide sequence accession numbers*:** nucleotide sequences determined in this study were deposited in the GenBank database under accession numbers JF461095-JF461251.

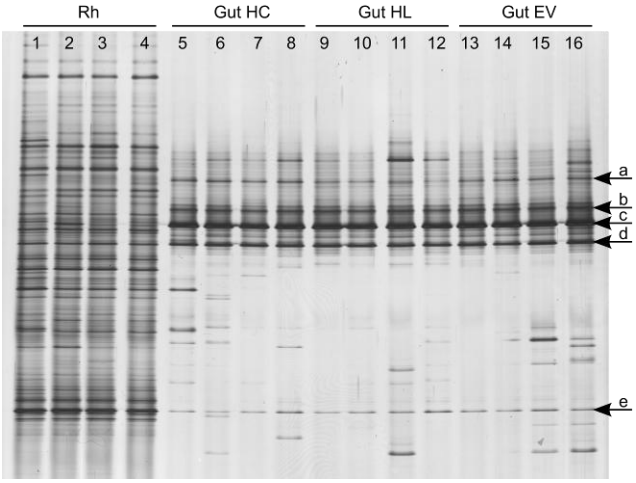
## Results

### *ITS-DGGE fingerprinting to investigate the gut flora*

In order to identify the most appropriate molecular marker for typing the gut-associated fungi of the WCR, ITS and SSU (18S) rRNA fingerprints were compared. 18S-DGGE profiles performed according to Oros-Sichler *et al*. (2006), displayed a very low diversity within the fungal gut flora with only one dominant band which occurred indistinctly in the fingerprints of all gut samples (Supplemental Information Fig. S1). In contrast, ITS-DGGE profiles showed complex band patterns ranging in the fungal electrophoretic mobility. Thus, the ITS region was used in our investigations for a better discrimination power of the fungal communities in WCR gut. Another critical factor evaluated and optimized was the individual number of guts needed to obtain reproducible ITS-DGGE profiles. Fingerprints of microorganisms associated to individual gut were highly variable among replicates and often not reproducible (Supplemental information Fig. S2). We speculated that the amount of the DNA target extracted from individual gut fell below the detection limit of the PCR. Thus, DNA extracted from pooled gut of 10 individuals was tested. Due to the stability and reproducibility of the DGGE patterns obtained, DNA extracted from composite samples of 10 gut was used in our analyses.

### *Gut-associated fungi of WCR larvae and influence of the soil type*

In order to investigate the influence of the soil type on gut-associated fungi, total community DNA extracted from gut of larvae sampled in three different soil types was analyzed by ITS-DGGE fingerprinting. DGGE profiles revealed five dominant fungal populations (“a” , “b”, “c”, “d” and “e”) which occurred in all gut samples (Fig. 1a). Cluster analysis showed that the fungal communities in the gut of larvae grown in different soil types grouped together sharing more than 80 % similarity. Statistical analysis revealed that gut-associated fungi were not significantly affected by the soil type.




Band a: *Candida sake*

Band c: *Fusarium* spp.

Band d: *Gibberella zeae*

Band e*: Verticillium dahliae*

Fig.1. ITS-DGGE profiles showing the comparison of fungal rhizosphere communities of maize grown in Haplic Chernozem (Rh) and of gut fungal communities of WCR larvae feeding on maize roots grown in Haplic Chernozem (Gut HC), in Haplic Luvisol (Gut HL) and in Eutric Vertisol (Gut EV).

***Identification of gut-associated fungi in WCR larvae***

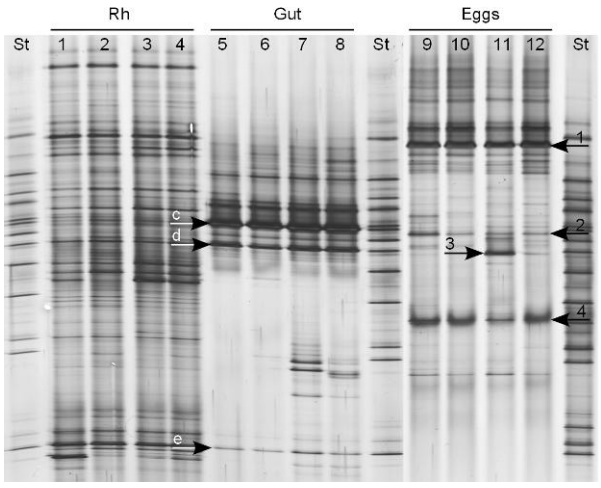
To identify the most dominant fungi in the gut of WCR larvae grown in the three different soil types, a clone library of the PCR product from the first ITS amplification (PCR products from total gut DNA were pooled according to the soil type) was generated. ITS inserts from about 50 clones per soil type were re-amplified and analyzed by DGGE to check the electrophoretic mobility. Most inserts showed the same electrophoretic mobility of band “c” (68.3 %) and band “d” (17.3 %). Only two cloned inserts co-migrated with band “a” and only one with the band “e”. None of the cloned ITS fragments co-migrated with band “b”. The remaining clones were carrying inserts with electrophoretic mobility not corresponding to the most relative dominant bands in the ITS-DGGE fingerprinting. A maximum of eight clones per soil type carrying the insert representative of bands “a” “c”, “d” and “e” (Fig 1a) were selected for sequencing. The operational taxonomic unit (OTU) behind band “c” was affiliated by blast analysis of the ITS sequences to *Fusarium* spp. with 98 % identity (ID) (accession numbers JF461095, -97, -99, -102, -103, JF461106, -109, -111, JF461113, -114), while the ITS sequences of the OTU behind band “d” showed maximal identity with *Gibberella zeae* (JF461098, -110, -112). The OTUs behind

band “a” and “e” were identified as *Candida sake* (JF461105, -115) and *Verticillium dahliae* (JF461104) with 99 % and 97 % ID, respectively.

***DGGE fingerprinting of fungi associated to the rhizosphere, to the gut and eggs of WCR.***

In order to study whether the most dominant fungi detected in the WCR gut were transovarially transmitted rather than taken up during the root larval feeding from the rhizosphere, ITS fingerprints of gut, rhizosphere and egg samples were compared. The ITS-DGGE of fungal communities in the rhizosphere of maize pants grown in Haplic Chernozem (HC), in the gut of larvae grown in the same soil type and in WCR surface-sterilized eggs is exemplary shown in Fig. 2.

The dominant band identified as *Verticillium dahliae* in the ITS-DGGE of guts (band e, Fig. 2) occurred also in the fungal fingerprinting of the maize rhizosphere. No corresponding bands were found in the ITS-DGGE of eggs. Thus, we assumed a potential origin of *V. dahliae* in the maize rhizosphere from where it might have been ingested. In contrast, bands “c” and “d” in Fig. 2, identified as *Fusarium* spp. and *Gibberella zeae* in the gut fingerprints were observed as a faint band in both rhizosphere and egg samples. This suggested that *Fusarium* spp. and *Gibberella zeae* were either parentally transmitted via the eggs or ingested from the rhizosphere and enriched in the gut of WCR larvae.




Band 1: *Mortierella gamsii*

Band 2: *Fusarium* spp.

