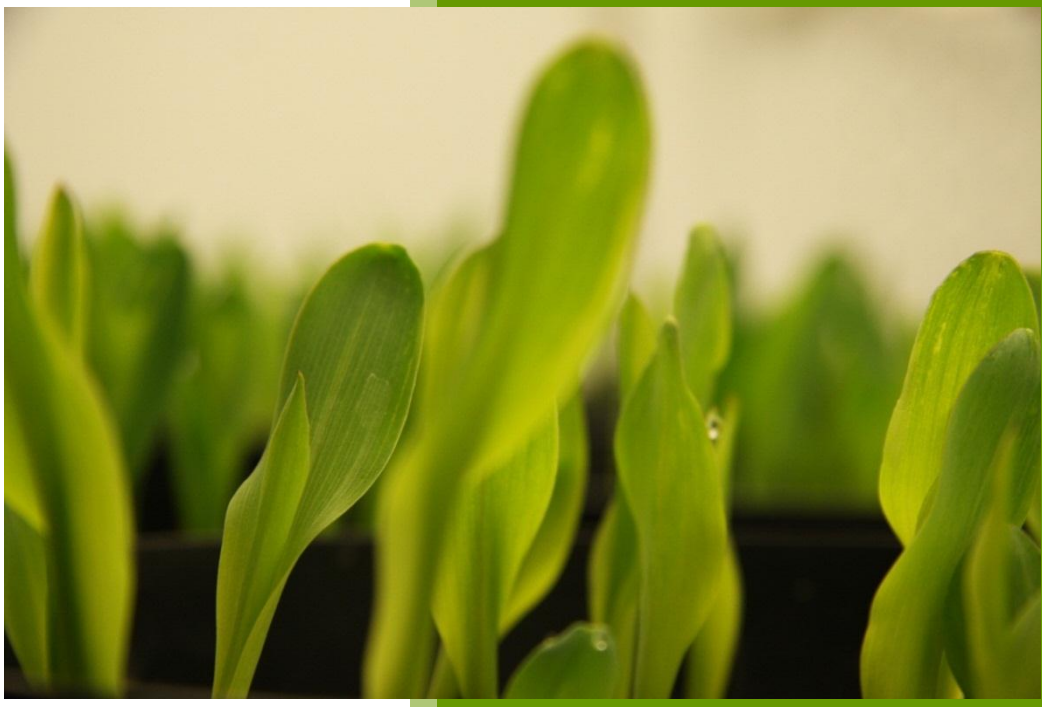
Direct and indirect effects of the rhizobacteria *Pseudomonas putida* KT2440 on maize plants



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"Direct and indirect effects of the rhizobacteria *Pseudomonas putida* KT2440 on maize plants"

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Summary

To protect themselves against biotic and abiotic stresses, plants have developed a broad range of defense mechanisms that are constitutively present or that can be induced in response to a stress. Such induced defense can be the result of a resistance elicitation by non-pathogenic organisms that are present in soil and is then called induced systemic resistance (ISR). ISR confers plant resistance against a large variety of attackers such as pathogens and herbivores. In monocotyledonous plants this phenomenon has been less studied than in dicots such as Arabidopsis. Hence, the aim of this thesis was to enhance our knowledge on ISR in monocots and more specifically in maize plants.

To facilitate plant root manipulation for our experiments, we established a soil-free system for growing maize plants. Based on an existing system for root observation, we adapted a system that is convenient for working with beneficial as well as pathogenic microbes.

ISR establishment necessitates the local recognition of the beneficial microbe by the plant. Thus, we studied the reaction of maize after inoculation with a well-known maize colonizer rhizobacterium *Pseudomonas putida* KT2440. The presence of these bacteria activated plant immunity early in the interaction. We hypothesized that KT2440 manipulates root defense to be able to colonize roots. We observed that KT2440 had a beneficial effect on plant growth, showing their capacity to be a plant growth promoting rhizobacteria.

After analyzing the local plant response to KT2440 inoculation we tested the efficiency of KT2440 to induce a systemic defense against various types of attacks. We demonstrated that ISR triggered by KT2440 was efficient against an hemibiotrophic fungus, *Colletotrichum graminicola*, and a generalist herbivore, *Spodoptera littoralis*. However, the efficacy of ISR induced by KT2440 was dependent of the host-plant specialization of the leaf herbivores as ISR triggered by KT2440 did not affect the specialist *S. frugiperda*. Our transcript and metabolite analyses revealed the involvement of phenolic compounds as well as ethylene-dependent signaling in maize ISR. However, mechanisms involved in ISR induced by KT2440 in maize remain to be further investigated.

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CHAPTER 1 Introduction

Induced resistance in monocots

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Abstract

Plants have evolved elaborate mechanisms to recognize the different type of stress they have to deal with and to specifically defend themselves against these threats. Plant defense mechanisms that are induced during pathogenic or beneficial interactions, such as systemic acquired resistance or induced systemic resistance have been in the focus of interest for a long time but most studies were conducted with dicotyledonous plants such as cucumber, tobacco or in recent years with Arabidopsis. Monocots, including the economically important cereals, did not receive the attention they deserve within this field of research. This has led to a gap in knowledge about induced resistance (IR) in monocots that is however rapidly closing thanks to the availability of modern technologies allowing tackling novel questions in an efficient and quite rapid manner. Here, we present recent advances in IR against biotic and abiotic stresses in monocots.

Introduction

Plants are constantly challenged by their environment. Biotic stress due to microbial pathogen attack or herbivores can threaten a plant's survival. Abiotic environmental factors such as drought or salinity can also negatively influence a plant's fitness. To counteract these stresses and to ensure reproduction, plants have evolved sophisticated tools to defend themselves. Physical barriers and chemical defense substances constitutively present in the plant represent a first level of defense that is not specific for a given aggression. If these defense layers are overcome by the aggressor, plants then have the capacity to induce specific defenses in response to the stress factor. Following recognition of the type of attack, biochemical signaling cascades are activated that can result in local and systemic induced resistance. It has been known for over a century that plant defenses can be preconditioned. In 1961, for the first time, Ross introduced the concept of localized acquired resistance (LAR, Ross 1961a) and systemic acquired resistance (SAR, Ross 1961b). He showed that tobacco plants were more resistant against Tobacco mosaic virus if they were previously challenged locally (on the same leaf) or systemically (on a distant leaf) with the same necrotrophic pathogen. Since then, the term SAR has been extended to resistance observed upon a previous pathogen infection or a treatment with chemical SAR-inducers that fulfill specific criteria as described by Uknes et al. (1992). Plant defenses can also be associated with the colonization of roots by certain beneficial microorganisms. This is called induced systemic resistance (ISR). ISR can be triggered by plant growth promoting rhizobacteria (PGPR), endophytic fungi or mycorrhiza. Commonly, the inducible defense networks are regulated pivotally by phytohormones, which serve as specific chemical signals (Balmer and Mauch-Mani 2012). ISR is mediated by a jasmonate (JA)- and ethylene (ET)-sensitive pathway and SAR is mediated by a salicylic acid (SA)-dependent pathway and is associated with the production of pathogenesis related (PR) proteins. ISR is mainly counteracting necrotrophic pathogens and pests (Van Loon 2007), whereas SAR is mainly effective against biotrophic pathogens (Vlot et al. 2008). SAR and ISR confer a plant protection against a wide variety of attackers. In addition to their effect against biotic stresses, some chemicals and ISR-inducers are potentially capable of protecting against abiotic stress, leading to induced systemic tolerance (IST).

In the last decades, research on SAR and ISR was performed mostly on dicotyledonous plants, such as Arabidopsis, tobacco, cucumber and tomato. The further understanding of the IR mechanisms promoted the development of bacterial formulations and synthetic inducers for use in agriculture. Although monocotyledons include economically important plants, mainly for food or ornamental industries (Table 1), the knowledge about induced resistance in these plants is still elusive (Kogel and Langen 2005). A better understanding of SAR and ISR in monocots such as cereals could help to improve crop management in a sustainable manner. In this chapter, we present the current knowledge of IR against biotic as well as abiotic stresses in monocots, from the plant recognition to the systemic response upon a stress.

Table 1. Examples of economically important monocotyledonous plants

Family	Members (examples)	Uses
Amaryllidaceae	Amaryllis, daffodil	Ornamentals
Arecaceae	Oil palm, date palm, coconut	Food
Bromeliaceae	Pineapple, bromelia	Food, ornamentals
Liliaceae	Lily, tulip, onion, garlic, leek, asparagus	Food, ornamentals
Musaceae	Banana	Food
Orchidaceae	Orchids, vanilla	Ornamentals, spices
Poaceae	Cereals, sugarcane, turfgrass, native grass, bamboo	Food, ornamentals, wood
Zingiberaceae	Ginger, curcuma	Spices

Plant stress recognition and innate immunity

Appropriate classification of given threats is crucial for plant survival. Plants perceive abiotic stresses through physical properties of the plasma membrane such as the lipid or fatty acid composition. However, these signal receptors remain to be identified (Huang et al. 2012). Plants are equipped with cellular tools to perceive pathogen-derived structures, allowing an induction of specific defense mechanisms adapted to an attacker (Spoel and Dong 2012). The initial defense response takes places at the frontline of plant-attacker interaction: plants recognize pathogen- or microbe-associate molecular patterns (PAMPs/MAMPs) such as flagellin or chitin with the help of pattern recognition receptors (PRRs) that are located at the plasma membrane (Zipfel 2009). MAMP-perception leads to diverse downstream responses such as calcium influx, oxidative burst or the activation of transcription factors, a resistance-mediating process commonly named “PAMP-triggered immunity” (PTI). As dicots and monocots both encounter similar enemies including bacteria, fungi and herbivores, the PRRs of both families are likely to be conserved. Consequently, the Arabidopsis receptor-like kinase flagellin insensitive 2 (FLS2, Chinchilla et al. 2007), which perceives amino acids from bacterial-derived flagellin, has been found in rice (Takai et al. 2008). FLS2 homologues are confirmed in all higher plants where genomic data is available. In addition, monocots are also known to perceive bacteria via lipopolysaccharides from their cell wall (Desaki et al. 2006), or bacterial proteins such as Ax21, a 194-amino acid protein conserved in *Xanthomonas* strains (Lee et al. 2009). Ax21 is recognized by the plant receptor XA21. *Xa21* homologues are present in different *Poaceae* such as rice, maize, sorghum and Brachypodium (Tan et al. 2012). Additional examples of defense-related receptor kinases are RPG1 in barley (Chen et al. 2006) or Yr36 in wild wheat (*Triticum turgidum* L. ssp. *graminis*). Moreover, monocots are also able to recognize elicitors secreted from non-pathogenic

rhizobacteria, for example exopolysaccharides from *Pantoea agglomerans* (Ortmann and Moerschbacher 2006), or pyocyanin produced by *Pseudomonas aeruginosa* 7NSK2 (De Vleesschauwer and Höfte 2009). Both factors trigger ISR in wheat and rice, respectively. However, the mechanism of perception has yet to be elucidated for these elicitors. To counter fungi, plants are capable of sensing chitin. In rice, this is mediated by the plasma membrane glycoprotein CEBiP and the chitin elicitor receptor kinase 1 (CERK1). Both dimerize together to induce downstream defense signaling cascades such as the generation of reactive oxygen species (ROS) and the expression of *PR* genes (Shimizu et al. 2010).

Beside PAMPs and MAMPs, plants are also able to perceive damage-associated molecular patterns (DAMPs) during pathogen and pest attacks. DAMPs can be composed of polysaccharides that are released from destroyed cell walls, or they can be endogenous peptides. Recently, an ortholog of AtPep1, an endogenous elicitor against pathogens, has been discovered in maize (Huffaker et al. 2011), indicating that DAMP-mediated signaling is conserved in both monocots and dicots. Similarly, plants are also able to recognize herbivores by perceiving herbivore-associated molecular patterns (HAMPs). An example of a HAMP in monocots is volicitin, a hydroxyl fatty acid-amino acid conjugate present in the oral secretions (OS) of *Spodoptera exigua* (Alborn et al. 1997). Volicitin triggers the induction of volatile defense compounds in maize. Maize cells perceive volicitin with the help of a plasma membrane protein (Truitt et al. 2004). Other well-described HAMPs are caeliferins, disulfooxy fatty acids derived from *Schistocerca americana* OS which are triggering IR in maize (Alborn et al. 2007).

Despite being recognized by plant cells, successful pathogens and pests are competent in overcoming the first defense lines. By delivering avirulence (Avr) proteins, also called effectors, into plant cells, bacteria, fungi, oomycetes and pests can suppress PTI through the manipulation of host cellular defense mechanisms in favor of a subsequent infection. For example, the pathogen Blumeria graminis f. sp. hordei utilizes AVRK and AVRA10 proteins to suppress PTI in barley cells (Ellis et al. 2007). In turn, plants employ the secondary step, which takes places mostly in the cytoplasm. There, nucleotide-binding leucine rich repeat (NB-LRR) proteins (Elmore et al. 2011), which are encoded by plant resistance (R)-genes, recognize and attenuate pathogen effectors, resulting in an effector-triggered immunity (ETI, Jones and Dangl 2006). Some NB-LRRs can directly recognize pathogen molecules (effectors), while other R genes indirectly recognize metabolic perturbations due to the presence of the pathogen, thus triggering ETI. This mechanism usually includes the generation of ROS and the activation of TGA and WRKY transcription factors (Jones and Dangl 2006).

Genes encoding NB-LRRs are amongst the largest and widest conserved gene families in plants (Jones and Dangl 2006). In rice, NB-LRRs that confer resistance to *Magnaporthe grisea* are well described (reviewed in Chen and Ronald 2011). NB-LRRs also promote resistance to herbivores, such as Bph14 that is active against effectors from the brown planthopper (Du et al. 2009). Some *R* genes in rice do not exhibit NBS or LRR domains, as

shown for xa13 (Antony et al. 2010). Despite the large number of known NB-LRRs in rice, most of their targets are unknown. Only a few *M. grisea* effectors that are targeted by NB-LRRs are described, such as AvrPiz-t (Shang et al. 2009). In other monocot species than rice, including *Zea mays*, *Sorghum bicolor* and *Brachypodium distachyon*, conserved NB-LRR genes were found (Kim et al. 2012).

In conclusion, PTI and ETI mechanisms are conserved in monocots and dicots. Although they have been extensively studied in the dicot model plant *Arabidopsis thaliana*, recent advances in high-throughput genomics helped gathering more knowledge about this first line of inducible defenses in monocots. Identification of novel NB-LRR gene clusters represents a promising tool for future crop breeding programs.

Systemic acquired resistance (SAR)

In case of a local inoculation with leaf pathogens, which generate a hypersensitive response by the plant, a long-lasting and broad-spectrum disease resistance is observed in distal plant parts. This phenomenon, called SAR, has been widely studied in the dicotyledonous model plants Arabidopsis and tobacco but information on SAR in monocots is scarce. Only a few reports describe biological SAR phenomena in monocots. Infection of rice by *P. syringae* pv. *syringae* led to a systemic resistance against *M. grisea* (Smith and Métraux 1991). In *Lilium formonasum*, a previous infection with *Botrytis elliptica* suppressed a secondary infection with the same pathogen in systemic tissues (Lu et al. 2007). In wheat, SAR against stem and leaf rust has been noted (Barna et al. 1998). Some pathogen-derived proteins that are responsible for mediating SAR in monocots have been identified. PemG1, from the fungal pathogen *M. grisea*, and fragments of HpaG(Xooc), a harpin from *Xanthomonas oryzae* pv. *oryzicola*, were shown to induce disease resistance in rice plants against *M. grisea* (Peng et al. 2011; Chen et al. 2008).

Various synthetic chemicals can mimic the biological activation of SAR by necrotrophic pathogens such as INA (2,6-dichloroisonicotinic acid), BTH (*S*-methyl benzo-1,2,3-thiadiazole-7-carbothioate) and probenazole (3-allyloxy-1,2-benziso-thiazole-1,1-dioxide). Chemical SAR inducers were also found to be active in monocots, such as BTH and INA in maize (Morris et al. 1998) and BTH in wheat (Görlach et al. 1996) and in barley (Faoro et al. 2008). BTH was also reported as a SAR inducer in non-cereal monocots such as in banana against *Colletotrichum musae* (Zhu et al. 2007), in curcuma against *Pythium aphanidermatum* (Radhakrishnan et al. 2011) and against *Colletotrichum falcatum* in sugarcane (Ramesh Sundar et al. 2005). Probenazole is a well-known SAR-inducer for rice plants. Oryzemate (trade name of probenazole) has been used against rice blast and bacterial blight for years. Despite its wide use in disease control, mechanisms underlying this resistance still need to be understood. A recent study shows that probenazole treatment did not alter SA level in plants. However, treated plants showed a strong up-regulation of

OsSGT1, that is responsible for SA conversion into SA-*O*-*β*-*glucoside* (SAG), which can be converted back to SA when needed (Umemura et al. 2009). Probenazole is also efficient in other monocots, for example in *Lilium formonasum* against *Botrytis elliptica* (Liu et al. 2008). Saccharin, a metabolite of probenazole, is also known as SAR-inducer in dicots as well as in monocots such as rice or barley (Srivastava et al. 2011).

In Arabidopsis, several mobile signals involved in systemic resistance have been described such as methyl salicylate (MeSA; Park et al. 2007) and azelaic acid (Jung et al. 2009). In monocots, the presence of MeSA and azelaic acid in maize leaves and roots was detected but their role in SAR still needs to be elucidated (Balmer and Mauch-Mani, unpublished). SAR is generally associated with SA accumulation that leads to the activation of the transcription factor activator NPR1 (nonexpressor of PR genes). NPR1 triggers extensive changes in the defense gene transcriptome (Maleck et al. 2001). NPR1 has been confirmed in all monocots where genomic data are available (Kogel and Langen 2005). In rice, overexpression of *AtNPR1* (Chern et al. 2001) resulted in an enhanced resistance to *Xanthomonas oryzae* pv*. oryzae*. Transcriptomic analysis showed that *OsNPR1* is dominantly involved in the down-regulation of genes and in the SA-mediated suppression of ABA-responsive genes (Sugano et al. 2010). The role of SA during SAR in monocots has yet to be elucidated. Rice contains high endogenous levels of SA (Silverman et al. 1995) and pathogen infection does not lead to increased levels. However, transgenic rice plants expressing the SA-degrading enzyme salicylate hydroxylase (NahG) exhibit a diminished resistance against *M. grisea* (Yang et al. 2004), although *PR* gene expression profiles were found to be unaltered. The role of SA in other monocot models is less studied. Some reports on wheat and barley showed a LAR, in which SA levels were found to be unaffected (Thordal-Christensen and Smedegaard Petersen 1988, Jørgensen et al. 1998). In contrast, a recent study of *P. syringae* pv. *tomato*-induced LAR in barley demonstrated extensive homology between gene expression profiles during LAR in barley and SAR in Arabidopsis (Colebrook et al. 2012). Moreover, leaf and root infection of young maize plants with the hemibiotrophic fungus *Colleotrichum graminicola* triggers local and systemic PR gene induction and SA accumulation (Balmer and Mauch-Mani, unpublished results), suggesting that the role of SA in monocots is ambiguous.

Typical gene markers of SAR in dicots are *PR1* and *PR5,* which are up-regulated during a SAR response. Similarly to Arabidopsis, BTH-treatment of maize triggers the accumulation of PR proteins such as PR1 and PR5 (Morris et al. 1998). In barley, resistance induced by a combination of defense elicitors (BTH, ß-aminobutyric acid and cis-jasmone) was associated with an up-regulation of *PR1-b* (Walters et al, 2011). Monocot and dicot PR protein sequences were found to share extensive similarities. However, when performing an unrooted phylogenetic tree analysis using *PR1* homologues from different species, dicot *PR1* genes grouped together in a cluster distant from monocot sequences (Lu et al. 2011). Thus, *PR1* likely underwent major changes after the monocot-dicot separation.

SAR is a general plant defense mechanism observed in both monocots and dicots. However, the signaling process during SAR is highly conditional, corroborated by the lack of reproducibility by independent laboratories (Kogel and Langen 2005). SAR phenomena depend on multiple factors such as type of attackers, age of plants and growth conditions. The timing for SAR establishment varies and can take up to several days, depending on the SAR system (Faoro et al. 2008). Moreover, a field assay conducted with several barley cultivars showed that SAR efficiency also varies among cultivars (Walters et al. 2011). Thus, particular molecular or chemical SAR factors have to be specifically assessed for a given pathosystem, which might explain the differing mode of action of certain SAR regulators between dicots and monocots.