Band 3: *Cylindrocarpon olidum*

Band 4*: Thrichocladium asperum*

Fig. 2. Comparison of ITS-DGGE profiles obtained from rhizosphere samples of maize plants grown in Haplic Chernozem (Rh), from WCR gut samples of larvae feeding in Haplic Chernozem (Gut) and from WCR egg samples (Eggs).

***Identification of the relative most abundant fungi colonizing WCR eggs***

In order to identify the OTUs in WCR eggs a clone library of the PCR products of the first ITS amplification obtained from WCR eggs was generated. The inserts of positive transformants were re-amplified and analyzed by DGGE. The majority of the clones (67 clones) carried a fragment corresponding to the most dominant band (band 1, Fig. 2). The cloned ITS fragments with the electrophoretic mobilities of bands 2, 3 and 4 (Fig. 2) were obtained as well. Sequencing and blast analysis of band 1 sequence showed that the most dominant fungal population present in the eggs of WCR shared 98 % ID with *Mortierella gamsii* (JF461176 to JF461178). The sequencing of band 2 revealed in the WCR eggs 99 % ID with *Fusarium* spp. (FJ461124 to JF461129). Band 3 and 4 sequences were affiliated to *Cylindrocarpon olidum* with 98 % ID (JF461162 to JF461134) and to *Trichocladium asperum* with 100 % maximal identity (JF461170 and JF461160), respectively.

***Phylogenetic analysis of gut- and egg-associated fungi***

In order to investigate whether fungal communities in the gut of WCR larvae resemble that one present in WCR eggs a phylogenetic analysis of all cloned ITS sequences obtained from gut and egg samples was performed. Ten reference sequences chosen to be the higher related to the sequences found in the eggs and in the guts of WCR larvae were included in the analysis. The dendrogram in Fig. 3 showed distinct clusters for gut and egg sequences. The biggest cluster consisted in the ITS reference sequence of *Mortierella gamsii* (DQ093723.1) and 67 sequences corresponding to the most dominant population in WCR eggs (band 1, Fig. 2). The reference sequence of *Trichocladium asperum* (AM292050.1) grouped together with two sequences derived from eggs, showing maximal identity. In the third cluster one sequence from the gut grouped together with the ITS sequence of the *Verticillium dahliae* reference strain (DQ282123). These sequences showed little variation between each other. The fourth cluster included egg sequences clustering with *Cylindrocarpon olidum* (AJ677294) even though they showed slight differences. The last cluster consisted of two subgroups: one was represented exclusively by gut sequences clustering together with *Gibberella zeae* reference sequence (AB250414.1); while the second one included both gut and egg sequences plus the reference sequences of *Fusarium solani* (FJ460589) and *Fusarium* sp. 18014 (EU750687.1), *Fusarium* sp. 14005 (EU750680.1) and *Fusarium* sp. 19001

(EU750688). Although the *Fusarium* sequences were highly similar to each other, the ITS sequences from gut still clustered separately from those from eggs, indicating a different origin of these sequences.

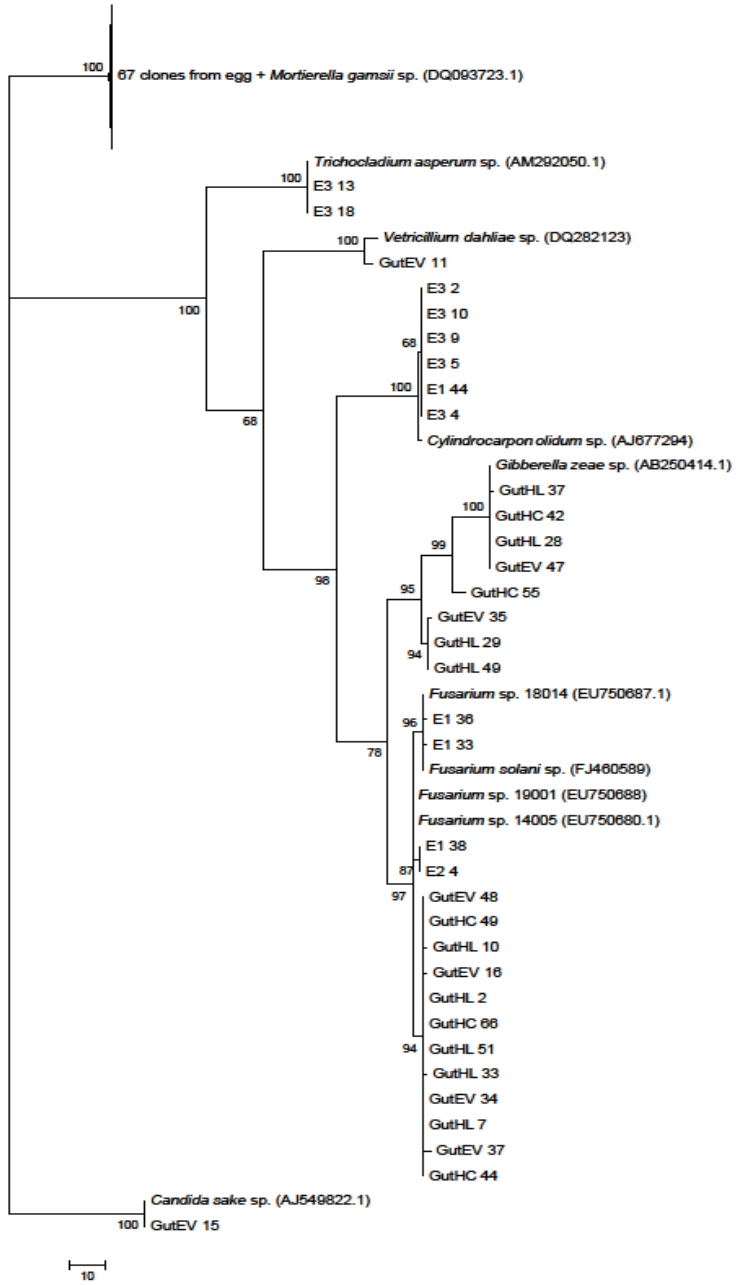



Fig. 3 Maximum parsimony tree derived from ITS sequences amplified from egg (E) and gut samples obtained from WCR larvae feeding in different soil types (Gut HC, Gut HL and Gut EV). The dendrogram generated with MEGA 4 software was rooted on *Candida sake* sequence (AJ549822.1), which clustered together with a corresponding gut sequence (JF461105). The branches show boostrap values higher than 60.

***Influence of the soil type on gut-associated bacteria of WCR larvae***

16S DGGE analysis was carried out in order to study the soil type effect on the bacterial populations inhabiting the digestive tract of WCR larvae. Total bacterial communities and four different bacterial taxonomic groups (*Alphaproteobacteria, Betaproteobacteria*, *Pseudomonas* and *Actinobacteria*) were investigated. The DGGE profiles of the total bacterial communities and of the *Alphaproteobacteria* in the gut were very similar to each other: both DGGE fingerprints showed highly similar patterns among replicates and among gut samples from larvae collected in different soil types. In particular, only one dominant band with identical electrophoretic mobility was observed in all gut samples (data not shown). Statistical analysis revealed that the total bacterial populations and the *Alphaproteobacteria* in WCR gut were not significantly influenced by the soil type. Similarly, also the betaproteobacterial DGGE showed just one dominant band with the same electrophoretic mobility in all replicates and gut samples independently from the soil type from where the larvae originated (Fig. 4). Statistical analysis showed that the soil type did not influence significantly the *Betaproteobacteria* in the WCR gut. Pseudomonas and actinobacterial communities in the gut showed high variability among replicates in DGGE gels. This suggested either a low abundance of these bacterial groups to be PCR-amplified or a transient association with the intestine of WCR larvae. No influence of the soil type on those microbial populations was observed by statistical test.

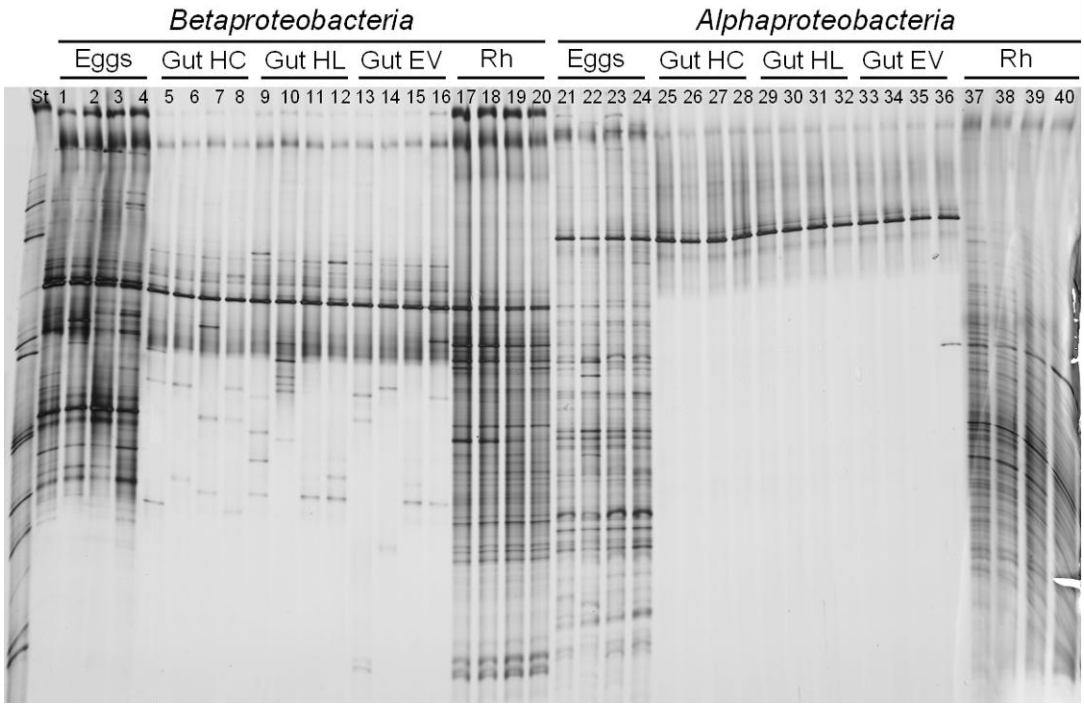



Fig. 4. Alpha- and betaproteobacterial DGGE of eggs (Eggs) and gut of WCR larvae grown in Haplic Chernozem (Gut HC), in Haplic Luvisol (Gut HL) and in Eutric Vertisol (Gut EV). The fingerprinting of the alpha- and betaproteobacterial communities in the rhizosphere of maize grown in Haplic Chernozem is reported as well (Rh). St: 16S standard. The gel shows no soil type effect on the *Alpha*- and *Betaproteobacteria* in the digestive tract of WCR larvae .

***Identification of the dominant gut-associated bacteria of WCR larvae grown in different soil types***

The sequencing of the dominant band in the 16S-DGGE fingerprints of the total bacterial communities revealed in WCR gut the symbiotic species *Wolbachia* (99% ID). The same species was identified by sequencing of the dominant band in the 16S-DGGE fingerprints of the *Alphaproteobacteria* (JF461204 to JF461209). According to the DGGE fingerprinting, no soil type effects on the total bacterial- and alphaproteobacterial communities were observed. The sequencing of the dominant band in *Betaproteobacteria* fingerprints revealed in all gut samples a bacterial species affiliated to *Herbaspirillum* sp. with 98% ID (JF461196 to JF461203). This bacterial population was identified in the WCR gut independently from the soil type in which the larvae were feeding. Because the fingerprintis of *Pseudomonas* and

actinobacterial populations in WCR larval guts showed high variability among replicates (no common populations in the gut were observed), no specific bands from those communities were investigated.

***DGGE fingerprinting of bacteria associated to the rhizosphere, to the gut and eggs of WCR.***

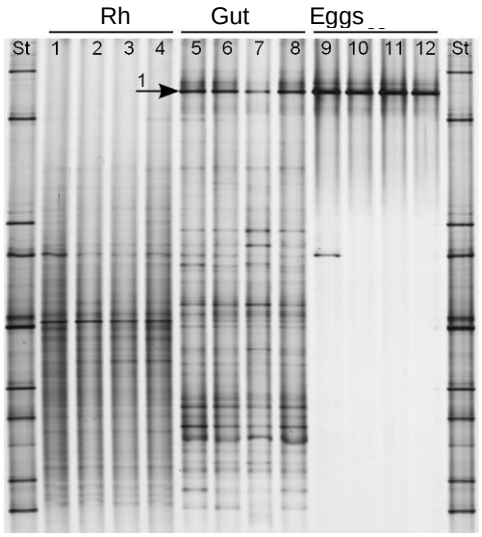
In order to understand the origin of the dominant gut-associated bacteria of WCR, the total bacterial communities in the maize rhizosphere, in the guts and in the eggs of the WCR were compared by 16S-DGGE. The DGGE patterns revealed a dominant band (band 1, Fig. 5a) with identical electrophoretic mobility in egg and gut samples, indicating a transovarial transmission of the bacterial population responsible of band 1. The band in the gut fingerprints was previously identified as *Wolbachia* sp. The absence of band 1 in the rhizosphere fingerprinting indicated in WCR eggs the presence of a symbiotic species.

The same comparative analysis has been performed for all four group-specific bacteria. 16S-DGGE of the *Alphaproteobacteria* showed in the egg samples a dominant band (band 2, Fig. 5b) with identical electrophoretic mobility of the band identified as *Wolbachia* sp. in the alphaproteobacteria fingerprinting of gut samples. This suggested a parental transmission of *Wolbachia* sp., while the absence of a corresponding band in the rhizosphere fingerprintis indicated a symbiotic origin of this bacterial species. Betaproteobacterial DGGE showed one band common in the rhizosphere, gut and egg samples (band 3, Fig. 5c). We hypothesized that the population observed in the gut is a non-symbiotic species which might have either an external environmental origin or a transovarial origin. In the egg profiles a second dominant band (band 4, Fig. 5c) was observed.