Induced systemic resistance (ISR)

Plant resistance can be systemically induced by the presence of beneficial root-colonizing microorganisms such as plant growth promoting rhizobacteria (PGPR), endophytic fungi or mycorrhiza (De Vleesschauwer and Höfte 2009; Pozo and Azcón-Aguilar 2007). ISR enhances the capacity of the plant to resist against a broad spectrum of attackers, mostly necrotrophic pathogens and chewing insect herbivores (Pineda et al. 2010). Studies on ISR were predominantly conducted on model dicots such as Arabidopsis or tomato. However, different beneficial microorganisms are known to induce systemic resistance in monocotyledonous plants as well. Table 2 indicates the most representative PGPR species that trigger ISR in monocots. ISR has been reported to be efficient in monocots against a wide range of attackers: viral (Harish et al. 2009), bacterial (Chithrashree et al. 2011) and fungal (De Vleesschauwer and Höfte 2009) pathogens, insect herbivores (Saravanakumar et al. 2007) and parasitic nematodes (Elsen et al. 2008). In addition to cereals, ISR was also demonstrated in non-cereal monocot plants: banana (Sangeetha et al. 2010), orchid (Wu et al. 2011), lily (Liu et al. 2008), date palm (El Hassni et al. 2004), onion (Karthikeyan et al. 2005), gladiolus (Shanmugam et al. 2011) and grasses (Crawford et al. 2010).

ISR mechanisms in dicots usually include the generation of ROS and the production of defense compounds such as defense proteins or phytoalexins. ROS accumulation and phenolic compound formation was observed for ISR in rice plants after infection with *M. oryzae* (De Vleesschauwer et al. 2008 and 2009). Different studies conducted on cereals (rice and wheat) associated ISR with an increased accumulation of plant defense proteins such as chitinase and peroxidase at the site of pathogen infection (Chithrashree et al. 2011; Saikia et al. 2006; Sari et al. 2008) or herbivore attack (Saravanakumar et al. 2007). The presence of *Bacillus pumilus* SE34 or *B. subtilis* GB03 in rice plants induced resistance against the bacterial pathogen *Xanthomonas oryzae* pv. *oryzae* via an increased activity of phenylalanine ammonia lyase (PAL), an enzyme involved in the formation of plant defense compounds such as phenolics or phytoalexins (Chithrashree et al. 2011). Benzoxazinones such as DIMBOA (2,4-dihydroxy-7-methoxy-2H-1,4-benzoxazin-3(4H)-one) are well-known phytoalexins that

are specific to cereals. A recent study showed that accumulation of DIMBOA was induced by the presence of mycorrhiza in maize roots and that its content is enhanced during ISR against a fungal infection (Song et al. 2011).

Table 2. Examples of the widely represented PGPR species that induce ISR in monocotyledons against pathogens and herbivores

PGPR species	Plant species	Plant attackers	References
Pseudomonas fluorescens	Rice	*Cnaphalocrocis medinalis* *Rhizoctonia solani* *Magnaporthe oryzae*	Saravanakumar et al. 2007 Radjacommarea et al. 2004 De Vleesschauwer et al. 2008
	Wheat	*Fusarium graminearum* *Gaeumannomyces graminis* var. *tritici*	Henkes et al. 2011 Sari et al. 2008
	Barley	*Fusarium graminearum*	Petti et al. 2010
	Pearl millet	*Sclerospora graminicola*	Raj et al. 2004
Pseudomonas aeruginosa	Rice	*Rhizoctonia solani* *Magnaporthe oryzae*	Saikia et al. 2006 De Vleesschauwer et al. 2006
	Wheat	*Blumeria graminis* *Cochliobolus sativus*	Muyanga et al. 2005
Bacillus pumilus	Rice	*Xanthomonas oryzae* pv. *oryzae*	Chithrashree et al. 2011
	Pearl millet	*Sclerospora graminicola*	Raj et al. 2003
Bacillus subtilis	Rice	*Xanthomonas oryzae* pv. *oryzae*	Chithrashree et al. 2011
	Pearl millet	*Sclerospora graminicola*	Raj et al. 2003
Bacillus cereus	Maize	*Cochliobolus heterostrophus*	Huang et al. 2010
	Sorghum	*Pythium utlimum*	Itris et al. 2008
	Formosan Lily	*Botrytis elliptica*	Liu et al. 2010

Different studies showed the efficacy of ISR in monocots against necrotrophic pathogens but only few demonstrated which signaling pathways are responsible in ISR. In

dicot plants, ISR requires signaling pathways that are SA-independent but JA- and ET-dependent. In rice, De Vleesschauwer et al. (2008) identified that ISR induced by *P. fluorescens* WCS374r against *M. oryzae* was dependent to a JA/ET-modulated signal but was independent from a SA-signaling. The involvement of the JA-signaling pathway in ISR was also shown in maize (Djonovic et al. 2007; Song et al. 2011) and barley (Petti et al. 2010). Interestingly, ISR triggered by *T. harzianum* 22 in maize seems to be associated with priming of genes involved in the production of volatile compounds called green leaf volatiles (Djonovic et al. 2007). Mycorrhized maize plants challenged with *Rhizoctonia solani*, showed an induction of defense-related genes involved in DIMBOA synthesis and SA- as well as JA-signaling pathways (Song et al. 2011). As in dicot plants (Van Wees et al. 2008), in some cases, PGPR or PGPF (plant growth promoting endophytic fungi) enhanced resistance against biotrophic pathogens similar to SAR, with a SA-dependent signaling and the accumulation of PR proteins (Molitor et al. 2011).

In conclusion, recent studies on ISR triggered by PGPR, PGPF or mycorrhiza in monocotyledons and more specifically in cereals tend to show mechanisms that are also common in dicotyledonous plants. However, ISR in both monocots and dicots is highly variable depending on the host-PGPR combination and on the type of attack. *P. aeruginosa* 7NSK2 and *Serratia plymuthica* IC1270 induced resistance against *M. oryzae* in rice but they enhanced disease severity caused by *R. solani* (De Vleesschauwer et al. 2006 and 2009). However, other *Pseudomonas* strains were able to induce ISR in the latter plant-pathogen system (Saikia et al. 2006, Radjacommarea et al. 2004). This variability in plant response makes it more difficult to fully understand the mechanisms underlying ISR, but a general scheme on the function of ISR is slowly emerging.

Induced resistance against insect herbivores

Plants are confronted with a wide variety of insect herbivore attacks. Upon perception of an herbivore, IR mechanisms are mediated by different defense-related hormones. Plant-induced defenses against phloem-feeding herbivores seem to share common plant reaction to biotrophic pathogens by activating SA-dependent pathways associated with the production of PR proteins (Alagar et al, 2010) and callose deposition at the feeding site (Hao et al. 2008). In rice, defense induced by an attack of the phloem-feeding brown planthopper is mediated by a SA-related signaling and is associated with an accumulation of PR proteins and an HR (Zhou et al. 2009). In resistant wheat cultivars, but not in susceptible ones, infestation by gall insects induces changes in SA levels (Tooker and De Moraes 2011). In contrary, plants induce JA and ET-dependent pathways against chewing herbivores. In maize, JA and ET are important in plant defense against *S. frugiperda* (Shivaji et al. 2010; Harfouche et al. 2006). JA was also shown to have an important role in IR of wheat against pests (El-Wakeil et al. 2010). In rice, the JA-dependent pathway induces resistance against insect herbivores and suppression of JA activity results in an improved larval performance of the

striped stem borer and leaf folder (Zhou et al. 2009). Ethylene is another key player in fending off herbivores. ET emission induced by elicitors of *S. frugiperda* OS influences the expression of direct defenses such as defense proteins and secondary metabolites (Harfouche et al. 2006). In rice, the ethylene responsive factor ERF3 mediates between SA, JA and ET pathways and thus orchestrates the response to chewing or phloem-feeding insects (Lu et al. 2011b).

After herbivore attack, plants can induce defenses that will directly act against insect herbivore. The maize insect resistance 1-cysteine protease (Mir1-CP) content increases in roots and leaves in response to larvae feeding on leaves, conferring a systemic induction of plant defense against herbivores (Lopez et al. 2007). Trypsin proteinase inhibitors are important defense compounds against herbivores such as the striped stem borer and leaf folder in rice (Zhou *et al*., 2011; Wang et al. 2011). Secondary metabolites, such as the hydroxamic acids in cereals, can also have a direct negative effect on insect herbivores (Chen 2008). Direct local defense can enhance direct plant defense systemically. Infestation of rice plants with *S. frugiperda* for example increases resistance against a subsequent attack by the rice water weevil (Hamm et al. 2010). Similarly, root infestation of maize by *Diabrotica virgifera virgifera* induces resistance in the leaves against *S. littoralis* and the necrotrophic pathogen *Setosphaeria turcica* (Erb et al. 2009). This illustrates that an induction of belowground defenses can induce aboveground resistance in maize.

Many plants respond to insect herbivory or wounding by emitting blends of volatile organic compounds (VOCs). VOCs release is an important cue for systemic defense signaling within an attacked plant as well as for plant-plant communication. Exposure of a maize plant to VOCs from infested plants primes the defense response against the generalist *S. littoralis* (Ton et al. 2007). Green leaf volatiles, specific VOCs emitted by plants upon wounding damages, can also activate defense mechanisms in neighboring intact plants (Ruther and Furstenau 2005).

Induced tolerance against abiotic stresses

Plant environmental stress such as drought conditions, high salinity in soil or too cold or too hot temperatures represents the most important economic problem for crop production worldwide. In plants, abiotic stress is often accompanied by an excess in ROS levels that leads to oxidative damage. Thus, plant tolerance to abiotic stresses involves adaptive changes in plant morphology, and physiological and biochemical processes to minimize stress-induced oxidative injury (for review see Mahajan and Tuteja 2005). ROS play a dual role in plant stress response: they are toxic by-products that accumulate in cells but they are also important signal transduction molecules. The signal perception of an abiotic stress by the plasma membrane is followed by the generation of second messengers such as calcium, ROS and inositol phosphates. These messengers modulate the calcium level in the cells. This

change is recognized by calcium sensors, resulting in the expression of major stress responsive genes, and finally leading to a physiological response. Certain chemical treatments can induce an increase of plant stress responses in monocotyledonous plants. Application of secondary messengers such as calcium or inositol to plants enhances their tolerance towards abiotic stress. Seed priming with $CaCl_2$ reduced chilling injury of maize. Plant growth was improved; antioxidant activity was enhanced and soluble sugars accumulated to a higher level after calcium treatment (Farooq et al. 2008). Onion plants treated with inositol showed less salt-induced damage of the cell division pattern in roots (Chatterjee and Majumder 2010). Ascorbic acid pretreatment of sugarcane results also in higher salt tolerance triggered by enhanced antioxidant enzymes activities (Munir and Aftab 2011).

Phytohormones play also a key role in abiotic stress signaling. In response to ROS accumulation, the level of SA and ET increases in stressed plants (Kiffin et al. 2006). The main function of ABA seems to be the regulation of plant water content and osmotic stress tolerance. ABA is responsible for the stomatal closure in plant response to drought stress. ABA-deficient mutants of tobacco, tomato and maize have growth problems under drought stress and not able to survive persistent drought periods (Huang et al. 2012). Exogenous application of phytohormones can increase abiotic stress tolerance in treated plants as in turfgrass where ABA treatment increased drought stress tolerance) (Lu et al. 2009). In maize plants, ABA application induced tolerance against chilling (Pal et al. 2011) and drought stress (Zhang et al. 2011). Treatment of maize plants with brassinolides enhances water stress tolerance and is associated with NO production followed by ABA biosynthesis (Zhang et al. 2011). In maize, the ABA-induced stress response leads to a decrease in SA levels under cold stress (Pal et al. 2011). This indicates a possible trade-off between ABA- and SA-related stress responses in monocots. However, SA application can increase plant abiotic tolerance associated with ABA production. In wheat, SA treatment reduced changes in IAA and cytokinin during salt stress tolerance resulting in ABA accumulation (Shakirova et al. 2002). Activation of antioxidants in SA-primed wheat plants confers a better tolerance to salinity (Afzal et al. 2011). Wheat drought tolerance has also been induced after SA treatment, involving solute accumulation (Loutfy et al. 2012). Interestingly maize drought tolerance induced by SA treatment was associated with an increased level of H_2O_2 (Saruhan et al. 2012), whereas similar hormonal treatment against salt stress in wheat led to a lower amount of H_2O_2 (Erdal et al. 2011). SA treatment induced also cold tolerance in banana (Kang et al. 2007) and maize (Kang and Saltveit 2002). These results illustrate the protective role of SA in the defense mechanisms against abiotic stresses.

In addition to their ability for inducing plant defenses against a broad variety of biotic stresses, beneficial microorganisms also induce plant tolerance against various abiotic stresses in dicotyledonous as well as in monocotyledonous plants. The presence of mycorrhiza in banana roots promotes growth under salt stress conditions (Yano-Melo et al. 2003). Abiotic stress tolerance of barley can be enhanced by the PGPF *Piriformospora indica*

(Achatz et al. 2010). Different PGPR induce salt tolerance in maize and wheat (Nadeem et al. 2010). PGPR are able to increase plant tolerance via different mechanisms such as lowering ethylene concentration in plants, producing phytohormones, regulating nutrient uptake, inducing and augmenting stress-response gene expression or the production of antioxidants. Beneficial effects of drought tolerant *Pseudomonas* strains on drought-stressed maize plants were observed at the morphological and physiological level. Interestingly, antioxidant enzyme activities were lower in bacteria-inoculated plants compared to control plants. This shows that the biochemical response of inoculated plants correspond to a less stressed plant (Sandhya et al. 2010). In wheat, salt-tolerant rhizobacteria induced phenolics and quercetin accumulation leading to an enhancement of plant growth under saline stress (Tiwari et al. 2011). PGPR containing ACC-deaminase can improve maize plant growth under salt stress conditions through better nutrient uptake (Nadeem et al. 2007) and exopolysaccharides produced by PGPR allow maize plants to tolerate salt stress by binding Na^+, resulting in a reduced salt uptake by the plant (Ashraf et al. 2004).

In some cases, plants that have been exposed to a previous abiotic stress have an increased tolerance for a subsequent abiotic stress. Halopriming enhanced sugarcane tolerance to salt and drought stress (Patade et al. 2009). This phenomenon has also been shown in banana fruits. A heat pretreatment induced an enhanced PAL activity conferring a chilling tolerance (Chen et al. 2008).

Abiotic stress tolerance is a very complex process involving diverse metabolic pathways and physiological adaptations. Our understanding of the plant abiotic stress responses is limited for monocots as well as for dicots. Several synthetic chemical plant-inducers are available against pathogens and pests. However, little information concerning such chemicals for helping plant against abiotic stresses is available. For now, the use of PGPR seems the most promising tool to manage crop production under environmental stresses, but further studies under field conditions are necessary.

Conclusion

For decades, the majority of research on IR has been performed in dicot model plants. Recently, cereal and non-cereal IR model systems have been established that will allow further understanding of induced resistance if combined with improvements in biotechnology such as reliable plant transformation methods for monocots. Essentially, IR represents a good alternative to synthetic chemical use in sustainable agriculture because of the wide spectrum of defense activity by IR inducers and the lack of selection pressure on pathogens after repeated IR treatment (Bousset and Pons-Kuhnemann 2003). Moreover, IR influences fitness costs in a positive way in plants under disease pressure (Walters et al 2008). Novel insights into the functioning of IR in monocots are expected and may lead to environmentally safe options for crop protection in the context of sustainable agriculture.

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Thesis outline

This PhD project was performed within the frame of a Swiss program of the National Centre of Competence in Research, called NCCR Plant Survival, in the work package concerning plant antagonists and mutualists. As previously described in the introduction part, there is a lack of knowledge concerning induced resistance in monocotyledoneous plants. The idea of the group project was to have a further understanding of induced resistance (IR) in maize plants and more specifically of inducible root defenses. In this context, the aim of this thesis was developed to study interactions between maize plants and beneficial rhizobacteria.

In a first phase a plant growth system that allows an easy access and observation of the maize root system while limiting damage on roots was developed. For this purpose, a soil-free plant growth system that is compatible with the study of beneficial as well as pathogenic root microorganisms has been set up (Chapter 2).

The second step was to test the direct changes induced byf the rhizobacteria *Pseudomonas putida* KT2440 on maize plants at different times during early plant development. *Pseudomonas putida* KT2440 were chosen for their well-known capacity to efficiently colonize maize seeds and roots. Different aspects have been tested: induction of growth promotion, defense gene expression, benzoxazinones quantification and metabolite profiling (Chapter 3).

The last step of the project was the analysis of possible induction of resistance triggered by *P. putida* KT2440. Two different biotic stresses were tested: a fungal pathogen, *Colletotrichum graminicola*, that causes corn anthracnose, and leaf herbivores that are specific or not to maize plants (*Spodoptera frugiperda* and *Spodoptera littoralis*). Expression of diverse defense genes and changes in secondary metabolites during systemic resistance induced by the rhizobacteria were investigated to better understand mechanisms involved in IR in maize (Chapter 4 and 5).

An appendix chapter presents preliminary results of additional experiments. A general conclusion of the thesis and perspectives for further investigations are addressed in Chapter 6.

CHAPTER 2

A soil-free root observation system for the study of root-microorganism interactions in maize

Adapted from

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Abstract

The root surface of a plant usually exceeds the leaf area and is constantly exposed to a variety of soil-borne microorganisms. Such root pathogens and pests, as well as belowground interactions with beneficial microbes, can significantly influence a plants' performance. Unfortunately, the analysis of these interactions is often limited because of the arduous task of accessing roots growing in soil. Here, we present a soil-free plant growth system (SF-ROBS) designed to grow maize (*Zea mays*) plants and to study root interactions with either beneficial or pathogenic microbes. The SF-ROBS consists of pouches lined with wet filter paper supplying nutrient solution. The habitus of maize grown in the SF-ROBS was similar to soil-grown maize; the growth of roots was even promoted in the SF-ROBS. SF-ROBS-grown roots were successfully inoculated with the hemi-biotrophic maize fungal pathogen *Colletotrichum graminicola* and the beneficial rhizobacteria *Pseudomonas putida* KT2440. Thus, the SF-ROBS is a system suitable to study two major belowground phenomena, namely root fungal defense reactions and interactions of roots with beneficial soil-borne bacteria. This system contributes to a better understanding of belowground plant microbe interactions in maize and most likely also in other crops.

Introduction

Soil-borne pathogens are estimated to cause an annual monetary loss of US$4 billion in the US (Okubara et al., 2005). Root physiology under biotic and abiotic stress conditions is a field of increasing importance, specifically in view of improving crop yield and diminishing the possible negative environmental impact of agricultural practices (Gewin, 2010). However, most studies on plant defense have been essentially focusing on aboveground plant parts. The root system plays a key role for the whole plant: roots are not only essential for nutrient and water uptake, they also contribute to adequately anchor the plant and have an important impact on its capacity to react to stress (Rasmann et al., 2008, Erb et al., 2009). In tobacco for example, nicotine is produced in the roots and translocated to the leaves in response to aboveground herbivore attack (Kaplan et al., 2008). *Colletotrichum graminicola* (Ces.) Wilson, the causal agent of corn anthracnose, infects both aboveground and belowground maize parts (Sukno et al., 2008). This pathogen is responsible for significant yield losses worldwide. Soil-borne pathogens, such as some species of *Fusarium*, *Phytophtora* or *Pythium,* have also an important economic impact. This stresses the need for studies focused on belowground plant interactions.

In addition to pathogenic interactions, beneficial interactions between microorganisms such as rhizobacteria or endophytic fungi and roots can have an impact on belowground stress reactions. The growing demand for sustainable alternatives to the massive input of pesticides in agriculture has led to an increase of interest concerning beneficial interactions between plants and soil-borne microbes. Such beneficial microbes are able to stimulate plant growth and to induce aboveground systemic resistance against different types of stresses (Pineda et al., 2010). For example, filamentous fungi such as *Trichoderma virens* or *Piriformospora indica* induce resistance against biotrophic and necrotrophic pathogens in some cereals (Deshmukh et al., 2006, Djonovic et al., 2007). Various rhizobacteria such as some *Pseudomonas spp.* or *Bacillus spp.* also protect plants against above- or belowground stresses (reviewed in De Vleesschauwer and Höfte, 2009). Selected rhizobacteria have been tested for their capacity to enhance defense reactions against biotic and abiotic stress and to promote growth of maize plants (Huang et al., 2010, Nadeem et al., 2009). *Pseudomonas putida* KT2440 has been recently tested for its close interaction with maize seeds and roots (Neal et al., 2012) and for its capacity to induce resistance in Arabidopsis against the pathogen *Pseudomonas syringae* pv. *tomato* DC3000 (Matilla et al., 2010).

Research focusing on belowground plant defense is limited by the difficult accessibility of roots growing in soil. For some plants such as Arabidopsis (Gibeaut et al., 1997, Hétu et al., 2005, Ishiga et al., 2011), tomato (Ahn et al., 2011), or rice (Kim et al., 2005) well-established growing systems circumventing the presence of soil are available as aeroponic, hydroponic, solid or semi-liquid cultures. A recently described aeroponic culture system allows even the study of root herbivory on Arabidopsis (Vaughan et al., 2011). For

maize plants, most of the soil-free systems are based on a solid substrate like quartz sand (Hund et al., 2009a, Schulze and Pöschel, 2004) or glass beads (Boeuf-Tremblay et al., 1995). However, none of these maize systems allows an easy access to the root during all steps of development. Moreover, these substrates tend to stick to the roots and can interfere with some measurements and manipulations.