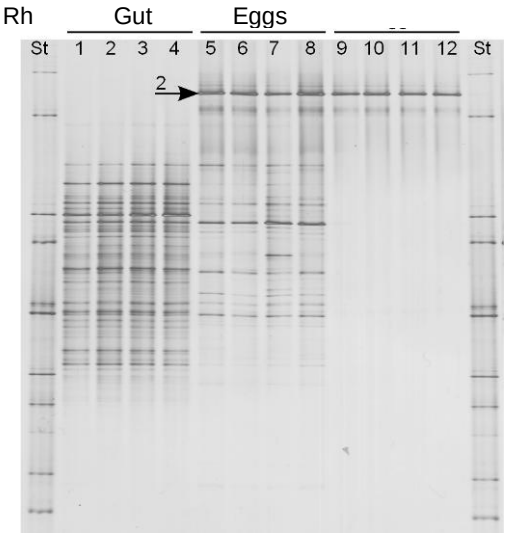
Pseudomonas DGGE profiles showed a band which was dominant and common among all rhizosphere and egg samples and few replicates of the gut samples (band 5, Fig. 5d). Actinobacterial DGGE profiles showed a similar situation, except for the electrophoretic mobility of the common band among rhizosphere, egg and gut samples which was lower (band 7, Fig. 5e). This suggested that the bacteria populations in the gut was either ingested during the root larval feeding from the rhizosphere where a similar band occurred as well, or parentally transmitted via the eggs. Other dominant bands were found exclusively in the fingerprints of Pseudomonas and Actinobacterial communities (band 6, Fig. 5d and band 8, Fig.

5e). The absence of corresponding bands in the gut fingerprints suggested a marginal role of these microbial populations in the larval gut.

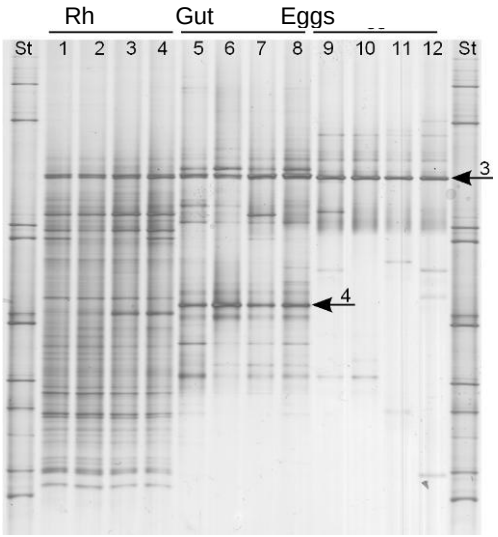
(a)



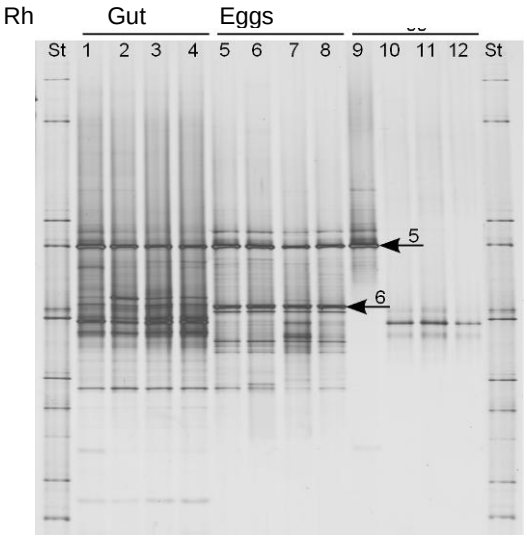

(b)



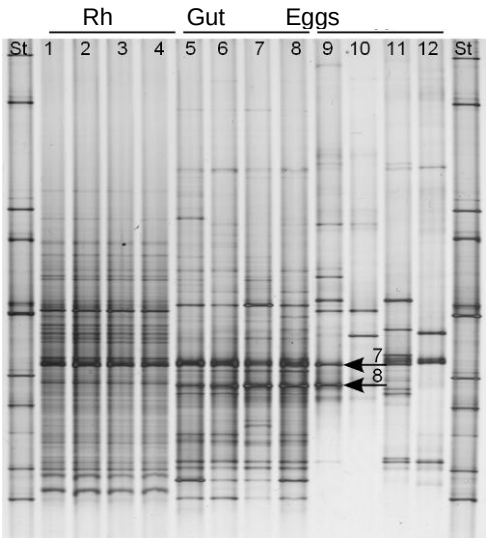

(c)




(d)



(e)




Band *1* *Wolbachia* sp.

Band 2: *Wolbachia* sp.

Band 3: *Herbaspirillum* sp. in the gut *and* *Duganella* sp. in eggs

Band 4*: endosymb*iont of *Mortierella elongata*

Band 5: *Pseudomonas* sp.

Band 6: *Lysobacter* sp.

Band 7: *Streptomyces* sp.

Band 8: *Tsukamurella* sp. in the gut and *Rhodococcus* sp. in eggs

Fig. 5. 16S rRNA DGGE fingerprints obtained from rhizosphere samples of maize plants grown in Haplic Chernozem (Rh), gut samples of larvae extracted from Haplic Chernozem (Guts) and egg samples (Eggs). (a) 16S-DGGE of the total bacterial communities, (b) 16S-DGGE of the *Alphaproteobacteria* populations, (c) 16S-DGGE of the *Betaproteobacteria*, (d) 16S-DGGE of the *Pseudomonas* communities, and (e)of the *Actinobacteria*. Arrows indicate bands excised from the gels for sequencing.

***Bacterial identification of dominant 16S-DGGE bands in WCR eggs and in corresponding gut bands***

DGGE dominant bands generated from gut and egg samples were excised from the acrylamide gel of all replicates, combined, cloned and sequenced.

Sequencing of band 1 (Fig. 5a) and band 2 (Fig. 5b) from the total bacterial and alphaproteobacterial DGGE patterns revealed in the egg samples the symbiotic species *Wolbachia* (100 % ID) (JF461210 and JF461211). Sequencing of the band 3 (Fig. 5c) excised from the DGGE gels of the betaproteobacterial communities revealed in WCR eggs the bacterial species *Duganella* sp. with 99-100 % ID (FJ461212 to FJ461218). In the gut the corresponding band was identified as *Herbaspirillum* sp.. Band 4 was identified as an unknown population sharing 96 % ID with a bacterium endosymbiont of *Mortierella elongata* (JF461219 to JF461216). Sequencing of band 5 excised from *Pseudomonas* DGGE gels revealed *Pseudomonas* sp. (99-100 % ID) (JF461237 to JF461245) in the WCR eggs. The sequence of the corresponding band in one of the replicates of the gut samples displayed 99 % ID with *Pseudomonas* sp. as well (JF461248 to JF461251), indicating a parental transmission. Band 6 was affiliated to *Lysobacter* sp. (99 % ID) (JF461239 to JF461247). *Lysobacter* sp. was amplified using specific primers for *Pseudomonas.* This indicates that the primers specificity is reduced in presence of high abundance of *Lysobacter* sp.

The sequencing of the band 7 excised from the DGGE gel of the actinobacterial communities revealed *Streptomyces* sp. with 100 % ID (JF461221 to JF461224) in the WCR eggs. The corresponding band in one of the gut samples revealed a bacterial population belonging to the same genus (JF461232 and JF461233). *Rhodococcus* sp. was identified by sequencing of the band 8 with 99-100 % ID (JF461226 and JF461230). The corresponding band in one of the gut samples revealed the genus *Tsukamurella* with 98-100 % ID (JF461231, JF461234 to JF461236).

***Phylogenetic analysis of gut- and egg-associated bacteria of WCR larvae***

In order to investigate whether bacterial communities in the guts of WCR larvae resemble the ones present in WCR eggs a phylogenetic analysis was performed. The analysis comprised cloned 16S rRNA gene fragment sequences derived from gut and egg samples. In addition 16 reference sequences with the highest

similarities to sequences found in the guts and in the eggs of WCR were included. The analysis resulted in five main clusters (Fig. 6): *Alphaproteobacteria*, *Betaproteobacteria*, *Pseudomonas*, *Lysobacter* and *Actinomycetes*. The *Wolbachia* reference sequence (AY007551) clustered together with all *Wolbachia* OTUs identified in the eggs and in the guts. Because some sequences from eggs and from gut were identical to each other we could confirm a parental transmission of this species.

The sequences of the reference strains *Streptomyces graminearum* (EF37143)*, S. flavogriseus* (CP002475), *Pseudomonas lutea* (EU118771) *P. aeruginosa* (GU377209) and *P. putida* (EU834404) clustered with all the corresponding OTUs detected in both egg and gut samples. However, no identical sequences originating from eggs and gut were found. Thus, no parental transmission of *Streptomyces* sp. and *Pseudomonas* sp. could be confirmed. *Pseudomonas aeruginosa* clustered only with sequences from egg samples.

As expected the reference sequences *Herbaspirillum* sp. (EU341291) and *Tsukamurella pulmonis* (AB564289) clustered exclusively with gut samples. The reference sequences of the bacterium endosymbiont of *Mortierella elongate* (AB558492), *Lysobacter daejeonensis* (DQ191178), *L. gummosus* (FN600120) and *L. spongiicola* (AB299978), *Rhodococcus* sp. (AB458522 and AM497794) and *Duganella* sp. (EF592558) clustered exclusively with egg samples.

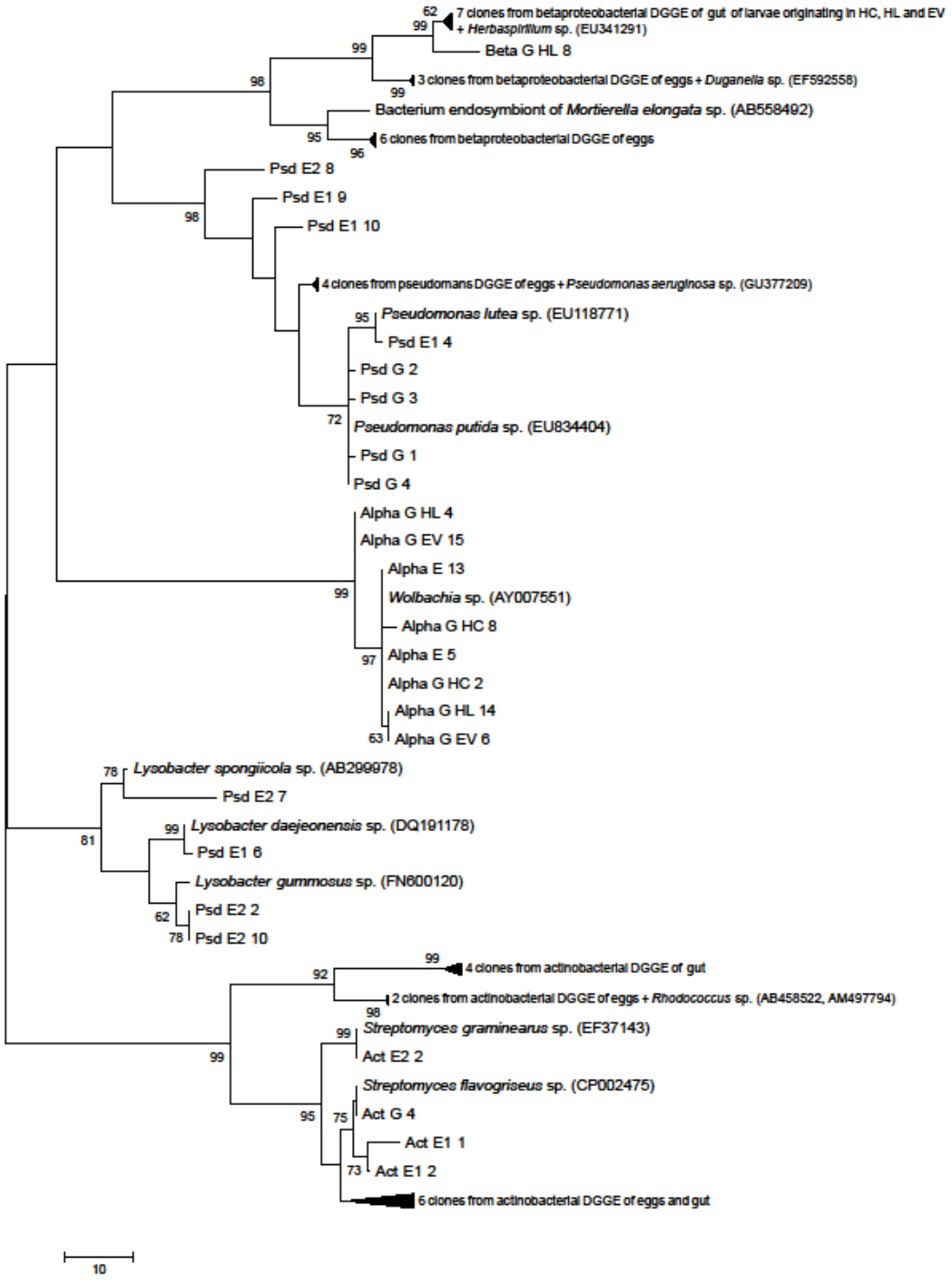



Fig. 6 Maximum parsimony tree (MEGA 4) derived from 16S rRNA sequences isolated from surface sterilized eggs (E) and gut samples obtained from WCR larvae feeding on maize plants grown in different soil types (Gut HC, Gut HL and Gut EV). 500 bootstrap replicates. The branches show bootstrap values higher than 60.

## Discussion

### *ITS-DGGE fingerprinting to investigate the gut flora*

The first aim of this study was to validate a previously published cultivation-independent method for an easy and fast detection of fungal communities in the digestive tract of insects. We proved that ITS-DGGE fingerprinting is a useful technique for typing the gut microflora.

### *Soil type effect on the total fungal and bacterial community composition and dominant microbial population in WCR gut*

The second aim of this study was to investigate the influence of the soil types Haplic Chernozem, Haplic Luvisol and Eutric Vertisol, on the microbial communities inhabiting the gut of WCR larvae. We hypothesized that different soil types, supporting different microbial populations, might modify the gut microflora of WCR larvae feeding on soil-surrounded roots. Surprisingly, our investigations led to the opposite conclusion: the soil type does not affect the fungal and bacterial composition of the intestinal environment. No similar studies have been reported until yet.

This work elucidated also the dominant fungal and bacterial populations intimately associated with the WCR gut. Although the DGGE fingerprinting is not a quantitative method, the dominant microbial populations in the gut samples appear as thick bands in the DGGE gel. Thus, we could report *Fusarium* spp. and *Gibberella zeae* as dominant fungal populations inhabiting the digestive tract of WCR. Molnár *et al*. (2008), investigating the gut flora of the WCR by DGGE of the D1 domain of the 26S rRNA gene for the yeast detection, identified these fungi as well. This indicated that gene fragments of *Fusarium* spp. and *Gibberella zeae* were a-specifically amplified by Molnár *et al*. (2008), most likely due to their high abundance in the gut system. No indication about their dominance in the gut system of WCR was previously reported.