The aim of our study was to establish a growth system well adapted to maize. Moreover, this system should allow microorganism-root interactions and an easy access to the root system for *in vivo* observations, root inoculations with microbes and harvesting of material with the smallest possible damage to the roots. The soil-free root observation system (SF-ROBS) we finally established is adapted from the model of Hund et al. (2009b) created for maize root morphology analysis. The system has been used to study the response of maize roots to abiotic stresses, such as extreme temperature (Hund et al., 2012), low water potential induced by polyethylene glycol (Ruta et al., 2009) or aluminum toxicity (Trachsel et al., 2010) but was not tested for its suitability to study plant-microbe interactions.

In the following we report on the effect of the SF-ROBS on maize plants. Plants from standard soil-pot conditions were compared to plants grown in the SF-ROBS. Different parameters including the general aspect (habitus) of plants and measurements of different plant parts were assessed. Since one of the main reasons for adapting the soil-free system was to obtain an easier access to the roots for specific studies in plant-microorganism interactions, we tested the suitability of the SF-ROBS for a pathogenic interaction with the hemibiotrophic fungus *C. graminicola* and a beneficial interaction with the rhizobacteria *P. putida* KT2440.

Material and Methods

Growth of maize in the soil-free system

For sterilization, maize seeds (var. Golden Jubilee, West Coast Seeds, Canada) were rinsed in 70% ethanol, incubated for 5 minutes in 10% bleach and washed three times with sterile distilled water. The sterilized seeds were pre-germinated for 2-4 days between humid filter paper sheets (Filterkrepp Papier braun, 100 g m^{-2}, E. Weber & Cie AG, 8157 Dielsdorf, Switzerland) in a plant growth chamber (Percival AR-95L, CLF Plant Climatics GmbH, Wertingen, Germany) with the following conditions: 16 hours day at 26°C, 8 hours night at 22°C, 60% relative humidity and an irradiance of 400 µmol m^{-2} s^{-1}. They were then transferred to a soil-free root observation system (SF-ROBS). The SF-ROBS is based on the model of Hund et al. (2009b) with the following modifications: a larger size of pouches, the quality and quantity of the nutrient solution, the type of filter paper and an increased number of plants per pouch. In details, the SF-ROBS consists of a 34x64 cm polyethylene foil (PE-Teichfolie WA-1200, 0.5 mm, Walser AG, 8575 Bürglen, Switzerland), which was folded

in half to form a 34x32 cm pouch (Figure 1). Each pouch had three 2x1 cm slits, allowing the growth of the shoots; 3 cm below that aperture, a bulge made with a polyurethane bumper (3M Bumpon protective products, 12.7 mm diameter, 3.5 mm depth, 3M Europe, 1831 Diegem, Belgium) was pasted to one side of the pouch to keep the seedling in place. The seedling was fixed between two 33x33 cm filter papers (Filterkrepp) in the closed pouch with two standard paper clips (43 mm long) on each side of the bulge. The pouch was attached to an aluminum rod with two foldback clips (Büroline, 51 mm, 69198 Schriesheim, Germany), one on each side of the upper edge of the pouch. The filter paper was humidified with maize nutrient solution (MNS). The MNS (Ruakura solution adapted from Smith et al., 1983) consists of the following solutions: macronutrient stock solution A (2.31 g $MgSO_4.7H_2O$, 16.78 g $Ca(NO_3)_2.4H_2O$,8.48 g NH_4NO_3, 2.28 g KNO_3, 2.31 g $(NH_4)_2SO_4$ per liter); macronutrient stock solution B (2.67 g KH_2PO_4, 1.64 g K_2HPO_4, 6.62 g K_2SO_4, 0.60 g Na_2SO_4, 0.33 g NaCl per liter); micronutrient supplement (128.80 mg H_3BO_3, 4.48 mg $CuSO_4$, 81.10 mg $MnCl_2.4H_2O$, 0.68 mg MoO_3, 23.45 mg $ZnCl_2$, 809.84 mg $C_6H_5FeO_7$ per liter); each solution was sterilized by autoclaving; 200 mL of each of the macronutrient stock solutions was mixed with 100 mL of the micronutrient supplement and finally diluted with deionized water to a volume of 4.5 L. The pouches were put into polypropylene containers (60x40x32.5 cm, Rako, Migros, Switzerland), containing 4.5 L MNS, so that the bottom of the filter paper protruding from the pouches was constantly submerged in the nutrient solution. The filter paper was replaced every 3-4 days by new, moistened paper. The plastic containers with the growing maize plants were placed in the climate chamber at the same conditions as for seed germination. The SF-ROBS is also explained in the video available in the online supplementary material. To test an alternative nutrient solution, plants were grown as described above in Hoagland's No. 2 (Sigma-Aldrich, 3050 Spruce St., St. Louis, Missouri 63103, H2395) and compared with plants grown in MNS. Shoot and root fresh weights were measured for each plant (10 replicates per treatment) and possible symptoms of nutrient deficiencies were recorded.

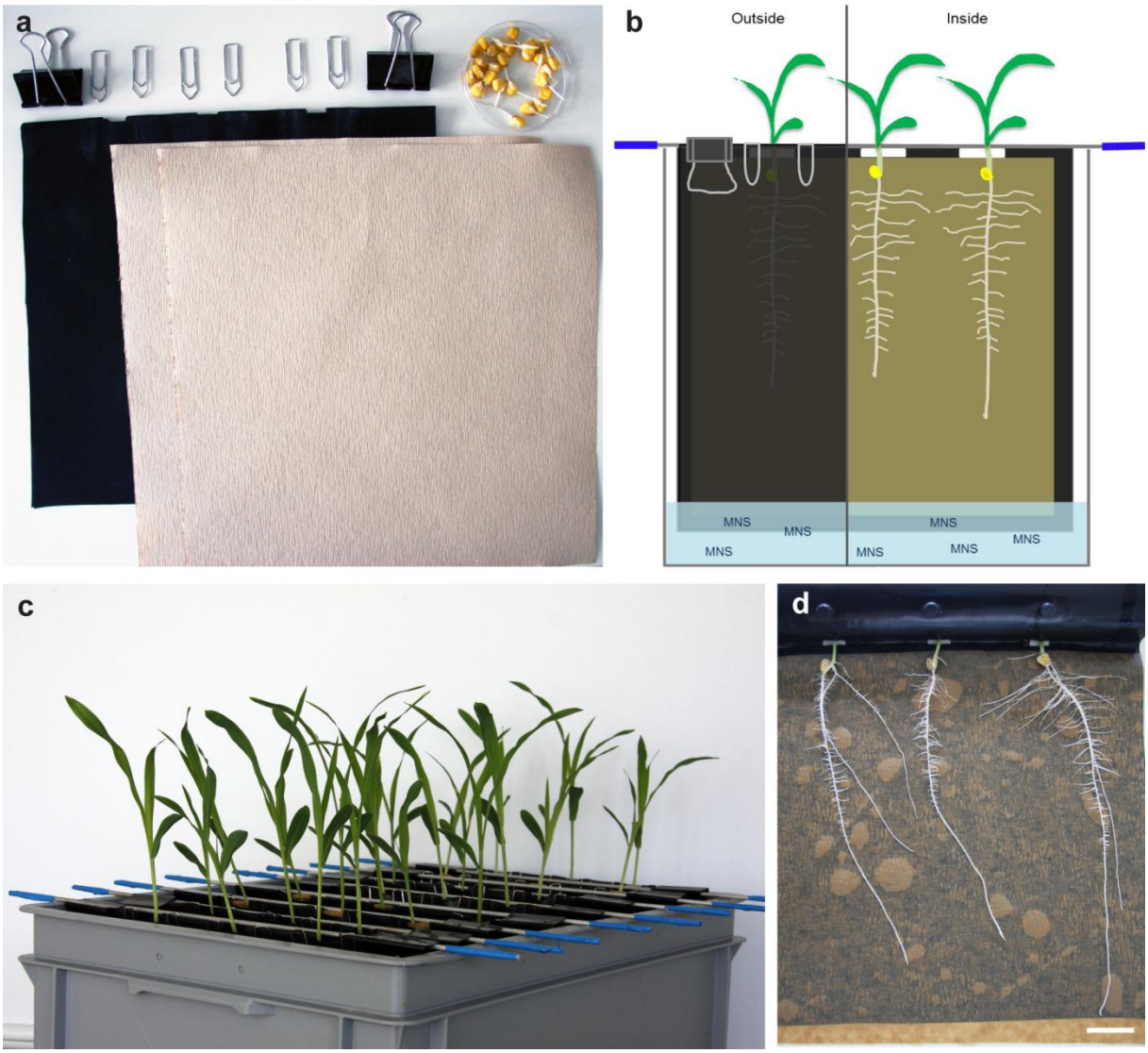

Figure 1. Details of the SF-ROBS. (a) Elements of the pouch system with germinated maize seeds in the upper right corner. The SF-ROBS consists in pouches formed from black PE foil folded on itself and two humid filter papers. The layers are held together by paper clips. These pouches are attached to an aluminium rod with two foldback clips and placed in polypropylene containers that contain the maize nutrient solution (MNS). (b) View of the outside and the inside of the black PE foil pouch in which plants are growing. (c) Nine-day old maize plants. (d) Root systems of 5-day old plants in the SF-ROBS. Bar = 2 cm.

Plant growth in soil

For experiments in soil, maize plants were potted in polypropylene pots, 11 cm high, 4 cm in diameter (Semadeni, 3072 Ostermundingen, Switzerland). Seeds were sterilized and pre-germinated as for the SF-ROBS. Germinated seeds were then transferred into a 50:50 (vol:vol) soil (25% compost, 12% sand and 63% peat; Ricoter Erdaufbereitung AG, 3270

Aarberg, Switzerland): sand (washed sand 0-4 mm, Jumbo, Switzerland) mixture. The soil:sand mixture was autoclaved one day before use. The plants were grown under the same conditions as for plants in the SF-ROBS.

Root infections with *Colletotrichum graminicola-gGFP*

To facilitate fungal detection and quantification a GFP-expressing strain of *C. graminicola* (gGFP; Erb et al., 2011) was used. *C. graminicola-gGFP* was maintained on potato dextrose agar under continuous light (70µmol m^{-2} sec^{-1}) and 25°C. Maize plants were removed from the SF-ROBS just before the inoculation process and the roots were inoculated by submerging the entire root system in a spore suspension (10^6 spores mL^{-1}, harvested from a 3 weeks old fungal culture) for 30 minutes in the dark at room temperature. Immediately afterwards, plants were put back in the SF-ROBS. Inoculations were performed at the end of a day period. Fungal root colonization was observed over a time period of 1-6 days post infection (dpi).

Plant inoculation with *Pseudomonas putida* KT2440

The rifampicin-resistant strain *Pseudomonas putida* KT2440 was grown on Luria-Bertani (LB; Difco LB, Becton, Dickinson and Company, 38800 Le Pont de Claix, France) agar supplemented with 100 µg mL^{-1} rifampicin (Fluka, Sigma-Aldrich, 9471 Buchs, Switzerland) at 25°C in the dark for 2-10 days. A single colony was picked and transferred to 100 mL of LB liquid medium with 100 µg mL^{-1} rifampicin for an overnight culture at 28°C, under continuous shaking at 150 rpm. The bacterial culture was divided in two, centrifuged at 3700 rpm, washed twice with sterile $MgSO_4$ 10 mM and the pellet was re-suspended in 25 mL of sterile M9 minimal medium (Sambrook and Russell, 2001). Maize seeds were first sterilized and pre-germinated for two days between Filterkrepp paper sheets as described above. The germinated seeds were then either mock-inoculated with sterile M9 minimal medium as control or with a fresh overnight bacterial suspension (1-3 x 10^{12} colony-forming units (CFU) mL^{-1}) by shaking for 30 minutes at 35-40 rpm at room temperature. Bacterial root colonization of such treated plants grown in the standard soil-pot system and in the SF-ROBS was then compared. All root types, except crown roots, from 11-day old plants were harvested and cleaned from remaining soil under running tap water. Roots were then quickly dried and 100 mg of fresh weight per sample were ground in 600 µL sterile $MgSO_4$ 10 mM. For each plant (12 replicates), two root samples were collected: one near the seed, for the upper part of roots, and the other one in the primary root tip area, for the lower root parts. Serial dilutions of each sample were plated on solid King's medium B (Pseudomonas agar F, Merck KGaA, 64271 Darmstadt, Germany) supplemented with 100 µg mL^{-1} rifampicin to quantify the rifampicin-resistant *P. putida* KT2440 strain. After 18-20 hours of incubation

at 25°C in the dark, the number of colony-forming units per gram of fresh root was determined.

Assessment of growth parameters

In order to compare plants from the SF-ROBS with plants grown in the standard soil system we measured shoot and root length as well as fresh weight of 12-day old plants. The part considered as shoot reached from the seed to the tip of the longest leaf, whereas the root part was from the seed to the tip of the primary root. After these measurements, roots and shoots were dried separately in an oven at 70°C (Hybridisation oven/shaker SI20H, Stuart Scientific, UK) in coffee filter papers until sample weight remained constant. The dry weight of shoots and roots was then assessed.

Imaging

Microscopy of *C. graminicola-gGFP*-infected roots was performed using a (Eclipse E800, Nikon Corporation, Tokyo, Japan) microscope and a dissecting microscope (C-BD230, Nikon Corporation, Tokyo, Japan). Images were captured using a digital sight device (DS-L1, Nikon Corporation, Tokyo, Japan). GFP fluorescence of the fungal structures was excited with blue light (430-470 nm).

Statistical analysis

For the comparison of plants from the soil-free system with plants from the standard system, measurements were analyzed using a Student t-test, after passing a Shapiro-Wilk test as a normality test. All analyses were performed using the R software v2.12.1 (R Development Core Team (2011). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL http://www.R-project.org/). For comparison of plants grown in different nutrient solutions and for bacterial root colonization, the data were processed in SigmaPlot 11.0 (Systat Software, Inc., San Jose California USA, URL http://www.sigmaplot.com). Depending on the distribution of data, a Student t-test or a Mann-Whitney Rank Sum Test were performed.

Results

The SF-ROBS favors maize root development

To evaluate the effect of the SF-ROBS on maize growth in comparison to soil, we assessed the habitus of the plants (Fig. 2a and b) and measured parameters linked to plant fitness: the plant length (Fig. 3a), fresh and dry weight (Fig. 3b and c). Both planting methods yielded healthy plants with a similar habitus (Fig. 2a). This visual observation is supported by no significant differences for either shoot length or weight measurements between both systems (shoot length $p = 0.607$; shoot fresh weight $p = 0.415$; shoot dry weight $p = 0.106$). Interestingly, plants from the SF-ROBS had a better developed root system with enhanced branching compared to plants from the soil system (Fig. 3). This was reflected in root length (44.82 cm), root fresh weight (1.68 g) and root dry weight (90.26 mg) of SF-ROBS-grown plants, which were significantly higher compared to soil-grown plants (root length 34.68 cm, $p = 0.002$; root fresh weight 0.77 g, $p = 3.325 \times 10^{-6}$; root dry weight 50.38 mg, $p = 7.576 \times 10^{-5}$).

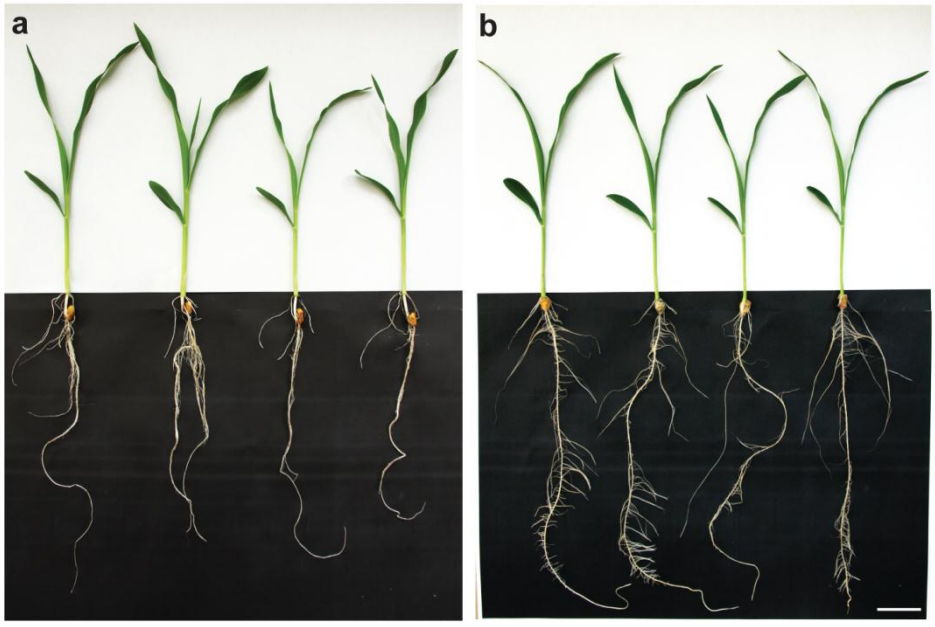

Figure 2. Comparison between plants grown in standard soil conditions or in the SF-ROBS. (a) Nine-day old plants up-rooted from standard soil conditions. (b) Plants grown in the SF-ROBS. Bar = 4 cm

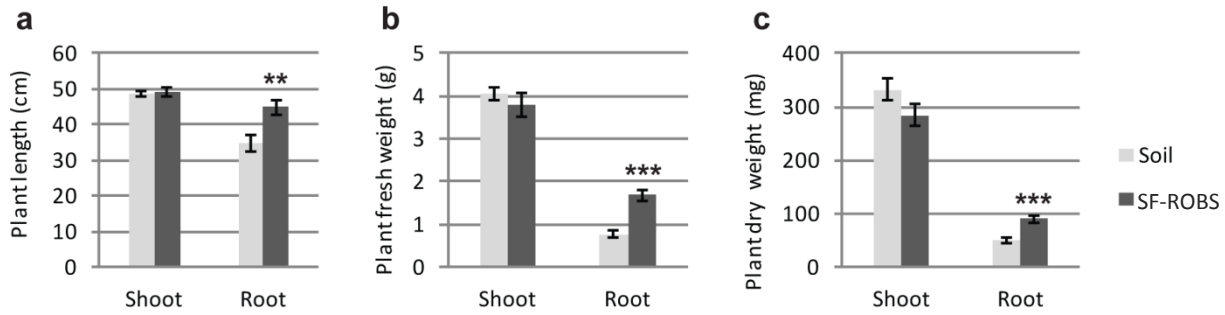

Figure 3. Comparison of root and shoot length and weight. Shoot and root dry and fresh weight of 12-days old maize plants grown in standard soil conditions or in SF-ROBS were assessed: (a) shoot and root length (cm), (b) shoot and root fresh weight fresh weight (g), and (c) shoot and root dry weight (mg). Error bars indicate the standard errors for the average values of 18 replicates. Asterisks indicate a significant difference in a Student t-test (** = $p < 0.01$, *** = $p < 0.001$).

Two different nutrient solutions were tested in the SF-ROBS: the MNS and the standard Hoagland's No. 2 solutions. Whereas plants with MNS were healthy, plants with Hoagland's No. 2 exhibited leaf chlorosis (Fig. 4). Moreover, plants grown in MNS had a significantly higher fresh weight of leaves (0.315 g; Mann-Whitney Rank Sum Test, $p = <0.001$) compared to plants grown in the standard solution (0.160 g). The root fresh weight of plants in MNS (0.496 g) was similar to plants in Hoagland's No. 2 (0.432 g; Student t-test, $p = 0.849$).

Figure 4. Leaves of 12 days-old maize plants (var. Jubilee) grown in the SF-ROBS containing Hoagland's No. 2 (H) or MNS. Leaves grown in Hoagland's No. 2 exhibit a chlorotic phenotype. Bar = 1cm.

The SF-ROBS facilitates colonization of maize root by *Colletotrichum graminicola*

To investigate whether the SF-ROBS can be used for fungal infection assays, roots were inoculated with the GFP-expressing pathogenic fungus *C. graminicola-gGFP* (Fig. 5). By monitoring the colonization over time, we found a characteristic infection pattern: four days after inoculation, the appearance of acervuli was observed (Fig. 5a, c and d). In later infection stages, epidermal cells packed with falcate conidia were detected (Fig. 5e). Mature roots, root caps and the root elongation zones were rapidly and consistently colonized by *C. graminicola-gGFP* (data not shown), suggesting that there was no fungal penetration preference for the different zones of the roots. During advanced infection stages (>4 dpi), colonized roots showed a brown discoloration (not shown).