Within the bacterial populations inhabiting the gut of WCR, *Wolbachia* sp. was the dominant one. Several studies revealed in the gut of WCR the presence of *Wolbachia* sp., an intracellular bacterium maternally transmitted to the offspring and responsible for reproductive incompatibilities between infected and uninfected individuals (Giordano *et al*., 1997; Clark *et al*., 2001; Roehrdanz & Levine, 2007). Recently Barr *et al*. (2010) showed that *Wolbachia* sp. colonizing the WCR insect is

responsible for the down-regulation of the maize plant defences suggesting an important role of this microorganism in the pathogenicity of the insect, which needs to be corroborated by additional studies.

DGGE analysis and sequencing of group-specific bacteria allowed us to identify minor populations which are not detectable in the total bacterial communities fingerprinting (Heuer *et al.,* 1997). Our study revealed *Herbaspirillum* sp. as dominant beta-proteobacterial population in WCR gut. *Herbaspirillum* sp. was detected in all gut samples, independently from the soil type in which the larvae were grown. Meyer and Hoy (2008) characterize *Herbaspirillum* sp. as a secondary symbiont in a citrus psyllid. However, our data clearly indicated that *Herbaspirillum* sp. is not transmitted via the eggs and it might have been originated from the rhizosphere in which the larvae were grown. Other studies reported *Herbaspirillum* sp. in the gut of insect (Zouache *et a*l., 2009; Ramírez-Puebla *et al*., 2010). However, no information about their biological role are available.

***Origin of the dominant fungal and bacterial populations in the guts of WCR larvae***

Comparative DGGE analysis of gut, rhizosphere and egg samples together with the sequencing of specific DGGE bands allowed us to investigate the origin of the major microbial populations in WCR gut.

Band “d” (Fig. 2) identified as *Gibberella zeae* in the gut fingerprinting occurred in the rhizosphere patterns as well. Unfortunately, due to the difficulties to sequence specific bands within complex DGGE patterns, we could not confirm that *G. zeae* in WCR gut originated in the rhizosphere. However, because the corresponding band was not found in the DGGE profile of eggs it is reasonable to assume an external environmental origin of this fungus (Fig. 2). To reach final conclusions further investigations are needed. For instance, the use of GFP-labeled fungi might help to better understand the pathway of those fungi from the rhizosphere to the insect’s gut and *vice versa.*

Band “c” (Fig. 2) identified as *Fusarium* spp. in WCR gut fingerprinting occurred in the rhizosphere and egg DGGE as thin band. The phylogenetic analysis of all ITS sequences obtained from gut and egg samples showed that *Fusarium* spp. found in the gut clustered separately for *Fusarium* spp. in the eggs suggesting an external environmental origin of this fungus. Because the pH in WCR midguts is approximately at pH 5.5 (Murdock *et al*., 1987; Wolfson, 1991; Gillikin *et al*., 1992)

and the majority of the *Fusarium* species are tolerant to acid and alkaline pHs, it is reasonable to speculate that the gut conditions of WCR larvae selected this fungus. The plating of gut homogenate of WCR larvae on *Fusarium* selective media revealed *Fusarium* species (Kurtz *et al.*, personal information), indicating that the fungus is viable in the gut of larvae. The finding that *Fusarium* spp. is dominant and viable in the digestive tract of the WCR clearly showed that WCR larvae might be viewed as vectors of potentially mycotoxin-producing *Fusarium* species. Thus, we can explain the increased colonization of maize roots by *Fusarium verticilloides* observed in presence of WCR larval feeding (Kurtz *et al*., 2010).

Band "1" (Fig. 5a and 5b) identified as *Wolbachia* sp. in the gut of WCR occurs as a dominant band only in the egg profiles. Phylogenetic analyses of 16S sequences in the gut and in the eggs of WCR larvae revealed the same *Wolbachia* sp. in both gut and eggs, confirming the maternal transmission of this species to the offspring.

Band "3" (Fig. 5c) identified as *Herbaspirillum* sp. in WCR gut profile occurred also in the rhizosphere and in the WCR egg fingerprinting. Because sequencing of this band from the egg DGGE revealed a bacterial population belonging to the genus *Duganella* we speculated that *Herbaspirillum* sp. in the gut originated either from the rhizosphere, where a band with the same electrophoretic mobility was observed, or from the plant roots. These microbes were identified as nitrogen-fixing endophytes in rice and maize plants (You *et al*., 2005; Balsanelli *et al*., 2010).

***Major fungal and bacterial populations in WCR eggs***

The last objective of this work was to identify the dominant fungal and bacterial populations in surface-sterilized eggs of WCR. The major fungal population identified shared 98 % similarity with *Mortierella gamsii* (*Zygomycota*). The relative high abundance of this fungus suggested an important role in the WCR biology which might be a matter of further investigations.

The dominant bacterial population identified in WCR eggs was *Wolbachia* sp. The role of *Wolbachia* sp. in WCR was discussed above. Together with *Wolbachia* sp., the eggs harbored *Duganella* sp., and a second beta-proteobacterial population which showed 96 % similarity with a bacterial endosymbiont of *Mortierella elongate*. The low sequence similarity with the bacterial endosymbiont of *Mortierella elongata* precluded a clear taxonomic identification, thus we suggest this to be a novel species. However, the finding of *Mortierella gamsii* as a dominant egg-associated

fungus suggested an interesting insect-fungi-bacteria interaction that should be studied in further experiments.

The other dominant bacterial populations identified in WCR eggs were *Pseudomonas aeruginosa, Lysobacter* sp., *Streptomyces flavogriseus, S. graminearum* and *Rhodococcus koreensis.* Several papers reported these microorganisms in the gut of earthworms (Fisher *et al*., 1995; Toyota & Kimura, 2004) or termites (Pasti *et al*., 1990). However, their biological role in WCR larvae is still unknown.

***Conclusions***

To conclude, the soil type does not influence bacterial and fungal communities in the gut of WCR larvae; relatively simple communities dominated for fungi by *Fusarium* spp. and *Gibberella zeae,* and for bacteria by *Wolbachia* sp. and *Herbaspirillum* sp. The WCR gut is a highly selective environment. The finding of *Fusarium* spp. in WCR gut is important in view of spreading potential mycotoxin-producer fungi. The major fungal populations identified in the eggs and potentially amenable to future manipulation were *Wolbachia* sp. and *Mortierella gamsii.*

**Acknowledgments**

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**Supplemental information**

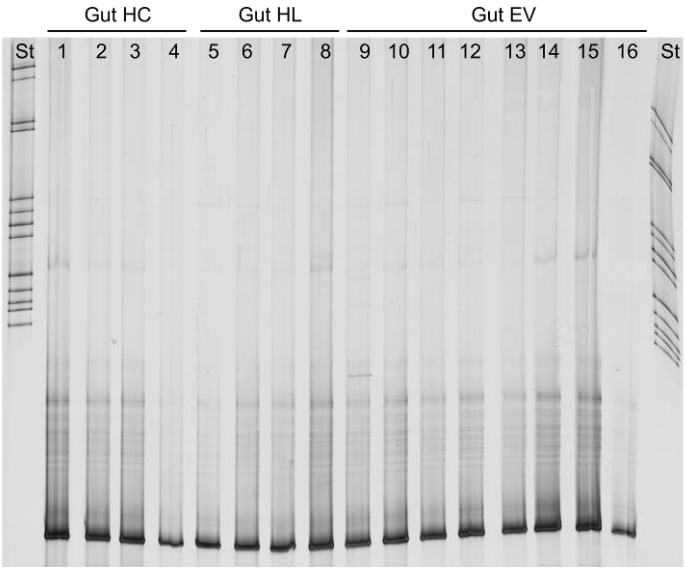


Fig. S1. 18S-DGGE fingerprins obtained from single gut of WCR larvae grown in Haplic Chernozem (GutHC: lane 1 to 4), in Haplic Luvisol (GutHL: lane 5 to 8) and in Eutric Vertisol (GutEV: lane 9 to 16). St: 18S-standard.