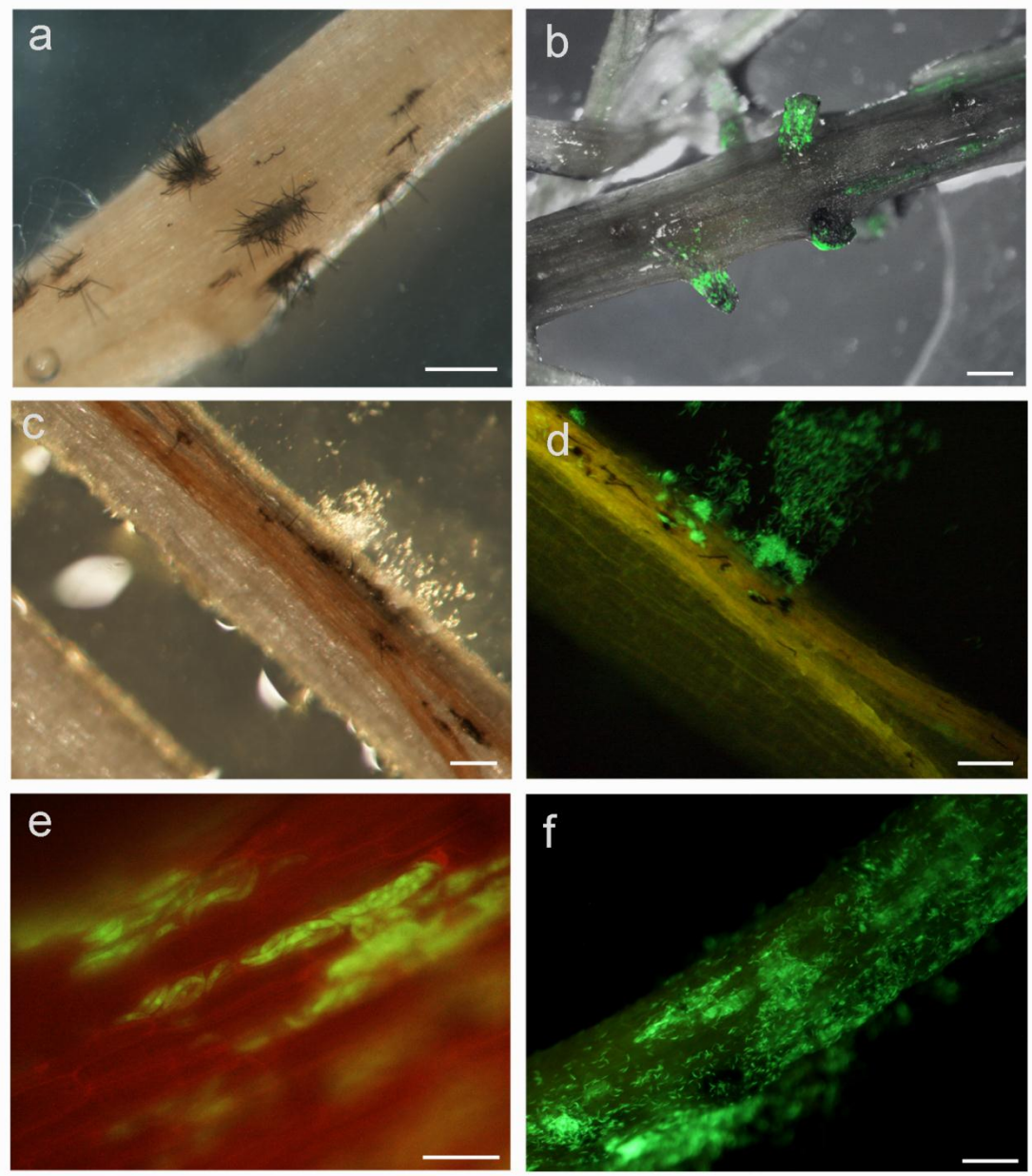

Figure 5. *C. graminicola-gGFP*-infection of maize roots grown in the SF-ROBS. Roots of 10-days old plants were inoculated by dipping in a spore suspension and kept in the SF-ROBS for further development. Colonization was observed under epifluorescence (b, d, e and f) and bright field illumination (a, c). (a) Acervuli with characteristic setae on infected roots, 4 dpi. (b) Early infection stage on an inoculated root (1 dpi); the fluorescence-image is superimposed over the bright field picture. (c, d) The same infected root viewed under UV and visible light at 5 dpi, showing conidia flowing out of acervuli. (e) Root epidermal cell packed with falcate conidia, 5dpi. (f) Heavily infected root at late stage, 6dpi. Bars = 100 µm (a, b, c, d, f) and 30 µm (e).

The SF-ROBS provides accurate conditions for root colonization by rhizobacteria

In order to test whether the SF-ROBS can also be used in research with beneficial root colonizing bacteria, the plants were inoculated with *P. putida* KT2440. The number of colony-forming units of *P. putida* KT2440 extracted from such inoculated roots of 11-day old maize plants was assessed to evaluate the capacity of the rhizobacteria to efficiently colonize roots in the SF-ROBS compared to the soil-pot standard system. There were no significant differences in root colonization of plants in the SF-ROBS (3,53 x 10^5 CFU g^{-1} of fresh roots) and in soil (2.5 x 10^5 CFU g^{-1} of fresh roots; Mann-Whitney Rank Sum Test, p = 0.29). The amount of bacteria differed between the upper and lower root parts. This difference was observed in both growing systems. There were more bacteria present in the upper parts (3.47 x 10^6 CFU g^{-1} of fresh roots in the SF-ROBS and 5.85 x 10^5 CFU g^{-1} of fresh roots in soil) than in the lower parts (3.60 x 10^4 CFU g^{-1} of fresh roots in the SF-ROBS and 8.7 x 10^4 CFU g^{-1} of fresh roots in soil; Mann-Whitney Rank Sum Test, p = < 0.001 for SF-ROBS and p = 0.002 for soil).

Discussion

The SF-ROBS facilitates the growth of young maize plants

Several culture methods have been described to allow growth of plants without soil. However, soil-free systems to study plants with higher biomass than Arabidopsis and demanding greenhouse care such as the monocotyledon model plant maize are less established. Gunning and Cahill (2008) described a method by which *Lupinus angustifolius* was cultivated in a system using blotting paper that was embedded between two plastic plates with a given spacing. They reported to have successfully tested maize in this system. Nevertheless, we found that this system was less viable in our hands for growing maize compared to our system. A major advance in this field was achieved with the development of a soil-free phenotyping platform for maize (Hund et al. 2009b). This system facilitates non-destructive digital assessments of the root morphology. Modification and adaptation of this method led to the development of the SF-ROBS presented here. Three critical elements of the SF-ROBS were identified: the pouches, in which the roots are growing, the filter paper providing the nutrients for the roots, and the nutrient solution itself. The pouches should shield the roots from light, and their surface should remain as aseptic as possible and not be toxic to plants. PE foil, which is commonly used to make garden ponds, was chosen since it satisfied these criteria. Choice of the right filter paper was found to be even more crucial. Standard white filter or blotting paper inhibited growth (data not shown), therefore we used filter paper that had not been treated with bleaching chemicals. Similarly, the nutrient solution had to be optimized for maize. Modified Ruakura solution (adapted from Smith et al. 1983) was identified as the most appropriate solution. Commonly used standard media such as Hoagland's No. 2 were found to be insufficient leading to visual chlorosis of the

leaves. The leaf color and growth rate were similar in both SF-ROBS and soil conditions. Roots grown in the SF-ROBS were longer and exhibited enhanced branching. Consistently, the fresh weight and dry weight of roots was found to be higher for SF-ROBS- grown plants, indicating that the SF-ROBS favors root development. The reason for this may lie in the reduced contact between the roots and the substrate in the SF-ROBS compared to soil. To counteract this situation, the plant will enhance the root surface contact for nutrients uptake by inducing a higher production of roots. Taken together, we demonstrate that the SF-ROBS is a convenient system to cultivate young maize plants. The main advantage of the SF-ROBS is the easy root handling. Roots can be accessed during any early developmental stage, and they can easily be removed from the system to perform treatments such as inoculations or microscopic observations. Moreover, harvesting roots for further experiments such as gene expression analysis or metabolomic fingerprinting is simplified using the SF-ROBS. Removing soil residues from roots is time-consuming and often leads to tissue damage, which might interfere with downstream experiments. However, the SF-ROBS requires regular changes of filter papers and a growth environment to limit the risk of contamination favored by the presence of a constant humid filter paper. Another limiting factor of the SF-ROBS is the root growth and age of the plants. Limited by the size of the pouch in our system, we were able to keep the plants no longer than about 21 days in the SF-ROBS (corresponding to a maize plant with four developed leaves). The short growth period does not allow studies plant-microbe interactions which need a longer time to establish. An extended culture time would require a size modification of the pouches to accommodate a larger root system. Efforts to increase the size of such paper-based rhizotron systems are in progress.

The SF-ROBS is convenient to study fungal root infections

Thus far, described culture methods of soil-free systems in combination with pathogens are scarce and often limited to *in vitro* analysis. Here, we present a soil-free growth system allowing *in vivo* observations of fungal root infections of maize. The *C. graminicola* infection assays performed in the SF-ROBS resulted in a colonization behavior and pattern which are similar to observations made for soil-grown plants (Sukno et al. 2008), indicating that the SF-ROBS does not alter the natural infection process. We observed an earlier development of acervuli on infected roots in the SF-ROBS compared to published data from a soil system (Sukno et al. 2008). This suggests that the infection process might be favored in the SF-ROBS, possibly through the constant humidity of the system but also by the enhanced infection efficiency or a decreased antiphytopathogenic potential in the paper compared to natural soil. Normally, maize roots are inoculated by either soaking seedlings (2 days after germination) in a spore suspension or by growing older seedlings in vermiculite mix containing agar plugs from *C. graminicola* cultures (Sukno et al., 2008). Especially the agar plug method makes it difficult to control the colonization of a specific root part, which results in a less efficient infection rate. The SF-ROBS allows the infection of specific root

parts and enables easy sampling for downstream plant-pathogen interaction analysis such as gene expression profiling or hormone quantification.

The SF-ROBS allows the study of root-bacteria interactions

Despite the fact that plant-beneficial microbe interactions are an emerging research field, the molecular and chemical mechanisms underlying these interactions remain largely unknown. As for root-pathogen interactions, the arduous accessibility of roots is also an issue when studying beneficial root microbes.

The ease of root harvesting makes the SF-ROBS an advantageous system to study the interactions of roots and beneficial root-colonizing microbes. Instead of a bacterial inoculation of soil, it is possible to inoculate the roots directly without damaging them. Here, we show that the SF-ROBS is suitable for cultivating maize roots inoculated with the root beneficial bacterium *P. putida* KT2440. *P. putida* KT2440 was successfully recovered from 11-day old roots grown either in the SF-ROBS or in the soil, showing that the SF-ROBS does not inhibit bacterial development in plant roots. A similar amount of bacteria was found in roots of SF-ROBS-grown plants as well as in roots of soil-grown plants, indicating that growth conditions do not affect the potential of bacterial root colonization. Bacterial colonization along the length of the root was similar in maize plants grown in the SF-ROBS as for plants grown in soil and as previously described in other systems (Simons et al., 1996) with a decreased gradient of bacteria from the root base towards the root tip. Furthermore, the density of *P. putida* KT2440 on roots is critical for bacterial contribution to plant defense. Raaijmakers et al. (1995) showed that a bacterial density of approximately 10^5 CFU per gram of root is required for direct disease suppression and induction of plant resistance. Hence, the SF-ROBS could be used for studies on maize resistance mechanisms induced by rhizobacteria.

Since the availability of nutrients for the plant is controlled through the supply by the MNS growth medium in the SF-ROBS, it would be also easily possible to study the importance of selected nutrients or combinations thereof on root colonization, on direct bacterial inhibitory effects or on plant induced resistance mechanisms.

To sum it up, we have presented here a soil-free growth system that allows the non-destructive study of interactions of roots with pathogenic and beneficial microorganisms. Moreover, our system is suitable for crop plants such as maize and could therefore contribute to a better understanding, and finally management, of belowground stress situations of plants.

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CHAPTER 3

Direct changes induced in maize plants by root inoculation with *Pseudomonas putida* KT2440

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Abstract

Plants interact with many different microorganisms in their immediate environment. Beneficial interactions between plants and microbes necessitate a mutual recognition and adapted responses to establish an equilibrated relationship. The rhizobacteria *Pseudomonas putida* KT2440 are known for their capacity to adhere to maize seeds and to colonize maize rhizosphere. However, there is no information available concerning the reaction of maize plants to root colonization by KT2440. We have tested the direct effect of KT2440 on maize at the transcriptomic and metabolomic level. The plants reacted stronger to the presence of KT2440 early in the interaction. Bacterization led to an up-regulation of jasmonic acid- and abscisic acid-signaling pathway, respectively, and a down-regulation of the salicylic acid-signaling pathway. Moreover, reduced level of HDMBOA-Glc, an important defensive secondary metabolite, and an increased level of phosphatidylcholine were observed in bacterized plants. These changes suggest that KT2440 could manipulate plant defense immunity to efficiently colonize roots and establish a plant accommodation state. Following recognition and acceptance of the rhizobacteria by maize plants, a beneficial effect on plant biomass was observed, pointing to the growth promotion capacity of these bacteria on maize plants.

Introduction

Plants are regularly challenged by a wide variety of organisms present in their direct environment such as pathogenic bacteria, virus, fungi, oomycetes, nematodes and herbivores but beneficial interactions between plants and soil-borne microorganisms are also frequent in nature. Plants therefore have to be able to rapidly recognize the interacting partner and to activate an adapted response.

The best-studied beneficial organisms are mycorrhizal fungi and rhizobia. Mycorrhizal fungi deliver nutrients to the plant and rhizobia, responsible for nodule formation in legumes, fix atmospheric nitrogen for the plant (Spaink, 2000; Harrison, 2005). Rhizobacteria as well as certain fungi that are associated to plant roots are also known to induce beneficial effects in plants. Some beneficial microorganisms, called plant growth promoting rhizobacteria (PGPR) or fungi (PGPF), are able to directly or indirectly positively affect plant growth and protection (reviewed in Lugtenberg and Kamilova, 2009). The establishment of such beneficial interactions requires recognition and accommodation of the microbe by the plant. Interestingly, there is increasing evidence indicating that plants react in a similar way towards pathogenic and non-pathogenic microbes: both are recognized based on microbe-associated molecular patterns (MAMP). Several MAMPs of beneficial microbes have been shown to activate MAMP-triggered immunity (MTI). Lipopolysaccharides (LPS) and exopolysaccharides are known as MAMPs of rhizobia (Zamioudis and Pieterse, 2012). LPS, flagellin and the siderophore pyoverdin of different *Pseudomonas* strains induced early defense responses in tobacco cell cultures such as: reactive oxygen species (ROS), medium alkalinization, increase in cytoplasmic calcium concentration and defense gene expression (van Loon et al., 2008). Some proteins and secondary metabolites of the PGPF *Trichoderma* were identified as MAMPs (reviewed in Hermosa et al., 2012). However, in order to establish a beneficial interaction involving an efficient bacterial colonization, there is a necessity to suppress MTI following recognition of the microbe (Gutjahr and Paszkowski, 2009; Zamioudis and Pieterse, 2012).

The establishment of a beneficial plant microbe interaction seems to involve a two-phase process. First, the presence of microbes is considered as a threat by the plant, inducing an MTI process. Second, beneficial microbes are able to suppress the plant reaction via the release of effector molecules such as mycorrhizal Myc factors and rhizobial nodulation factors. In the case of PGPR, no effectors have been identified yet (Zamioudis and Pieterse, 2012). As well-known for rhizobia and some pathogenic bacteria, certain PGPR possess a type three secretion system (TTSS), showing their potential to inject effectors into plants. However, the TTSS of rhizobia and PGPR seem to have only an assisting role in the suppression of MTI (Zamioudis and Pieterse, 2012). Another common mechanism involved in MTI suppression is the manipulation of the plant hormonal balance. As described for some pathogens, such as *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst*) in Arabidopsis, beneficial microbes are able to exploit plant hormone crosstalk to overcome MTI. *Pst* produces and

delivers by TTSS the effector molecule coronatine, that is structurally close to the plant hormone jasmonic acid isoleucine. The plant will respond by inhibiting the antibacterial salicylic acid- (SA) mediated defense pathway and inducing jasmonic acid- (JA) and abscisic acid- (ABA) mediated pathways, responsible for stomatal closure (Zheng et al., 2012). This manipulation promotes bacterial entry through open stomata, disease symptoms and systemic plant susceptibility. This example is a good illustration of pathogen virulence by inhibiting host defense signaling. It has been shown previously that inhibition of pathogen-associated molecular-pattern-triggered responses occurs also at the plant root level (Millet et al., 2010). Beneficial microbes also do exploit plant hormone signaling to enhance their colonization. Root colonization by the PGPF *Piriformospora indica* was associated with a JA-dependent signaling during its biotrophic phase, followed by the involvement of gibberellic acid (GA) during the cell death associated phase (Jacobs et al., 2011). Colonization of rice roots by the mycorrhizal fungus *Glomus intraradices* was associated with local and systemic up-regulation of the JA-dependant pathway and the down-regulation of *PR* gene expression (Campos-Soriano, 2011). In leaves of tomato plants colonized with *Trichoderma atroviride* P1 and *T. harzianum* T22 SA regulation was altered (Tucci et al., 2010). Plants associated with PGPR showed also some local and systemic hormonal changes. As hypothesized in Zamioudis and Pieterse (2012), PGPR may produce phytohormones to attenuate SA signaling via hormonal crosstalk mechanisms. Jasmonic acid signaling was up-regulated in wheat in response to *Pseudomonas fluorescens* Q8r1-96 (Okubara et al., 2010). The local transcriptional response of Arabidopsis roots upon *P. fluorescens* GM30 colonization showed an up-regulation of the ABA- and ethylene- (ET) dependent pathway (Weston et al., 2012). In contrary, the interaction between *P. fluorescens* SS101 and Arabidopsis resulted in an SA-dependent response (Van de Mortel et al., 2012). These examples show the variability of the hormonal manipulation by PGPR.

Several changes in plant physiology have been described to occur during beneficial interactions between plant and soil-borne microbes. Moreover, these changes occur in a bacterial strain-plant cultivar dependent manner. For example, the inoculation of maize with *Azospirillum* strains induced different changes of defense compounds depending on the bacterial strain and maize cultivar involved in the interaction (Walker et al., 2011). During beneficial interactions, as well as for pathogenic interactions, many changes occur in membrane lipids (Cowan, 2006). Plant primary metabolism is affected by beneficial interaction triggered by PGPR such as *Azotobacter* (Kumar, 2007) and *P. fluorescens.* Levels of the amino acids tryptophan and phenylalanine were altered in the shoots of Arabidopsis following root inoculation with *P. fluorescens* GM30 or Pf-5 (Weston et al., 2012). Secondary metabolites are important for plant defense but their presence also fluctuates in response to beneficial interactions. Benzoxazinones (BXD) are well-known defense compounds of the *Poaceae* family that are efficient against herbivores, aphids and fungi (reviewed in Niemeyer, 2009). Significant changes of BXD content were observed in maize plants upon mycorrhizal or rhizobacterial colonization. Whereas the presence of *Glomus mosseae* induced a systemic accumulation of DIMBOA (Song et al., 2011), interaction with *Azospirillum* caused a local

decrease of DIMBOA, DIMBOA-Glc and HDMBOA-Glc (Walker et al., 2011). Other phytoalexins such as coumarin and flavonoids also quantitatively changed in plants that were in association with rhizobacteria (Dardanelli et al., 2010; Van de Mortel et al., 2012; Drogue et al., 2012). These effects on metabolite profiles suggest the establishment of complex responses during beneficial interactions.

Pseudomonas putida KT2440 (KT2440) are gram-negative rhizobacteria that have the capacity to adapt to various niches such as soil or polluted environments (Wu et al., 2010). These rhizobacteria are also able to colonize a wide range of plants and were extensively studied for their capacity to adhere to maize seeds (Espinosa-Urgel and Ramos, 2004). A detailed analysis of the genome of KT2440 enhanced the presence of encoding genes involved in pathways linked to plant growth promoting properties, such as the production of phytohormones (Wu et al., 2010). In addition, KT2440 were able to induce systemic resistance in Arabidopsis against *Pst* (Matilla et al., 2010) and to enhance defense responses of maize after wounding and JA treatment (Neal and Ton, 2013). These beneficial effects could make KT2440 an interesting biological agent for agricultural use. Despite extended studies on KT2440, the response of plant upon KT2440 colonization has not been investigated yet. In our study, maize plants were inoculated with KT2440 and the plant response to this rhizobacteria-plant interaction was investigated at the transcriptional and metabolomic levels. We found that KT2440 elicited a host plant response that could benefit the rhizobacterial colonization. We also examined the potential of KT2440 of promoting maize growth.

Material and Methods

Plants and bacteria

Prior to germination, maize seeds (var. Golden Jubilee, West Coast Seeds, Canada) were first rinsed in 70% ethanol 3-5 times, then sterilized in bleach 10% for 5 minutes and washed five times in sterile distilled water. Seeds were then placed for germination in humid rolled filter paper (Filterkrepp Papier braun, 100 g m^{-2}, E. Weber & Cie AG, 8157 Dielsdorf, Switzerland) for two days in a growth chamber (16 hours day at 26°C, 8 hours night at 22°C, 60% relative humidity and an irradiance of 400 µmol m^{-2} s^{-1}). After two days, seedlings were transferred to a soil-free root observation system (SF-ROBS) as described in Planchamp et al. (2012). Seedlings were grown in the same conditions as for germination.

The rifampicin-resistant strain *Pseudomonas putida* KT2440 stock was kept in glycerol 50% at -80°C until use. For each experiment, a bacterial culture was made from a fresh stock on Luria-Bertani (LB; Difco LB, Becton, Dickinson and Company, France) agar supplemented with 100 µg mL^{-1} of rifampicin. After two to five days of growth in the dark at 25°C, a single colony of KT2440 was picked and transferred to 100 mL of LB liquid medium supplemented with 100 µg mL^{-1} of rifampicin for an overnight culture at 28°C, under continuous shaking at

150rpm. Pre-germinated seeds were inoculated with a bacterial suspension, for bacterized plants, or sterile M9 minimal medium, for control (or mock-treated) plants, as previously described (Planchamp et al., 2012).

In order to assess the effect of bacterial root colonization over time, maize roots and leaves of nine plants (per treatment) were collected at different times (3, 5 and 10 days) after seedling inoculation with KT2440. This material was used to test the bacterial colonization over time and for gene expression and metabolite analysis. Material of two independent experiments were pooled together to minimize a possible biological variation (representing 18 plants per treatment, per collection time point).

Quantification of bacteria in roots

For bacteria-inoculated and mock-inoculated seedlings, roots from six plants were collected and quickly blotted dry. Hundred mg of fresh weight per sample were separately ground in 600 µL of sterile $MgSO_4$ 10mM. Serial dilutions were plated on King's medium B supplemented with 100 µg of rifampicin and were incubated at 25°C in the dark for 18-20 hours. The colony-forming units (CFU) per gram of fresh root were then determined.

Gene expression analysis

The whole root system (except crown roots) and leaf material (second and third leaves) from six biological replicates (one replicate representing a pool of three plants) were flash-frozen after collection and separately ground with a mortar and a pestle taking care to keep the plant material frozen. RNA extraction, cDNA synthesis, qRT-PCR analysis and relative gene expression calculation were then performed as described by Balmer et al. (2013). Target gene expression was calculated by comparison with two housekeeping gene: *Zm-GAPc* and *Zm-Actin*. Primers for qRT-PCR that were used in this study are listed in Balmer et al. (2013). Relative gene expression was calculated (following the Pfaffl model; Pfaffl et al., 2002) and a statistical analysis was performed by using REST 2009 (Qiagen).

Metabolomic analysis and quantitative measurements of benzoxazinones

Material prepared for gene expression analysis was also used for a metabolomic analysis and a quantitative measurement of benzoxazinones (BXD). Metabolites were extracted from 100 mg of frozen ground tissue using 1 mL of extraction solvent (methanol 70%, water 29.5%, formic acid 0.5%). Metabolite extracts were diluted ten times in order to quantify BXD. Diluted and non-diluted extracts were analyzed using a UHPLC-QTOF-MS system (Waters, Milfors, USA) equipped with an electrospray (ESI) interface. An Acquity BEH C18 column (2.1

x 1.7 µm, Walters) was used for separation at a flow rate of 400 µL min^{-1}. The mobile phase consisted of two solvents: solvent A (water and formic acid 0.05%) and solvent B (acetonitrile and formic acid 0.05%). The gradient program was employed as follows: 0-3.5 min 2-27.2% B, 3.5-8 min 27.2-100% B, holding at 100% B for 1.5 min, reequilibration at 2% B for 1.5 min. The column temperature was maintained at 40°C and the injection volume was 2.5 µL. UV spectra were acquired over the range 210–400 nm (at 20Hz and 1.2 nm of resolution). Trace at 264 nm was extracted for BXD quantification. Further detection was performed by QTOF-MS in ESI positive ion mode over an *m/z* range of 85–600 Da for BXD quantification and 85–1200 Da for metabolite fingerprinting with a scan time of 0.4 sec. QTOF-MS settings were: capillary voltage at +2800 V, cone voltage at +25 V, source temperature at 120°C, desolvation temperature and gas flow at 330°C and 800 L h^{-1}, respectively. A 400 ng mL^{-1} solution of peptide leucine enkephalin was used as an internal reference for the MS. MassLynx™ V.4.1. was used to control the system.

The following extracted ion chromatograms were used for BXD quantification (peak height measurement; ± 0.01 Da): *m/z* 194.045 for DIMBOA-Glc (retention time (RT) 2.14 min) and DIMBOA (RT 2.38 min) and *m/z* 166.050 for HDMBOA-Glc (RT 2.71 min). Absolute quantification of BXD was performed by using calibration curves obtained from purified standards of DIMBOA, DIMBOA-Glc and HDMBOA-Glc (Glauser et al., 2011) at the following concentration: 0.5, 2, 5, 10, 20 and 50 µg mL^{-1}. MS or UV traces were used for quantification whenever BXD concentration in our metabolite extracts was below or above 5 µg mL^{-1}, respectively.

For fingerprinting analysis, data were processed as in Balmer et al. (2013). Interesting markers were tentatively identified as follows: molecular formulae were computed according to mass and spectral accuracies using the elemental composition calculator provided by MassLynx™. Proposed molecular formulae were then imported and verified into different metabolite databases such as the Dictionary of Natural Products and the KNapSAcK Core system to verify if those could correspond to known compounds. The putative structures were then matched with the MS/MS fragments obtained at high collision energy using MassFragment™ (Waters).

Assessment of growth parameters

Seeds were sterilized and then inoculated with a bacterial suspension as described for pre-germinated seeds. Twenty five plants per treatment were grown for seven days in humid rolled filter paper. Plant material was collected and different growth parameters were recorded for shoots and roots: length, fresh weight and dry weight. Shoots were defined as starting at the seed up to the tip of the longest leaf. Roots reached from the seed to the tip of the primary root. Following measurements on fresh material, roots and shoots were dried separately in an oven at 70°C in coffee filter papers. When sample weight remained constant

between two measurements, the dry weight was assessed. The percentage of water content in shoots and roots was calculated following the formula: ((fresh weight – dry weight)/fresh weight)*100.

Statistical analysis

Statistical analysis of metabolite and gene expression analysis were performed as described above. The rest of the comparisons were accomplished using SigmaPlot 11.0. Depending on the data distribution, a Student t-test or a Mann-Whitney Rank Sum Test were used for comparison between two groups of data. The comparison of bacterial amount in roots was achieved with a one-way ANOVA on Rank, followed by a Tukey Test ($p < 0.05$).

Results

Bacterial root colonization during plant growth

In order to test root colonization by *Pseudomonas putida* KT2440 during maize growth, the number of colony-forming units of these bacteria extracted from inoculated roots was assessed at 3, 5 and 10 days post bacterial inoculation (dpi). In mock inoculated roots, no bacteria were found. In bacterized roots, there were no statistical differences between 3dpi (2×10^7 CFU g^{-1} of fresh roots) and 5dpi (8×10^6 CFU g^{-1} of fresh roots; one-way ANOVA on Rank, followed by a Tukey Test, $p > 0.05$). In comparison to 3 and 5dpi, there were fewer bacteria present in roots at 10dpi (1×10^5 CFU g^{-1} of fresh roots; one-way ANOVA on Rank, $p < 0.05$).

Local and systemic changes in plant gene expression upon KT2440 inoculation

The plant response upon KT2440 inoculation during maize growth was tested at the transcriptional level locally (in roots) as well as systemically (in leaves). The relative expression of 21 stress-related genes was calculated by comparing control and bacteria-inoculated plants at different time points (3, 5 and 10dpi). Only few changes were observed (Figure 1). The local and systemic responses were different and more important transcriptional changes were observed in roots compared to leaves. The majority of down-regulated genes in roots were linked to the salicylic acid (SA) pathway (genes coding for the endochitinase *PR3* and the ribonuclease *PR14*). These down-regulations were observed from 5dpi. Earlier, at 3dpi, there was an up-regulation of *PR10.1*, a SA-related pathway gene. Genes involved in indole production, a defense-related volatile as well as a benzoxazinoid (BXD) precursor, *Bx1* and *IGL*, were up-regulated in roots at 3dpi and 10dpi, respectively. *IGL* was also up-regulated in leaves at 10dpi. *TPS23*, a gene coding for the volatile (*E*)-β-

caryophyllene synthesis, was up-regulated in roots at 3dpi. Abscisic acid (ABA)-related genes (*NCED* and *PIP1-5*) were up-regulated in roots at 3 and 10dpi and remained unchanged in leaves. *NCED* was only marginally 3.96 fold up-regulated in roots of KT2440-inoculated plants 10dpi ($p = 0.07$). JA-pathway related genes showed an overall slight induction in roots but only at 10 dpi in leaves. Interestingly, genes related to the auxin and ethylene pathway were only activated in leaves but not in roots. *SAUR52*, an auxin responsive gene, was down-regulated in leaves at 5dpi, but up-regulated later in plant growth (10dpi).

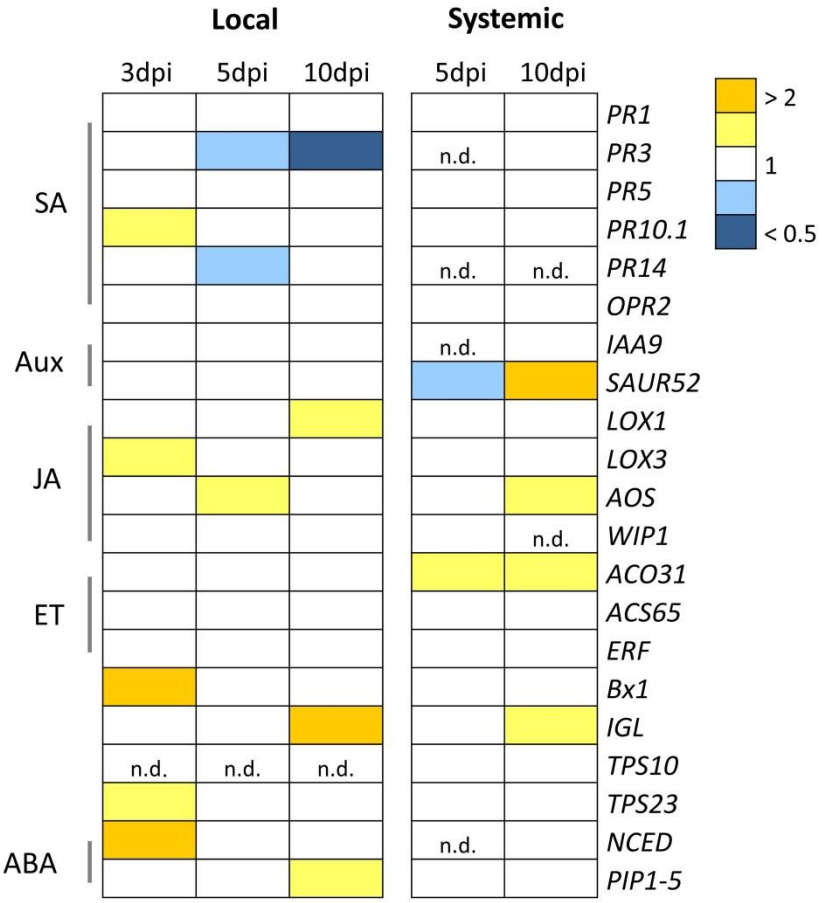

Figure 1. Local (roots) and systemic (leaves) relative gene expression profile of maize plants (var. Jubilee) upon KT2440 root inoculation. Plant material was collected 3, 5 and 10 days post inoculation (dpi). Results show the analysis of the plant material of two independent experiments pooled together (n=6 replicates, each replicate representing a pool of 3 plants). Yellow color indicates statistically significant gene up-regulation and blue color refers to down-regulated genes. n.d. = not detected. SA = salicylic acid, Aux = auxin, JA = jasmonic acid, ET = ethylene, BXD = benzoxazinoid, ABA = abscisic acid.

Local and systemic changes in plant metabolites upon KT2440 inoculation

To gain more knowledge of KT2440-related changes in maize plants, metabolite changes were also analyzed. The plant responses upon KT2440 inoculation were determined at the metabolomic level using a UHPLC-QTOF-MS system. Secondary metabolites were measured in root and leaf tissues at different time points after bacterial inoculation (3, 5 and 10 days) in order to obtain a general view of metabolomic changes and the identification of compounds that could serve as markers in the beneficial interaction between maize and KT2440.

A principal component analysis (PCA) was performed to test possible separations between treatments within time points. PCA of metabolomic fingerprinting of roots at 3dpi separated control from KT2440-inoculated plants on the first axis (PC1; Figure 2). This result indicates distinct metabolomic profiles between treatments.

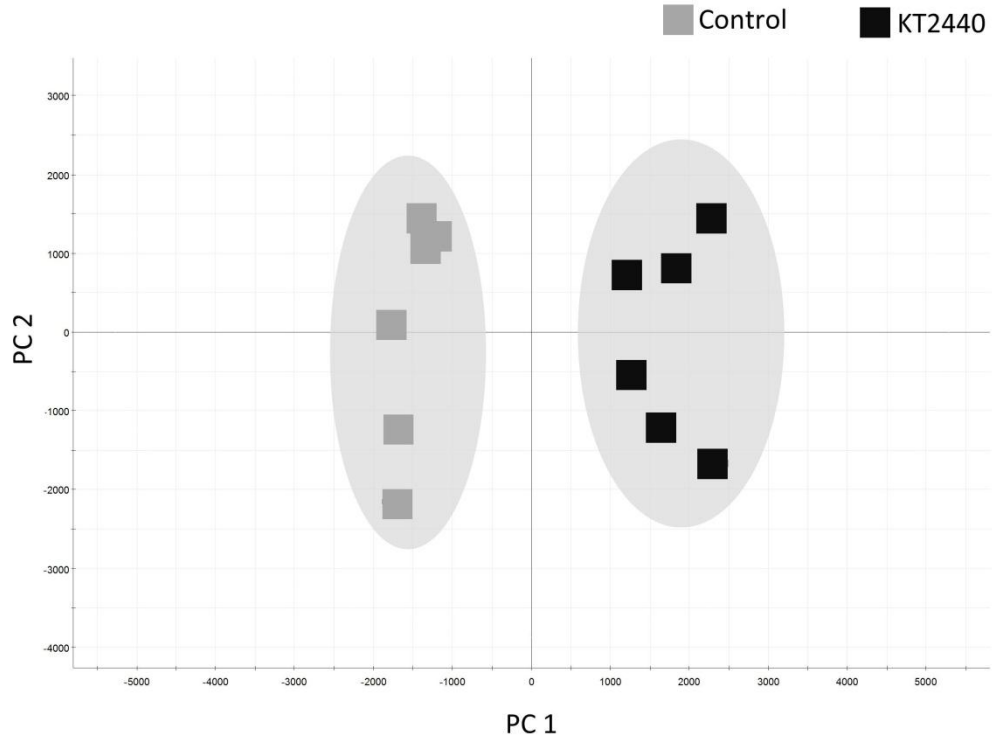

Figure 2. Principal component analysis (PCA) score plot of metabolite fingerprinting of maize roots (var. Jubilee) 3 days after root inoculation with KT2440 (n=6 replicates, each replicate representing a pool of 3 plants; plant material is coming from two independent experiments). Grey squares represent non-bacterized plants, black squares represent bacterized plants. Analysis was performed on EZinfo.

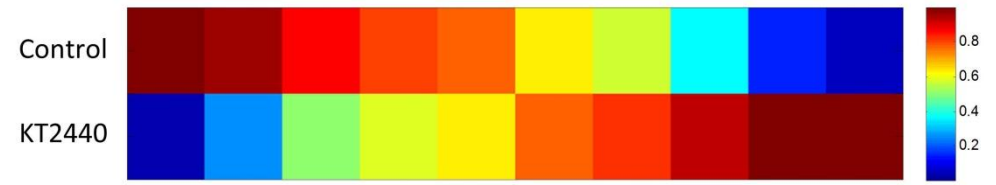

Figure 3. Cluster plot of metabolomic fingerprinting of control and KT2440-inoculated roots (maize var. Jubilee) at 3 days after inoculation. Two-hundred and eight significant markers were grouped in 10 clusters. The dark red color indicates a higher presence of selected metabolites, whereas dark blue color represents a small amount of these metabolites. The analysis was processed on MarVis.

Two-hundred and eight markers were identified (Figure 3) and clustered using the MarVis program. Compounds that were more abundant in KT2440-inoculated plants compared to control plants were found to be several phospholipids. The most representative marker had an *m/z* of 782.56 (Figure 4 and 5). This compound was significantly 14 fold more abundant in bacterized roots than in control (p=0.002; Mann-Whitney Rank Sum Test). This phospholipid was identified as a diacylglycerophosphocholine (also known as phosphatidylcholine).

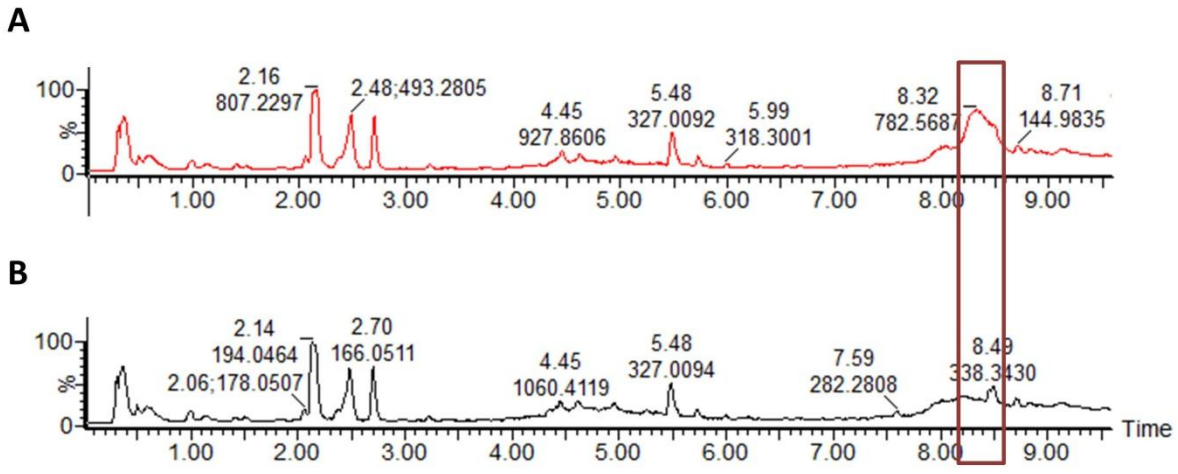

Figure 4. UHPLC-QTOF-MS fingerprints of maize roots (var. Jubilee) 3 days after bacterial or mock inoculation. **A** = KT2440-inoculated plants. **B** = control plants. The red rectangle highlights the area where compounds were significantly more present in KT2440-inoculated plants in comparison to control plants.

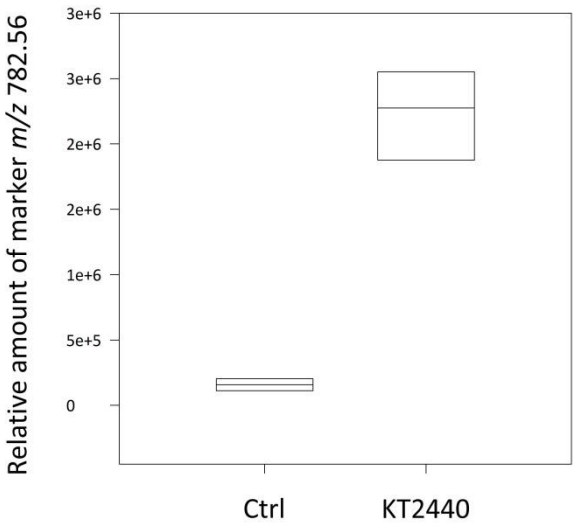

Figure 5. Relative amount of the marker *m/z* 782.56, a phosphatidylcholine, detected in roots (var. Jubilee) 3 days after inoculation with KT2440. Values were calculated from the relative median peak height of control (Ctrl) and KT2440-inoculated plants (n=6 replicates, each replicate representing a pool of 3 plants; plant material is coming from two independent experiments). The treatments were statistically different (p=0.002; Mann-Whitney Rank Sum Test)

PCA of the other time-points (5 and 10dpi) did not separate the treatments (Figure 6), showing no significant changes in secondary metabolite profiles in roots and in leaves later in KT2440-plant interaction, already 5 days after KT2440 inoculation.