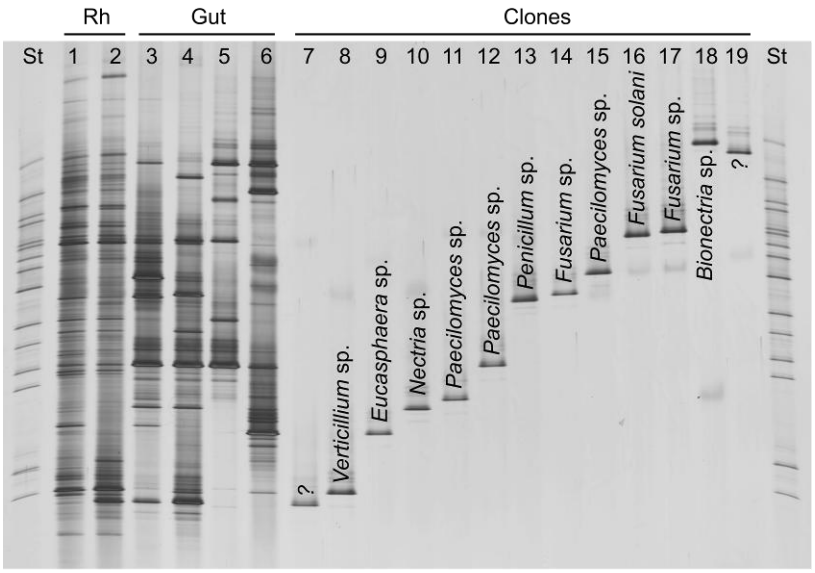


Fig. S2. ITS-DGGE fingerprints of the fungal communities in the rhizosphere of maize plants grown in Haplic Chernozem (Rh: lane 1 and 2), in single gut samples obtained from WCR larvae feeding on maize plants grown in the same soil type (Gut: lane 3 to 6), and DGGE profiles of cloned ITS fragments from single gut samples (clones: lane 7 to 19). The fungi identified by sequencing and blast analysis of cloned ITS fragments are reported above the corresponding DGGE band in the figure.

# Chapter VI

## General discussion and main findings

The Western Corn Rootworm (WCR, *Diabrotica virgifera virgifera* LeConte) is an important maize pest in North America and Europe. Larvae feed on the roots of maize plants causing bent stalks, plant lodging and yield reduction. This herbivorous insect showed a high adaptability to prevailing pest management strategies such as annual crop rotation with soybean (Gray *et al*., 2009) or WCR-resistant transgenic plants (Gassmann *et al.*, 2011). New or adapted pest management strategies urgently require a better understanding of the multitrophic interaction in the rhizosphere and endorhiza of maize.

The objectives of this thesis were:

- To investigate the effects of the root larval feeding of the WCR on the microbial communities;
- To study the complex interactions among WCR, *Glomus intraradices* (*G.i*.) and microbial communities in the rhizosphere and endorhiza of maize plants;
- To assess the effect of the soil type on the fungal and bacterial communities inhabiting the digestive tract of WCR larvae;
- To investigate the dominant microorganisms associated with gut and eggs of WCR, and their transovarial transmission.

These objectives have been assessed in the following chapters.

In **chapter 3** we investigated the effect of the root feeding of WCR larvae on microbial communities living in the maize rhizosphere. Rhizospheric microorganisms influence the plant fitness and the ecosystem functionality (Hayat *et al*., 2010). We hypothesize that different soil types and different maize cultivars support different fungal and bacterial communities in the rhizosphere which might affect WCR larval feeding and development. Larval feeding in turn could induce microbial community changes. The effect of different soil types on the larval feeding and development was investigated by Benedikt Kurtz (PhD thesis, University of Göttingen, 2010), while larval feeding effects on the microbes living in the rhizosphere was the main object of this chapter. In a greenhouse experiment maize genotypes KWS13, KWS14, KWS15 and MON88017 were grown in three different soil types in

presence and in absence of WCR larvae. Bacterial and fungal community structures were analyzed by DGGE of the16S rRNA gene and ITS fragments which were PCR amplified from the total rhizosphere community DNA. Differentiating 16S-DGGE bands were excised from the gel, cloned and sequenced in order to identify specific bacteria responding to WCR larval feeding. 16S- and ITS-DGGE analysis showed that WCR larval feeding affected the fungal and bacterial populations inhabiting the maize rhizosphere in a soil type and plant-genotype dependent manner. DGGE band sequencing revealed an increased abundance of *Acinetobacter calcoaceticus* in the rhizosphere of several maize genotypes in all soil types. *Acinetobacter calcoaceticus*, was recently described as a phenol degrading microorganism. Our findings suggest that phenolic compounds released upon WCR wounding led to the observed bacterial community changes. The consequences of such shifts on the rhizosphere microbes induced by WCR larval feeding remain to be explored.

In **chapter 4** we elucidated the complex interactions among WCR, *G.i.* and microbial communities in the rhizosphere and endorhiza of maize plants. Because the arbuscular mycorrhizal fungi (AMF)s are well known to influence plant-insect interactions (reviewed by Gehring & Bennett, 2009), we hypothesized that *G.i.* affects the larval fitness together with the community structure of the rhizospheric- and root-associated microorganisms. We also hypothesized that the WCR larval feeding alters the root-associated microorganisms. In order to test our hypothesis, four treatments were established: (C) the control: maize plants grown for 9 weeks in Haplic Chernozen soil (plant growth stage VT); (W): maize plants injected with ca. 200 non diapausing WCR eggs after 6$^{th}$ week of plant growing (plant growth stage V7); (G): maize plants grown in *G.i*.-inoculated soil; (GW): maize plants grown in *G.i*.-inoculated soil and injected with ca. 200 WCR eggs after 6$^{th}$ week of plant growing. After 20 days, larval number, developmental stage and root feeding of WCR were measured. Root colonization level by AMFs was estimated by microscopy. Dominant AMF species in soil and endorhiza were analysed by cloning of 18S rRNA gene fragments amplified from total community (TC) DNA, restriction fragment length polymorphism (RFLP) and sequencing. Quantitative Real Time-PCR was used to quantify *G.i.* in the roots. Bacterial and fungal communities in the rhizosphere and endorhiza were investigated by DGGE of 16S rRNA gene and ITS fragments, PCR amplified from total community DNA extracted from rhizosphere and root material.