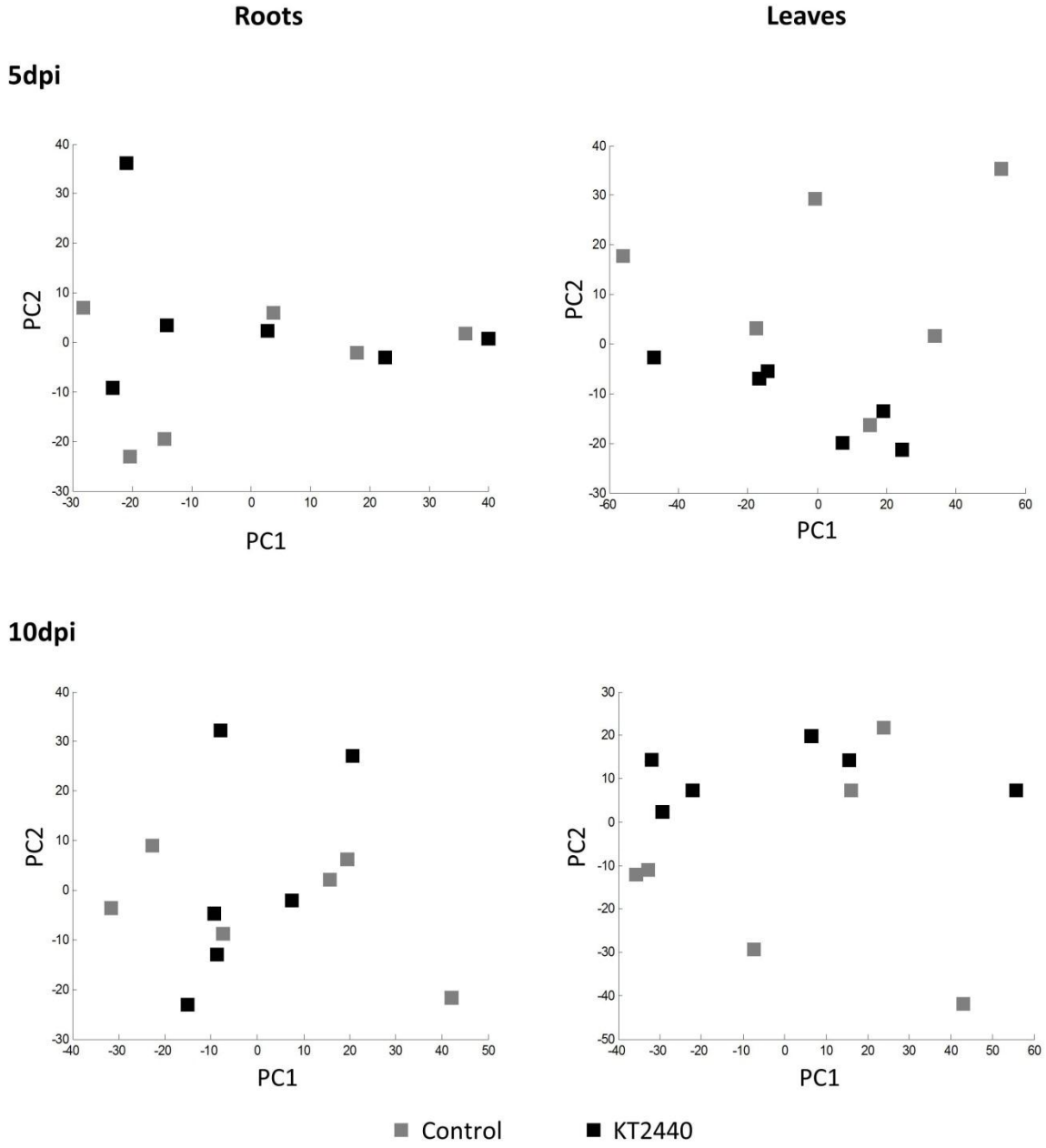

Figure 6. Principal component analysis (PCA) score plot of metabolomic fingerprinting of maize roots and leaves (var. Jubilee) 5 or 10 days after treatments (n=6 replicates, each replicate representing a pool of 3 plants; plant material is coming from two independent experiments). Grey squares represent non-bacterized plants, black squares represent bacterized plants. Analysis was performed on MarVis.

The clustering tool of MarVis was used to identify intensity profiles between treatments within the same time-point and plant tissue (Figure 7). The number of statistically significant biomarkers was higher for leaves (99 at 5dpi and 105 at 10dpi) than

for roots (75 at 5dpi and 15 at 10dpi). Clusters with higher intensities in leaves at 5dpi were present in bacteria-inoculated plants. However, the opposite observation, *i.e.* higher cluster intensity in control leaves was found at 10dpi. As differences between treatments were not remarkable, no further identifications were performed.

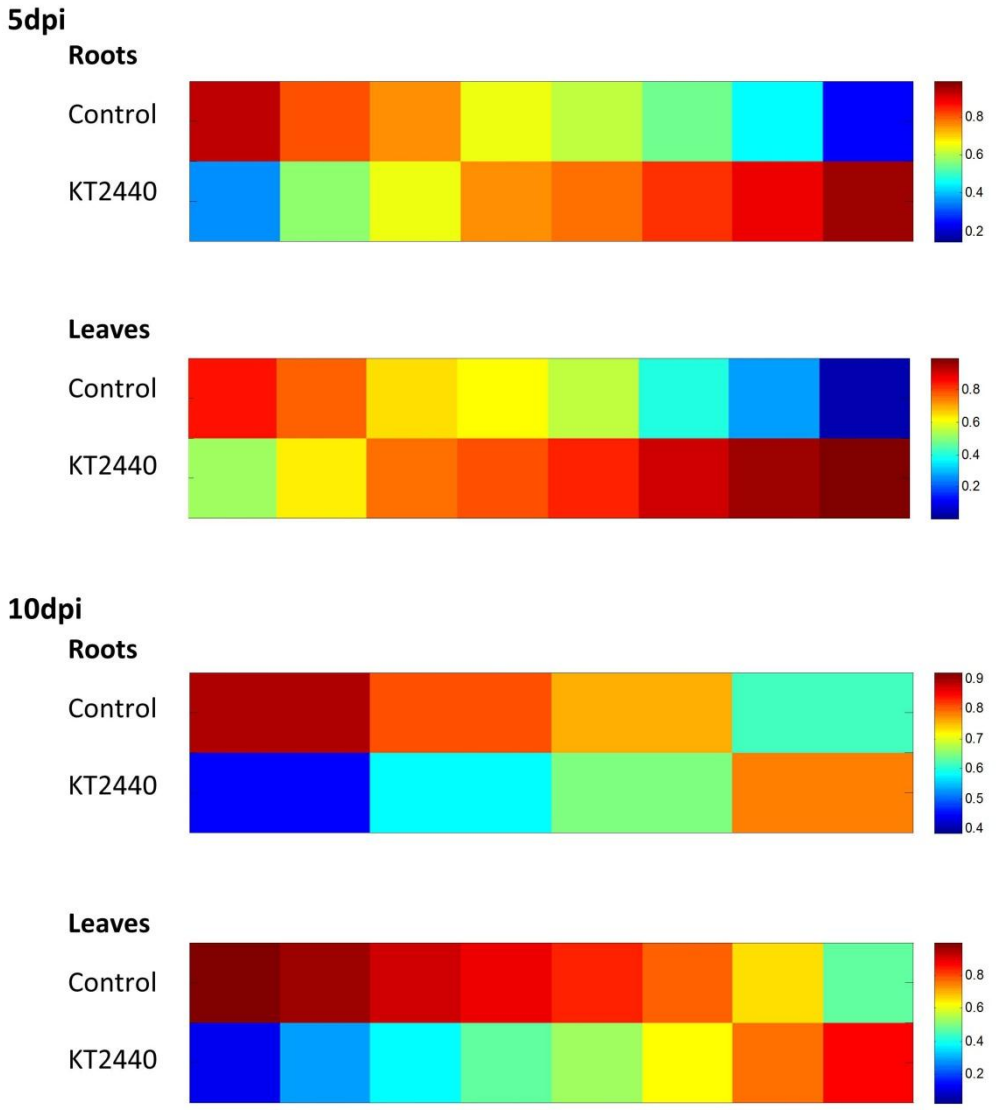

Figure 7. Cluster plots of metabolomic fingerprinting of control and bacteria-inoculated (KT2440) plants (maize var. Jubilee) detected in leaf or root tissues 5 and 10 days after bacterial inoculation (dpi) (n=6 replicates, each replicate representing a pool of 3 plants; plant material is coming from two independent experiments). Dark blue and dark red colors represent higher differences between treatments for markers present in the cluster. Analysis was performed on MarVis.

Changes in benzoxazinones content

The absolute changes of three BXD were measured in leaves and roots at different time points upon KT2440 inoculation, as described above (Figure 8). Changes between control and bacteria-inoculated plants were only observed for HDMBOA-Glc in roots at 3dpi. No other changes of BXD content were detected at later time points (t-test, $p>0.05$). Changes of BXD content pattern between leaves and roots and between the different time points were identical for control and KT2440-inoculated plants. In roots and leaves, DIMBOA-Glc decreased with age. In leaves, DIMBOA-Glc remained the most prominent BXD, whereas in roots it was HDMBOA-Glc. No DIMBOA was detected in leaves for both treatments.

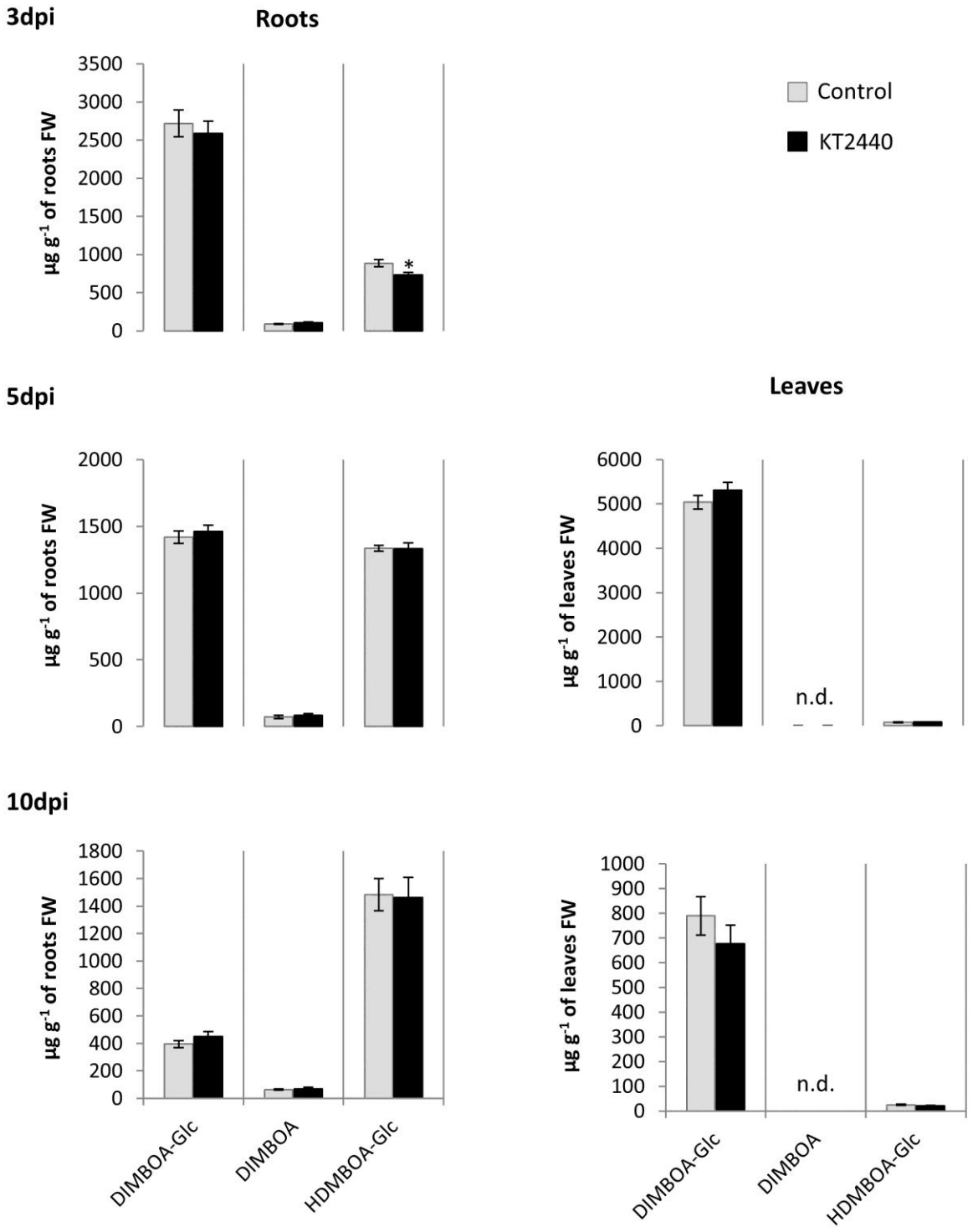

Figure 8. Absolute quantification of DIMBOA-Glc, DIMBOA and HDMBOA-Glc (µg g-1 of fresh material) in leaves and roots 3, 5 and 10 days after bacterial or mock inoculation (maize var. Jubilee, n=6 replicates, each replicate representing a pool of 3 plants; plant material is coming from two independent experiments). Star indicates a significant difference between control and bacteria-inoculated plants calculated by using a t-test: * if $p<0.05$. n.d. = not detected.

Growth promotion induced by KT2440

The consequences of KT2440 root colonization on plant physiology were assessed 7 days after mock or KT2440 inoculation (Table 1). No significant differences were observed for shoot or root length between the two treatments (Mann-Whitney Rank Sum test, $p > 0.05$) but there was a tendency of an increased shoot length of bacterized plants in comparison with control plants ($p = 0.051$). Shoot and root fresh weight, as well as dry weight, were significantly higher in KT2440-inoculated plants ($p \leq 0.007$). There were no changes in water content of roots between treatments but there was a slight decreased amount of water in leaves of bacterized plants (0.8% less, $p < 0.001$).

Table 1. Physiological measurements of control and KT2440-inoculated plants (n=25) 7 days after treatment. Median values were compared by using a Mann-Whitney Rank Sum test and p values are indicated (with a significant level fixed at $p < 0.05$).

Parameter	Control	KT2440	p value
Shoot length (cm)	12.6	13.2	0.051
Root length (cm)	22.6	22.8	0.327
Shoot fresh weight (g)	0.39	0.49	0.003
Root fresh weight (g)	0.23	0.30	0.007
Shoot dry weight (mg)	41.2	55.2	<0.001
Root dry weight (mg)	16.2	23.4	<0.001
Water content in leaves (%)	89.4	88.6	<0.001
Water content in roots (%)	92.3	92.2	0.900

Discussion

KT2440 have been well-studied for their capacity to closely interact with maize plants by adhering efficiently to maize seeds and being attracted by maize plants that release DIMBOA in the rhizosphere (Espinosa-Urgel and Ramos, 2004; Neal et al., 2012). However, little information was available concerning their direct effect on plant physiology. Here, we showed that efficient root colonization with KT2440 was accompanied by a plant reaction at the transcriptomic and metabolomic level.

Root colonization pattern of KT2440 in maize plants

As a preliminary phase, we investigated the colonization behavior of KT2440 following their application to maize seedlings. We have shown before that KT2440 cells are not evenly distributed along maize roots: stronger colonization was observed in basal root parts in comparison to root tips (Planchamp et al., 2012). Here, we investigated the maize root colonization of KT2440 during plant growth. Despite a decreased concentration of KT2440 during plant growth, in all the experiments we conducted, KT2440 cell number reached at least 10^5 CFU g^{-1} of fresh root 10dpi. This concentration corresponds to a normal bacterial colonization of the rhizoplane that ranges from $10^5 – 10^7$ CFU g^{-1} of fresh root weight (Benizri et al., 2001). Efficient root colonization by rhizobacteria is an important factor that can influence their capacity to induce plant resistance or to promote plant growth. Whereas a bacterial density of approximately 10^5 CFU g^{-1} of fresh root weight is necessary for an induction of plant resistance by the bacteria (Raaijmakers et al., 1995), Jacoud et al. (1998) showed that despite a rapid decline of *Azospirillum lipoferum* CRT1 cell numbers, a plant-growth promoting effect was present in previously inoculated maize plants. In a previous study, we showed that KT2440 was not affected by our growing system in comparison with a standard soil system (Planchamp et al., 2012). Moreover, KT2440 seems to be a competitive strain under non-sterile soil conditions (Neal et al., 2012). Then, the decrease of number of KT2440 cells is mostly due to a normal colonization stabilization instead of potential competition or non-adequate external conditions. This preliminary study on KT2440 root colonization pattern confirmed the efficiency of KT2440 to colonize roots of maize var. Jubilee and its fluctuation during plant growth before reaching a stable colonization.

Beneficial effects on plant physiology

Before our study, it was not known whether KT2440 were able to promote plant growth. Our study confirmed that KT2440 presence in maize roots improves plant physiology. An enhanced fresh and dry plant biomass in KT2440-inoculated plants and no changes for plant length were found. Considering that also no increase of water content was present in bacteria-inoculated plants, these results indicate an indirect evidence for an increase of secondary roots production in plants containing KT2440. Some plant hormones such as auxin (*e.g.* indole acetic acid, IAA) (Spaepen et al., 2009), ET (Nadeem et al., 2010), ABA and cytokinin (CK) (Nibau et al., 2008) are known to induce lateral root formation. KT2440 do not possess a functional ACC deaminase meaning that the rhizobacteria do not directly modulate the stress-related production of ET by degrading it (Wu et al., 2010). However hormone stimulation such as auxin for example would be possible to indirectly influence ET content in plant roots through an antagonistic effect. Our transcriptional analysis could indicate a potential involvement of ABA in lateral root formation and of auxin and ethylene in shoot parts. Quantitative analysis of the capacity of KT2440 to effectively induce changes in plant hormonal levels would be necessary to confirm our results.

Several bacterial mechanisms can directly or indirectly influence plant growth (reviewed by Lugtenberg and Kamilova, 2009). Eventhough we did not find growth promotion elicitors induced by the presence of KT2440, the genome of KT2440 (Wu et al., 2010) and previous studies can give some clues about PGPR properties of KT2440. KT2440 have broad capabilities for nutrient uptake and to tolerate a heavy metal polluted environment (Wu et al., 2010). KT2440 are potentially producers of hormones or volatiles that are phytostimulant such as IAA and butanediol but this remains to be experimentally confirmed. For now, records from either the literature (Leveau and Lindau, 2005) or our own experiments (data not shown) indicate that KT2440 failed to synthesize IAA from tryptophan. Further investigations using sensitive detection methods such as HPLC-MS would be necessary.

Rhizobacteria can also indirectly promote plant growth by controlling pathogen threat. Antibiotic compounds such as 2,4-diacetylphloroglucinol (DAPG) seem not to be produced by KT2440 (see Chapter 4) but antagonistic effects may be induced by KT2440 via other compounds. For example, production of the siderophore pyoverdine by KT2440 was confirmed by the appearance of a yellow fluorescent color when KT2440 is growing on an iron-limited medium (see Chapter 4). KT2440 is also able to control attacks via induction of systemic resistance (ISR). ISR triggered by KT2440 was demonstrated in Arabidopsis and maize (Matilla et al., 2010; Neal et al., 2013; Chapter 4).

Possible plant defense manipulation during maize root-KT2440 interaction

To investigate local and systemic plant responses upon KT2440 inoculation a transcriptional analysis of stress- and defense-related genes and a metabolite profiling were established. Transcriptional and metabolite changes mostly occurred in roots three days after KT2440 inoculation, compared to later time points and shoots. Specific differences were present in roots and in shoot indicating the presence of local and systemic responses to KT2440 root colonization that are tissue specific. Only few transcriptional changes and weak transcript responses were observed. This observation is in correlation with other similar studies involving ISR-triggered rhizobacteria in which the capacity of the rhizobacteria to induce plant resistance against attacks was not associated with extended transcriptional changes but by an enhanced (faster and/or stronger) plant response to attacks (Van Wees et al., 2008). Only few studies concerning early local and systemic changes in plant gene expression and metabolism induced during plant-beneficial microbe interactions have been conducted. Increasing evidence point to a hormonal signaling manipulation triggered in the roots and/or in the shoot upon first steps of the beneficial interaction (Zamioudis and Pieterse, 2012). Our transcriptional analysis revealed that maize roots respond to early steps of KT2440 colonization via a JA- and ABA-dependent signaling. Interestingly, during later steps, the SA pathway was suppressed. Considering the hormonal crosstalk existing between JA and SA pathways and that SA signaling is involved in plant defenses against pathogenic bacteria, it is

tempting to hypothesize that KT2440 exploit hormone-regulated defenses. It seems possible that KT2440 short-circuit SA-triggered responses by activating an SA counteracting signaling via JA-dependent pathway. Several examples in the literature show that attenuation of SA signaling via hormonal crosstalk mechanisms exist for a wide variety of beneficial microbes such as PGPF (Jacobs et al., 2011), mycorrhiza and rhizobia (Gutjahr and Paszkowski, 2009) and also PGPR (Okubara et al., 2010). However, in contrast to Arabidopsis response to *Pseudomonas fluorescens* GM30 (Weston et al., 2012) and WCS417 (Van Wees et al., 2008) no ET response was induced in maize roots upon KT2440 inoculation. Interestingly, the ABA-dependent pathway was locally up-regulated in maize 3 days after KT2440 inoculation. Weston et al. (2012) found that inoculation with *P. fluorescens* GM30 induced ABA-signaling 3 days after inoculation, whereas *P. fluorescens* Pf-5 did not, indicating a strain-dependent response of Arabidopsis roots. ABA has a well-known role in plant defense against abiotic stresses such as drought or salinity but recent studies showed also its involvement in plant hormone regulation (Pieterse et al., 2012). ABA works antagonistically with SA and synergistically with JA. However, the role of ABA in SA-JA crosstalk is still poorly understood. In addition to its role in hormonal balance for plant defense, ABA is also presumed to act in systemic signaling within plants. Recently, Balmer et al. (2013) hypothesized that ABA could act as a general root-shoot systemic resistance signal efficient against herbivores and hemibiotrophic fungi. So, the ABA-dependent pathway could play a role in the SA-JA crosstalk or in a root to shoot signaling during KT2440-maize interaction, or both.

During early reactions of maize roots to KT2440 inoculation, other typical defense genes were up-regulated. *TPS23* is the responsive-gene involved in (*E*)-β-caryophyllene synthesis, a plant volatile that is mainly known as an attractive substance for natural ennemies of herbivores (*e.g.* Rasmann et al., 2005). Interestingly, root emission of this volatile is also able to induce a systemic root response (Hiltpold et al., 2011). In addition to the attractive activity, (*E*)-β-caryophyllene emission may also have a direct antibacterial effect and an inhibitory role on ROS-mediated signaling (Huang et al., 2012). It is difficult to know how the presence of KT2440 can affect *TPS23* expression and the possible effect on systemic root resistance but it could be linked to the up-regulation of JA- and ABA-related genes. The pathogenesis-related protein PR10 has been associated with several constitutive and induced roles. Hence, *PR10* can be constitutively expressed and related to developmental regulation or activated by pathogens, wounding (Liu and Ekramoddoullah, 2006) or hormone application such as auxin, SA, JA, ABA and ET (Mathesius, 2009). *PR10* regulation seems to be also related to responses during beneficial interactions between plant and rhizobia (Mathesius, 2009), PGPR (Okubara et al., 2010), with the endophytic *Azocarus* (Miché et al., 2006) and mycorrhiza (Siciliano et al., 2007). Therefore, up-regulation of *PR10* in maize roots three days after KT2440 inoculation can be an illustration of its role in root development that could be promoted by the presence of KT2440, an effect of cell structure perturbation caused by the tissue colonization or a response to hormone crosstalk mediated by KT2440.

Benzoxazinones (BXD) are well-known secondary defense metabolites of the *Poaceae*. They are mainly present in the plant as glucoside derivatives such as DIMBOA-Glc and HDMBOA-Glc. BXD content varies in relation to the plant part and age (Cambier et al., 2000). In maize, content of DIMBOA-Glc is high after germination but then decreases with plant age. HDMBOA-Glc shows the same variation in shoot parts but remains at a constant level in roots becoming the dominant BXD form in roots. In our study, the same ratios as previously described were observed in roots and in shoots. Interestingly, ratio of BXD forms in plant is influenced by external attack: in response to pathogen or herbivore, DIMBOA-Glc is converted into HDMBOA-Glc (Ahmad et al., 2011; Glauser et al., 2011) or glucosides are hydrolyzed to an aglucone (*eg.* DIMBOA and HDMBOA) that is biologically more reactive than glucoside derivatives (Morant et al., 2008). HDMBOA-Glc content also increases following maize treatment with JA and MeJA. During the interaction with a non-pathogenic *Alternaria* strain there was also an increase of HDMBOA-Glc. Several studies also showed BXD ratio changes during beneficial interactions with mycorrhizal fungi or rhizobacteria. Song et al. (2011) showed that the presence of *Glomus mosseae* in maize roots induced an increase of DIMBOA content in roots. In response to inoculation with *Azospirillum* strains, DIMBOA content varied in a strain-dependent manner (increase or decrease) or did not vary compared to control (Walker et al., 2011). *P. fluorescens* F113, *A. lipoferum* CRT1 and *Glomus intraradices* JJ291 had a differential effect on BXD content on inoculated plants (Walker et al., 2012). These variations induced in beneficial interactions are very variable depending on the microbe strains and the plant cultivars that are involved. Considering also the low number of studies concerning BXD variation during beneficial interactions, it would be difficult to find a general plant response in such interactions.

The biosynthesis of BXD starts with the primary metabolite indole-3-glycerol phosphate that can be converted into indole by the indole-glycerolphosphate lyas (IGL) or the oxidative enzyme Bx1. Following indole formation, successive oxidation and methylation steps lead to the different BXD formation. The presence of up-regulations for either *Bx1* or *IGL* in roots of KT2440-inoculated plant roots could indicate an enhanced BXD production due to the plant-bacteria interaction. Several interactions between beneficial microbes and plants were associated with BXD ratio changes (see above). In addition, Neal et al. (2012) showed that KT2440 use DIMBOA exudated from maize roots as a chemotaxis signal and that KT2440 are well tolerant to this biocidal compound. Therefore, we hypothesized that KT2440 could manipulate BXD production in maize roots by increasing DIMBOA content in order to control rhizoplane colonization by favoring conspecific and by competing other rhizobacteria that would be less tolerant to DIMBOA presence. To verify this hypothesis, content of three representative BXD was quantified at the same time point as for gene expression analysis. In our study, despite the up-regulation of BXD-dependent pathway, no significant increase of BXD was found at any time point locally or systemically even though DIMBOA levels were slightly elevated in roots at 3dpi. Furthermore, HDMBOA-Glc was found to be reduced in KT2440-inoculated roots at 3dpi. This non-correlation between differential gene expression and effective BXD increased level in plant roots could maybe reflect the

release of some BXD out of the roots into root exudates. As concentration of BXD in roots does not necessarily correlate with concentration in root exudates (Niemeyer, 2009), it would be necessary to quantify BXD content in root exudates in order to reject or confirm our hypothesis.

It is noteworthy that changes in plant metabolites and transcripts mostly occurred early during the interaction. After several days, only weak or no changes were observed in contrary to more intricate beneficial interactions involving plants and mycorrhiza or rhizobia, where changes seemed to be longer-lasting. The same observation is valid for rhizobacteria with an endophytic life style. KT2440 that are rhizobacteria restricted to the rhizoplane and without an endophytic colonization (Wu et al., 2010) seem to induce changes only at the beginning of the interaction followed by an accomodation state of the plant to the presence of KT2440.

Importance of phospholipids in KT2440-maize interaction

During our metabolite profiling, phospholipids were found to be the most prevalent metabolites in roots 3 days after KT2440 inoculation. Phospholipids are structural components of biological membranes that also play a key role during perception of extracellular signals that are necessary during plant-microbe interactions. Phospholipases (PL), enzymes that hydrolyze phospholipids, are activated in response to an attack. In *Brassica napus* for example, the involvement of phospholipases in early response to systemic acquired resistance and induced systemic resistance inducers was demonstrated (Profotova et al., 2006). As reviewed by Cowan (2006), phospholipid-dependant mechanisms are linked to the mechanism of action of plant hormones involved in defense responses, such as ABA, ET, auxin, JA and CK.

Phosphatidylcholine (PC), the major compound found in KT2440-inoculated plants, is at the origin of production of important defense molecules. Hydrolyzation of PC by the phospholipase A (PLA) results in the production of fatty acids and lysophosphatidylcholine (LPC). Fatty acids are linked to JA-related pathway through their role in oxylipin and jasmonate biosynthesis. These defense compounds are generated via the activity of lipoxygenase (LOX). Interestingly, in our study *LOX3* was up-regulated concomitantly with an increased PC production, confirming a possible role of phospholipids in the maize-KT2440 interaction through JA-related pathway activation. Moreover, recently it was shown that plant lipid derivatives are key elements in cell-cell communication for KT2440. Hence, fatty acids activate a KT2440 gene that is important for population density control (Fernández-Piñar et al., 2012). This present study enhances the potential role of lipid derivatives during maize-KT2440 efficient colonization through an effect on plant immunity and bacterial density regulation.

LPC produced by PLA through PC hydrolization is proposed to have an important role in beneficial interactions and defense response. In cells of Californian poppy that were treated with elicitors, LPC activated a cytoplasmic acidification that is at the origin of phytoalexin biosynthesis (Viehweger et al., 2006). Drissner et al. (2007) demonstrated that LPC induce a strong alkalinization response by the plant, an important process in plant gene expression alteration, and proposed LPC as an important messenger that can transduce early signals during potato root colonization by mycorrhiza. PC hydrolyzation by PLD resulted in the formation of phosphatidic acid, an important second messenger for defense signaling that is also at the origin of ROS production and activation of protein kinase cascades. ROS generation and medium alkalinization are typical early defense signals that have been found to be induced in tobacco cells in response to beneficial bacterial elicitors (Van Loon et al., 2008), showing the importance of such signaling and the involvement of phospholipid-based signals in beneficial interactions between plants and rhizobacteria. It would be interesting to further elucidate the role of phospholipids in maize-KT2440 early interactions because, as for mycorrhizal interaction, phospholipids could play a role as messenger in signal transduction following KT2440 colonization (Drissner et al., 2007).

In summary, our results point to an involvement of ABA- and JA-signaling pathways in maize root MTI suppression upon KT2440 inoculation. It is speculated that the hormonal balance is manipulated by KT2440 in order to establish an efficient colonization by diminishing SA-triggered resistance of the plants. Changes in phospholipids content corroborate our hypothesis that plant resistance is activated in response to KT2440 inoculation. Changes in transcript and metabolite of maize plants mainly occurred at the beginning of the interaction and were followed by an accommodation state of the plant to the interaction. The local plant response to PGPR induces the production of a systemic signal that is currently not identified. Several putative root-to-shoot signal transduction messengers have been found in maize-KT2440 interaction: the plant hormone ABA and phospholipids. An implication of benzoxazinones in this early phase reaction would necessitate further investigations.

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Appendix

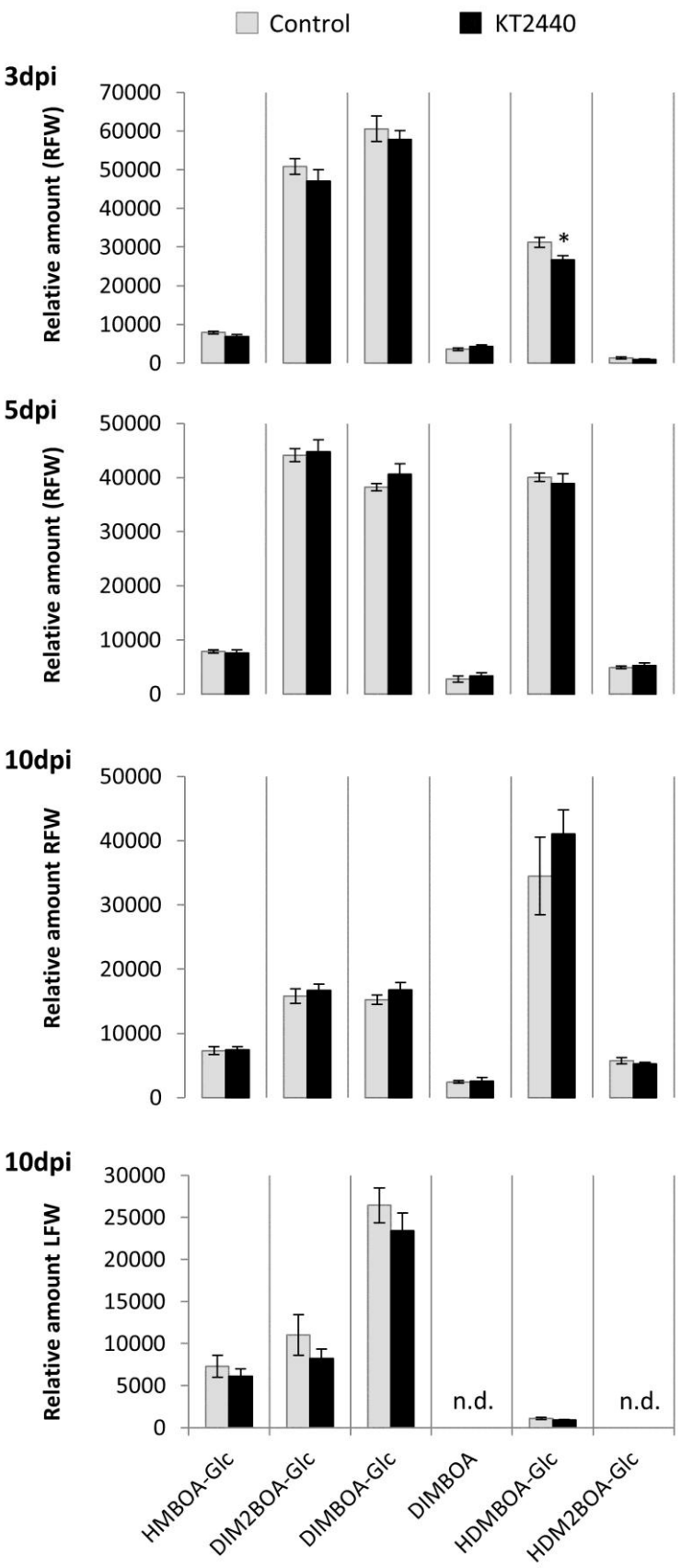

Figure S1. Relative quantification (MS traces) of six benzoxazinoids: HMBOA-Glc, DIM2BOA-Glc, DIMBOA-Glc, DIMBOA, HDMBOA-Glc and HDM2BOA-Glc in fresh roots or leaves at different time points. Star represents a significant difference between control and bacteria-treated plants: * if $p < 0.05$. n.d. = not detected.

Metabolite profiling (extra information)

Interestingly, a non-identified triglyceride (*m/z* 885.55) that was more present (more than 100 fold) in control plant roots 3dpi (88086.207 in control and 796.489 in KT2440-inoculated plants; p = 0.002, Mann Whitney Rank Sum test) was more present (600 fold) in KT2440-inoculated plant leaves 5dpi (282.957 in control plants and 169949.629 in KT2440-inoculated plants; p = 0.002, Mann Whitney Rank Sum test).

CHAPTER 4

Systemic resistance of maize plants induced by *Pseudomonas putida* KT2440 against anthracnose

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to be submitted

Abstract

In response to the biotic and abiotic stresses in their environment, plants have developed different defense strategies. Among those, the induction of defense responses only in presence of an attack has emerged as a cost efficient method. Induced systemic resistance (ISR) triggered by beneficial rhizobacteria or filamentous soil-borne fungi is associated with an enhanced plant capacity to respond to ethylene- and jasmonic acid-dependent pathways that are mostly involved in resistance to necrotrophic pathogens and insect herbivores. *Pseudomonas putida* KT2440 rhizobacteria (KT2440) are known to be associated with several economically important plants but almost nothing is known on their capacity to induce the systemic defense of such plants. Here, we demonstrate the ability of KT2440 to trigger ISR in maize plants against a biotic stress induced by the hemibiotrophic fungus *Colletotrichum graminicola*, the causal agent of maize anthracnose, by blocking or delaying the necrosis development stage. This defense state was reflected at the transcriptional and metabolomic level by a decreased defense response in KT2440-inoculated plants compared to mock-treated infected plants four days after infection.

Introduction

Beneficial interactions between plants and microorganisms occur frequently and are known to improve plant nutrition and/or help plants to overcome biotic or abiotic stresses. Mycorrhizal fungi and *Rhizobium* spp. are among the most studied beneficial microorganisms (Van der Ent et al. 2009; Gutjahr and Paszkowski, 2009). Mycorrhiza provide the host with an increase in root surface to absorb mineral nutrients such as phosphate, whereas rhizobia fix nitrogen from the atmosphere. Rhizosphere bacteria that stimulate plant growth and defenses, called plant growth-promoting rhizobacteria (PGPR), form another class of beneficial soil-borne microorganisms (Lugtenberg and Kamilova, 2009). In general, the most commonly occurring genera for PGPR are *Pseudomonas* spp., *Serratia* spp. and *Bacillus* spp.. These bacteria colonize the rhizosphere where they live off nutrients present in root exudates.

PGPR can directly influence plant growth through various mechanisms such as nitrogen fixation, ion uptake or the production of plant hormones or by acting indirectly against biotic threats. A direct effect of non-pathogenic rhizobacteria against an aggression has also been shown to be achieved through nutrient competition or production of antibiotics (Van Loon, 2007; Lugtenberg and Kamilova, 2009). Antifungal metabolites such as 2,4-diacetylphloroglucino (DAPG) or siderophores like pyoverdin can be produced by PGPR. Some PGPR prime plants to react faster and/or more effectively to an attack. The resulting defense state, called induced systemic resistance (ISR), is effective against a broad range of different stressors (see De Vleesschauwer and Höfte, 2009 and Van Hulten et al, 2010 for reviews). Plant growth promoting fungi (PGPF) such as *Piriformospora indica* (Waller et al., 2005) and *Trichoderma* spp. (Hermosa et al., 2012) may also protect plants from pathogenic aggressions. The plant defense mechanisms triggered by ISR are similar for the different inducers (Pineda et al., 2012).

Recognition of PGPR by plants through microbe-associated molecular patterns (MAMPs) such as siderophores, salicylic acid (SA), flagellin, lipopolysaccharides (LPS) or antibiotics, is followed by the suppression of MAMP-triggered immunity by the rhizobacteria via modulation of plant hormonal signaling (Zamioudis and Pieterse, 2012). Following microbe perception, an ISR signal is generated in the systemic tissue. Instead of inducing direct extensive changes in systemic plant parts, ISR is associated with priming for an enhanced expression of genes involved in jasmonic acid (JA)-, ethylene (ET)- and abscisic acid (ABA)-dependent pathways after an attack by a herbivore or a pathogen (Van Wees et al., 2008). Due to priming of the JA- and ET-dependent pathway, ISR is commonly recognized as as being effective against necrotrophic pathogens and herbivores. However, recent studies showed a more complex situation as specific transduction pathways activated during ISR depend on the host plant, the PGPR strains and the attacker. *Pseudomonas fluorescens* SS101 induces resistance against *Pseudomonas syringae* pv. tomato DC3000 in Arabidopsis via SA-signaling (Van de Mortel et al., 2012) and *Bacillus cereus* AR156 activates SA-, JA-, and

ET-dependent defense signaling in the same plant-pathogen system (Niu et al., 2011). In tomato plants, *P. putida* LSW17S induces resistance against a biotrophic and a necrotrophic pathogen (Ahn et al., 2011). However, De Vleesschauwer and colleagues (2009) showed that *Serratia plymuthica* ICI270-ISR was efficient against the hemibiotrophic fungus *Magnaporthe oryzae* but not against necrotrophs. In plant resistance induced by PGPR against herbivores still only little is known about the role of ISR (Pineda et al., 2010).

During ISR defense compounds such as phenylpropanoid pathway derivatives (phenolics) or indole derivatives are generated. Phenolic compounds were shown to rapidly accumulate during resistance against *M. oryzae* triggered by *P. fluorescens* WCS374r (De Vleesschauwer et al., 2008). Callose deposition is enhanced at the pathogen penetration site during ISR (Van Wees et al., 2008). Glucosinolates play a prominent role in resistance induced by *P. fluorescens* SS101 against a pathogen and an herbivore (Van de Mortel et al., 2012). Benzoxazinones (BXD) are important indole derivatives of the *Poaceae* family. The various forms of BXD are major defense compounds against herbivory and pathogenic attack (Ahmad et al., 2011; Marti et al., 2012). Furthermore, Ahmad et al. (2011) demonstrated that the BXD DIMBOA plays a key role for callose deposition, an important ISR-related mechanism. ISR triggered by mycorrhiza in maize plants illustrated that BXD also play a role in ISR (Song et al., 2011).

Most of the studies about induced resistance involve dicotyledonous plants. Although monocotyledons represent an economically important plant group, studies about wheat, maize or other cereals are less common. Induced systemic resistance mechanisms are effective against a wide range of pathogens (Djonovic et al., 2007) and herbivores (Saravanakumar et al., 2007) and the induced mechanisms seem to be similar in monocots and dicots. However, the regulation of induced resistance pathways and interaction with other plant pathways may be quite divergent (Kogel and Langen, 2005; Balmer et al., 2012).

While indirectly induced defenses of maize plants and herbivore-induced defenses are well documented (Turlings et al., 1995; Rasmann et al., 2005; Ton et al., 2006; Chen, 2008; Erb et al., 2009), only few studies report on direct systemic induced resistance such as ISR (Balint-Kurit and Johal, 2009; Balmer et al., 2012). The studied interactions between maize plants and beneficial microorganisms concern essentially growth promotion or improved tolerance against abiotic stresses (Nadeem et al., 2006), bioremediation and direct antagonistic effect against soil-borne pathogens (Wicklow and Poling, 2009). ISR has been documented in maize in response to the root colonizing fungi *Trichoderma harzianum* (Harman et al., 2004) and *T. virens* (Djonovic et al., 2007). The latter study demonstrated that *T. virens* systemic protection is associated with an induction of JA-biosynthetic genes and of genes involved in the production of green leaf volatiles. Mycorrhized maize plants showed an induction of SA- and JA-signaling pathways after being challenged by *Rhizoctonia solani*. This defense was also associated with an increased DIMBOA content and an up-regulation of DIMBOA-related genes (Song et al., 2011). Only two studies have reported the

elicitation of ISR in maize by PGPR. *Bacillus cereus* C1L induced maize resistance against southern corn leaf blight via the release of dimethyl disulfide (Huang et al., 2012) and the *Pseudomonas aurantiaca* JD37 induced resistance against the same pathogenic fungus (Fang et al., 2012). However, no ISR mechanisms have been described.