This work first showed inhibitory effects of the WCR larval development caused by the G.i. root mycorrhization. The effect observed was correlated to a *G.i.*-mycorrhization level of plant roots before WCR larval attack of about 9.5 $x10^5$ copy numbers of 18S/ITS fragments of *G.i*. per g root. *G.i.* affected mainly the fungal communities in the endorhiza. Populations of *Glomus* sp. were shifted in the maize roots. Less pronounced effects, although significant, were found also on the bacterial communities living in the endorhiza and in the rhizosphere of maize. In contrast, WCR feeding did mainly influence the bacterial communities in the rhizosphere and to a lesser extent in the endorhiza. No WCR effect on the endophytic and rhizospheric fungal communities was observed.

In conclusion, G.i. might be used in integrated pest management as it can delay larval development rendering WCR larvae more susceptible to predation by natural enemies. The mechanisms of this interaction remain unknown. However, our data showed that G.i. altered the interactions between the plant and the endophytic fungal and bacterial communities which might interfere with the WCR larval development. Thus, *G.i* could contribute to the control of WCR larvae either directly or indirectly through shifts in the endophytic microbial communities via plant-mediated mechanisms.

In **chapter 5** we investigated the effect of the soil type on the gut microbiome of WCR larvae. Microorganisms inhabiting the digestive tracts of insects can play important roles in the nutrition, development, survival, resistance to pathogens and reproduction of the insect host (Eutick *et al*. 1978, Fukatsu & Giordano *et al*., 1997; Brand *et al*., 1975; Brune, 2003; Moran *et al*., 2005). The genetic manipulation of microorganisms intimately associated with the insect gut is a novel approach to manage insect pests (Dillon *et al*., 2004; Riehle & Lorena, 2005; Douglas, 2007).

Different soil types can support different microbial communities. Because the larvae are feeding on maize root with tightly attached soil particles, we hypothesized that different rhizospheric microorganisms can be ingested from the external environment, modifying the gut microflora. DGGE technique and sequencing of ITS regions and 16S-rRNA gene fragments, PCR-amplified from total community DNA from gut of larvae grown in three soil types, were used to investigate the fungal and bacterial communities, respectively. In the same chapter we investigated the most dominant gut- and egg-associated microorganisms by DGGE technique and band sequencing. Comparative DGGE fingerprints and sequencing of microbial

communities in the gut and egg samples were used to investigate their transovarial transmission. Last but not least, we showed that ITS- DGGE fingerprinting is a useful technique for typing the fungal microflora of the WCR larvae.

This work showed that ITS-DGGE allows the characterization of the fungal communities inhabiting the digestive tract and eggs of WCR. Furthermore, we first reported that the dominant gut-associated fungi and bacteria of WCR larvae are not influenced by the soil type. The fungi *Fusarium* spp. and *Gibberella zeae* were dominant in the gut system and originated most likely from the external environment (rhizosphere or plant material). We speculated that those fungi can be enriched under the alkaline conditions of the WCR gut. We suggested that WCR larvae could serve as vectors of *Fusarium* spp. and *Gibberella zeae*. Within the bacteria, *Wolbachia* sp. and *Herbaspirillum* sp. were dominant in WCR gut. A transovarial transmission was observed only for *Wolbachia* sp., while *Herbaspirillum* sp. might have originated in the maize rhizosphere. In the eggs the bacterium *Wolbachia* sp. and fungus *Mortierella gamsii* were dominant.

To conclude, the soil type does not influence bacterial and fungal communities in the gut of WCR larvae; relatively simple microbial communities dominated the WCR gut: *Fusarium* spp. and *Gibberella zeae* were dominant within the fungi*,* while *Wolbachia* sp. and *Herbaspirillum* sp. were dominant within the bacteria. These findings suggested that the WCR gut is a highly selective environment for the microorganisms and that WCR larvae are vectors of mycotoxin producer-fungi. The major microbial populations identified in the eggs and potentially amenable to future manipulation were *Wolbachia* sp. and *Mortierella gamsii.*

**Main findings and conclusions**

The research described in this thesis revealed that:

- The root feeding of WCR larvae strongly affects the bacterial communities in the rhizosphere of maize. An increased abundance of the bacterial population *Acinetobacter calcoaceticus* in presence of WCR larvae was observed. Because this bacterium was recently described as a phenol degrading microorganism we suggested that the roots release, upon WCR wounding, phenolic compounds.

- WCR larval feeding does not affect the endophytic microbial populations.

- *G.i*. reduce the WCR larval development. Thus, G.i. might be used in integrated pest management of WCR.

- Shifts of the bacterial and fungal community composition in the rhizosphere and in root of *G.i.*-treated plants suggested that *G.i* could contribute to the control of WCR larvae either directly or indirectly through shifts in the endophytic microbial communities via plant-mediated mechanisms.

- The soil type does not influence bacterial and fungal communities in the gut of WCR larvae.

- Relatively simple microbial communities dominated the WCR gut: *Fusarium* spp. and *Gibberella zeae* were dominant within the fungi, while *Wolbachia* sp. and *Herbaspirillum* sp. were dominant within the bacteria. These findings suggested that the WCR gut is a highly selective environment for the microorganisms and that WCR larvae are vectors of mycotoxin producer-fungi.

- The major microbial populations identified in the eggs and potentially amenable to future manipulation were *Wolbachia* s*p*. and *Mortierella gamsii.*

In conclusion, the results acquired in this thesis provided additional insight into the multitrophic interaction among WCR larvae and rhizospheric- and root-associated microorganisms of maize plants. Furthermore, the potential role of Glomus intraradices as biocontrol agent of WCR larvae has been shown. Last but not least, a better knowledge of the gut and egg microbiota of WCR was acquired.

## References

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***Curriculum vitae***

Flavia Dematheis was born on the 4th June 1978 in Torino, Italy. After graduating high school in 1997, she started her M.Sc in plant biotechnology at Torino University. The main objectives of her M.Sc. thesis were the molecular characterization and the phylogenic analysis of *Fusarium oxysporum* f.sp. *lactucae.* During her studies she had a granted scholarship to develop a diagnostic preventive method for an easy detection of *Fusarium oxysporum* f.sp. *lactucae* on seed. In 2006 she worked "for fun" on the Department of aquaculture in Grugliasco (Torino) to molecular characterize trout populations in order to *restock* Italian rivers with *native species* of fish.

After the Mrs degree in biotechnology she had a one-year fellowship by the Centre of Competence for the Innovation in the Agro-Environmental Field (AGROINNOVA) of Grugliasco to molecular characterize specific strain of the yeast *Metschinikowia pulcherrima*, showing high biocontrol capability against phyto-pathogenic fungi.

In April 2007 she started her PhD in microbiology field in the Federal Research Centre for Cultivated Plants (JKI), Institute for Epidemiology and Pathogen Diagnostics, in Brauschweig (Germany). Her work was an interdisciplinary study between microbial ecology, plant pathology and entomology. The research aimed to investigate the effects of the root feeding caused by the pest of maize, *Diabrotica virgifera virgifera,* on the fungal and bacterial communities living in the rhizosphere and in the endorhiza of maize. The potential effect of *Glomus intraradices*-mycorrhized plants on the fitness of this soil-dwelling pest and on the microorganisms in the root zone (rhizosphere and endorhiza) was elucidated. Last but not least, her PhD work focused on the identification of the gut and egg-associated microorganisms of the insect.

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