Pseudomonas putida KT2440 (KT2440) are well-known rhizobacteria that have been mainly studied for their capacity to colonize maize seeds (Espinosa-Urgel and Ramos, 2004) and to regulate colonization via cell-cell communication (Fernández-Piñar et al., 2012). KT2440 show a specific ability to live in the maize rhizoplane: these bacteria are tolerant to DIMBOA, a biocidal compound released by maize plants into the rhizosphere, and they exploit this chemical as a chemotaxis signal (Neal et al., 2012). A recent study showed the ability of KT2440 to prime maize defense response upon an herbivore-mimicking treatment (wounding and jasmonic acid treatment). The aim of our study was to test the capacity of KT2440 to induce maize resistance against a hemibiotrophic pathogen, *Colletotrichum graminicola*. The mechanisms involved in ISR priming triggered by KT2440 were analysed at the transcriptional and metabolomic level.

Material and Methods

Biological material

In this study, the maize variety Golden Jubilee (West Coast Seeds, Canada) was used for its susceptibility to corn anthracnose (Venard and Vaillancourt, 2007). Maize seeds were rinsed in 70% ethanol 3-5 times and soaked in bleach 10% for 5 minutes. After sterilization, seeds were rinsed 5 times with sterile distilled water and placed in humid rolled Filterkrepp filter paper (E. Weber & Cie AG, Switzerland) for two days of germination in a growth chamber (16:8 Light:Dark 26°C:22°C, 400 µmol m^{-2} s^{-1}). Seedlings were then grown into the SF-ROBS system as described (Planchamp et al., 2012). Seedlings were placed for 10 days in the same growing conditions as for germination.

Pseudomonas putida KT2440, a rifampicin-resistant strain, was cultivated on Luria-Bertani (LB; Difco LB, Becton, Dickinson and Company, France) agar supplemented with 100 µg mL^{-1} of rifampicin, in the dark at 25°C. After two to five days, a single colony of KT2440 was transferred to 100 mL of LB liquid medium containing 100 µg mL^{-1} of rifampicin for an overnight culture (28°C, shaking at 150rpm). Pre-germinated seeds were inoculated with bacterial suspension, for bacterized plants, or sterile M9 minimal medium, for control plants, as described (Planchamp et al., 2012).

A GFP-expressing strain of the hemibiotrophic fungus *Colletotrichum graminicola* (*C. graminicola-gGFP;* Erb et al., 2011), the causal agent of corn anthracnose, was used for fungal infection tests. Fungal culture was maintained on potato dextrose agar (PDA; Difco PDA, Becton, Dickinson and Co., France) at 25°C, under continuous light (70 µM m^{-2} s^{-1}).

Antagonistic activity assays

The production of antibiotic compounds can be tested under *in vitro* conditions by growing bacteria on specific media. King's medium B (KB; Pseudomonas Agar F, Merck KGaA, Germany) was used to test the production of pyoverdines and malt agar (MA; Malt Agar, Biofile Italiana S,r,l., Italy) to test the induction of 2,4-diacetylphloroglucinol (DAPG). PDA and LB were used as control media. A 5 mm plug of a 3 week-old *Colletotrichum graminicola* culture was applied to the centre of plates containing solid medium and two different time points of bacteria application were tested: 2 days after pathogen application or on the same day. Four different treatments were tested: control (M9), an overnight culture of KT2440, a half concentrated overnight culture of KT2440 and a filtered bacterial overnight culture containing bacterial exudates (filter of 2 µm). Bacterial overnight culture and M9 were either tested separately or with the other treatments. The test-treatments were applied to 6 mm diameter filter discs (15 µl per disc) at 2.5 cm from the fungal plug and 7-8 mm from the plate border, in a cross alignment. Plates were kept at 25°C in the dark. Five days later, the appearance of a yellow or a brown coloration in KB or MA media, respectively, and inhibition zones were observed. Each experiment was repeated twice.

Induced resistance assays

Spores from a 3-week old *C. graminicola-gGFP* culture were collected in $MgSO_4$ (containing 0.01 % of Silwet L-77; Lehle Seeds, USA) and the spore concentration was adjusted to 10^6 spores mL^{-1}. Twelve-day old maize plants were infected or mock-treated (n=12 for fungal growth measurement; n=12 for metabolite and gene expression analysis) as follows: 20 µl drops of spore suspension or $MgSO_4$ were spread with a paintbrush on 2-4 spots on the surface of the second and the third maize leaves (var. Golden Jubilee). After inoculation, plants were kept at 25°C in the dark and high relative humidity (>90%) for 16 hours. After this incubation time, plants were returned to the growth chamber. Four days after infection, fungal colonization was quantified as described previously (Erb et al., 2011) by calculating a ratio between the fungus and the leaf on a predetermined area. Two independent experiments were pooled together (n=24 plants).

Before plant inoculation with *C. graminicola*, shoot parts of plants were measured with the aim to detect a possible promotion of plant growth induced by the presence of KT2440.

Bacterial quantification in roots and shoots

At the end of each induced resistance assay, roots and shoots from treated and control plants were collected to test for the presence of KT2440. Hundred mg of fresh root or 50 mg

of fresh leaf tissues were ground in 600 µL of $MgSO_4$ 10mM. Serial dilutions of these root or shoot extracts were plated on King's medium B containing 100 µg of rifampicin and incubated at 25°C in the dark. Eighteen to twenty hours later, colonies were counted and the colony-forming units (CFU) per gram of fresh root or shoot were determined.

Gene expression analysis

Four days after *C. graminicola* infection, plant material (root and leaf) was collected and flash-frozen in liquid nitrogen. Root and leaf material from 2-3 biological replicates (one replicate representing a pool of three plants; from plant material of a single experiment) were separately ground with a mortar and a pestle taking care to keep the plant material frozen. Steps from RNA extraction to relative gene expression calculation (by qRT-PCR) with the two housekeeping genes *Zm-GAPc* and *Zm-Actin* were performed as described (Pfaffl et al., 2002; Balmer et al., 2013).

Metabolomic analysis

Plant material from 3 or 4 biological replicates (one replicate representing a pool of three plants; from plant material of a single experiment) was collected and directly flash-frozen. Hundred mg of frozen ground tissue was mixed with 1 mL of extraction solvent (methanol 80%, water 19.5%, formic acid 0.5%) in order to extract metabolites. Metabolite analysis was processed by using UHPLC-QTOF-MS as previously described (Balmer et al., 2013). The resulting peak lists of the fingerprint analysis were subjected to principal component analysis on EZinfo (Umetrics, Umea, Sweden) after a Pareto scaling. A supervised data mining approach was applied by proceeding orthogonal partial least squares discriminant analysis (OPLS-DA or PLS-DA). Discriminant markers were tried to be identified first via the molecular formulae. They were computed according to mass and spectral accuracies using the elemental composition calculator provided by MassLynx™. Proposed molecular were verified into different metabolite databases (KNapSAcK Core system and Dictionary of Natural poducts). A relative quantification (peak area measurement) in leaves and roots four days after *C. graminicola* infection was done for the following benzoxazinones (BXD): DIMBOA-Glc, HMBOA-Glc and HDMBOA-Glc.

Statistical analysis

Statistical analysis of metabolite and gene expression analysis were performed as described above. The rest of the comparisons were accomplished using SigmaPlot 11.0. Depending on the data distribution, a Student t-test or a Mann-Whitney Rank Sum Test were used. The comparison of relative BXD quantification was achieved with a Kruskal-Wallis One Way

ANOVA on Ranks, followed by a Dunn's Method Test ($p < 0.05$), between the different treatment and within the same compound.

Results

Direct *in vitro* inhibitory effect of *Pseudomonas putida* KT2440 against *Colletotrichum graminicola*

Some rhizobacteria release substances that can directly affect a pathogen in the rhizosphere. Bacterial overnight culture and M9 (control) were applied on the same Petri dish as the other treatments (bacterial exudates and half amount of a bacterial overnight culture) (Figure 1) or in separated Petri dishes (Figure 2). There were no visible inhibitory zones around the bacteria on PDA, LB and MA (Figure 1 A, B and C). No DAPG, characterized by a brown coloration in the medium, was produced by KT2440 on MA (Figure 1C). On KB, an inhibition zone and a yellow coloration of the medium were clearly visible (Figure 1D, 2A and 2B). The same result was observed where treatments were in separated Petri dishes. The yellow color points to the presence of pyoverdine (also called pseudobactin or fluorescein), a siderophore produced by bacteria that is fluorescent under UV light (Figure 2). Repetitions of these experiments lead to the same results.

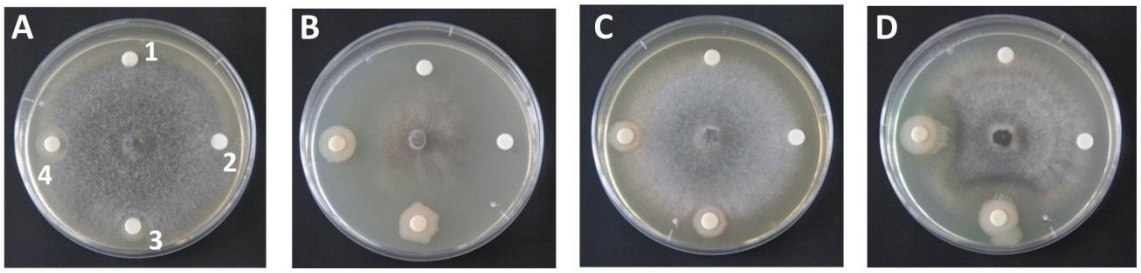

Figure 1. Test of a potential antagonistic effect of KT2440 against *C. graminicola* on different media (A = potato dextrose agar, B = Luria Bertani, C = malt agar, D = King's medium B). Four different treatments were tested: 1. control (M9 medium), 2. bacterial exudates, 3. overnight KT2440 culture and 4. half-strength overnight KT2440 culture. The plates were inculated with *C. graminicola* two days before treatment. An inhibitory zone was observed on picture D.

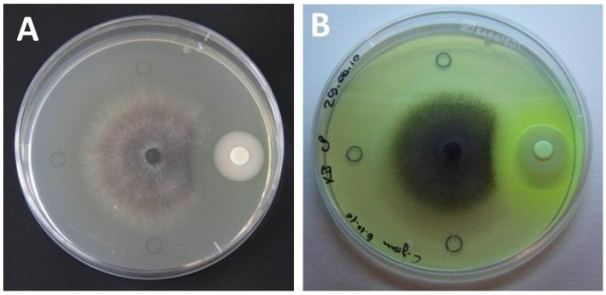

Figure 2. Presence of an inhibitory zone and production of pyoverdines by KT2440 on King's medium B after 5 days of growth: a yellowish fluorescent compound under normal (A) and UV light (B). Treatments were tested on separated plates. *C. graminicola* and treatments were applied to the media on the same day.

Induced systemic resistance (ISR) against *Colletotrichum graminicola*

To test the resistance induced by KT2440 against *C. graminicola* in maize leaves, fungal colonization of the tissue was measured four days after infection (dai), corresponding to the fungal necrotrophic phase. In addition, shoot length was measured in order to test for plant growth promotion induced by KT2440. Shoot length was not statistically different between control plants (21.6 cm) and KT2440-inoculated plants (21 cm; p = 0.875, Mann-Whitney Rank Sum test), indicating no induction of plant growth due to the presence of bacteria in the rhizosphere. However, the leaf area colonized by *C. graminicola* was twenty times smaller in bacterized plants compared to non-bacterized plants (p< 0.001, Mann-Whitney Rank Sum test; Figure 3). These differences are clearly visible in Figure 4. Mock-treated infected plants show necrosis at infection sites (marked by a black dot), whereas KT2440-inoculated plants show almost no necrosis.

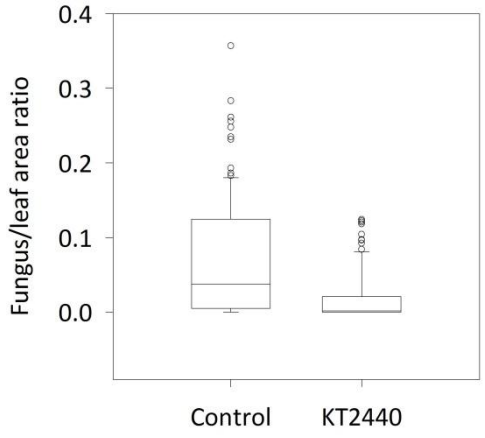

Figure 3. Twelve-day old maize plants var. Jubilee inoculated with a spore suspension of *C. graminicola*. Fungal growth in mock-treated (control) and KT2440-inoculated plants was measured 4 days after infection (during the necrotrophic phase) by calculating the ratio between fungal and leaf areas on a predetermined leaf area (n=24 plants per treatment, from two independent experiments). Treatments were statistically different (p<0.001; Mann-Whitney Rank Sum test).

Figure 4. Symptoms on maize leaves (var. Jubilee) due to infection by *C. graminicola* on mock-treated plants (A) and KT2440-inoculated plants (B) 4 days after inoculation (corresponding to the necrotrophic phase). Lesions are almost absent for KT2440-inoculated plants.

Four days after inoculation with *C. graminicola* bacterial presence in maize roots and leaves was quantified. No KT2440 were present in control plants and in leaves of KT2440-inoculated plants. In inoculated roots, 2.97 x 10^6 CFU g^{-1} of fresh root of KT2440 were counted.

Local and systemic transcriptional changes in plant during ISR against *Colletotrichum graminicola*

Gene expression (by qRT-PCR) following three different treatments was assessed four days after infection with *C. graminicola*: control plants that were mock-treated, control plants that were infected with *C. graminicola* and KT2440-inoculated plants that were infected with *C. graminicola*. Three different two-by-two comparisons of treatments were executed (Figure 5) to test for transcriptional changes of 44 defense-related genes as described (Balmer et al., 2013).

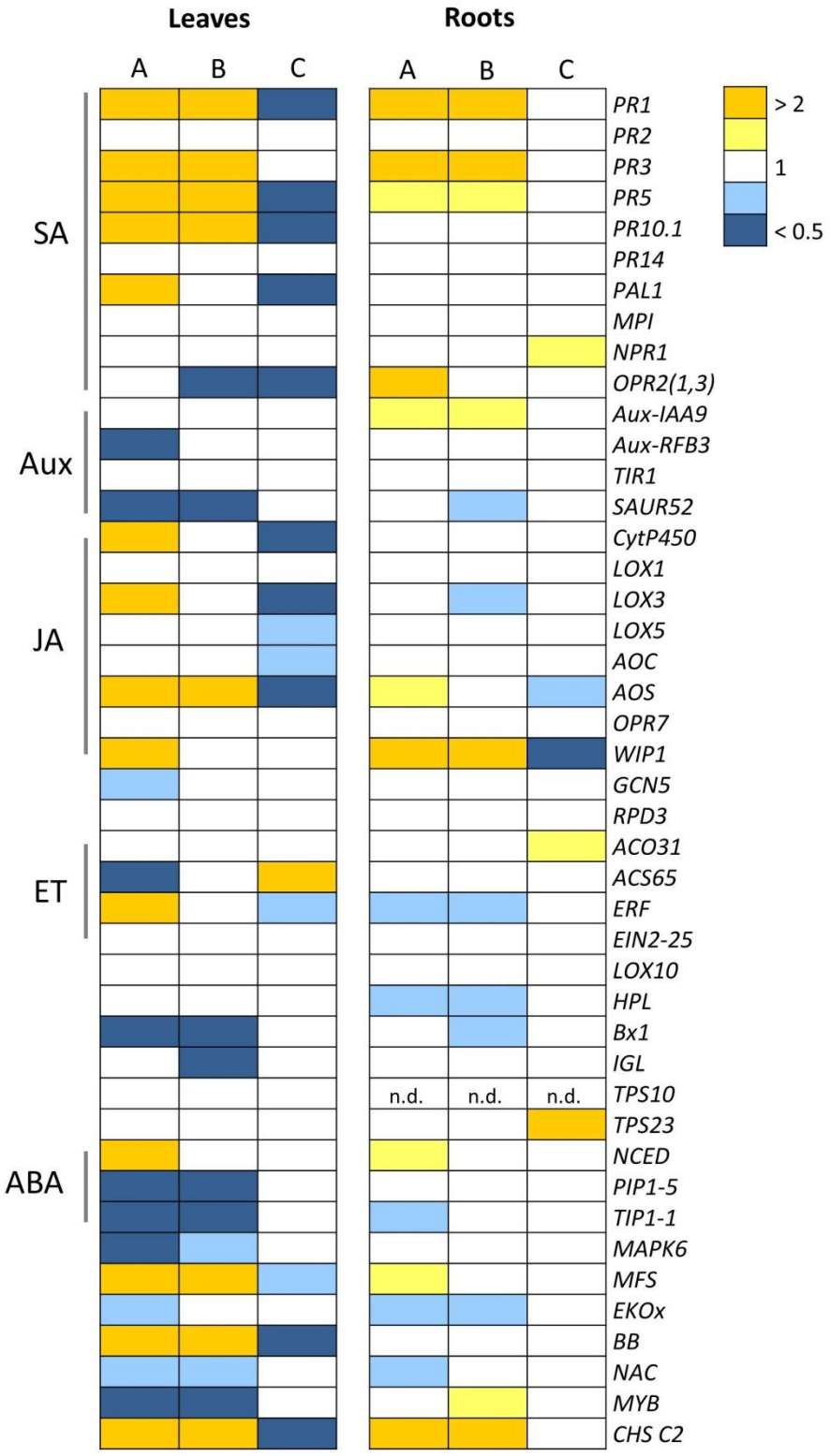

Figure 5. Local and systemic transcriptional changes in maize leaves (var. Jubilee) four days after inoculation with *Colletotrichum graminicola*. Relative gene expression was calculated by comparing control plants with either mock-treated plants infected with *C. graminicola* (A) or KT2440-inoculated plants infected with *C. graminicola* (B). In order to visualize the specific effect of the presence of the bacteria, mock-treated infected plants and bacterized infected

plants were compared (C). n=6 plants for control plants and n=9 for infected plants (plant material from a single experiment). Yellow color represents statistically significant up-regulated genes and blue color refers to down-regulated genes. Abbreviations: n.d. = not detected, SA = salicylic acid, Aux = auxin, JA = jasmonic acid, ET = ethylene, BXD = benzoxazinoid, ABA = abscisic acid.

In general, local and systemic transcriptional responses were similar between KT2440-inoculated plants and mock-treated plants challenged with *C. graminicola* (Figure 5, column A and B). However, some genes were specifically expressed in KT2440-inoculated plants or in controls in response to the fungal infection such as *LOX3* or *NCED*. Four days after infection, genes involved in the salicylic acid-dependent pathway were up-regulated in leaves of bacterized and non-bacterized plants. The jasmonic acid pathway was more activated in leaves of mock-treated infected plants than in bacterized infected plants. Even if responses were similar, there was a reduced induction of most of these genes when comparing control infected plants to KT2440-inoculated infected plants (Figure 5, column C). Only a gene of the ethylene-related pathway, *ACS65*, had an increased expression level in KT2440-inoculated plants four days after *C. graminicola* inoculation in comparison to control infected plants. There were fewer up-regulated genes in roots compared to leaves, showing a more intense local reaction to pathogen infection in leaves. In roots, some genes were specifically expressed in KT2440-inoculated plants or in control plants after infection. In leaves, the effect of KT2440 was associated with a general attenuated response. In roots, few changes were linked to the presence of KT2440 when comparing KT2440-inoculated plants to mock-treated plants after infection with *C. graminicola* (Figure 5, root part, column C): *NPR1*, *ACO31* and *TPS23* were up-regulated and the JA-related *AOS* and *WIP* were down-regulated.

Metabolomic profiling during ISR against *Colletotrichum graminicola*

To further understand the effect of bacterial presence in maize roots against *C. graminicola*, a metabolomic fingerprinting was undertaken. A principal component analysis of the metabolome was performed to compare non-infected control plants, plants that were infected with *C. graminicola* and KT2440-inoculated plants infected with the same pathogen (Figure 6). Groups of non-infected and infected plants were clearly separated for leaf material. Some compounds that were more present in infected or in non-infected plants were identified. Several defense markers were more abundant in infected plants, whereas in control plants, mostly molecule conjugates were present. Lysophosphatidic acid (*m/z* 483.26, LPA 16:0; *m/z* 481.25, LPA 16:1), a chlorogenic acid (*m/z* 353.08), citric acid (*m/z* 191.01) and a partially identified compound $C_{27}H_{45}N_2O_8P$ (*m/z* 555.28) were more abundant in infected plants. In contrary, the relative abundance of DIMBOA-Glc (*m/z* 372.09), the

cinnamate ester 2-*O*-caffeoyl-hydroxycitric acid (*m/z* 369.04), kaempferol-glucoside-rhamnoside (*m/z* 593.15) and maysin (*m/z* 575.14) were reduced in pathogen-infected leaves. In addition, a monogalactosyl diglyceride (MGDG) (*m/z* 707.43), containing linolenic acid (18:3) and $C_{12}H_{19}O_3$ (putatively 12-oxo-cis-9-dodecenoate) as glycerides, was absent in infected plants.

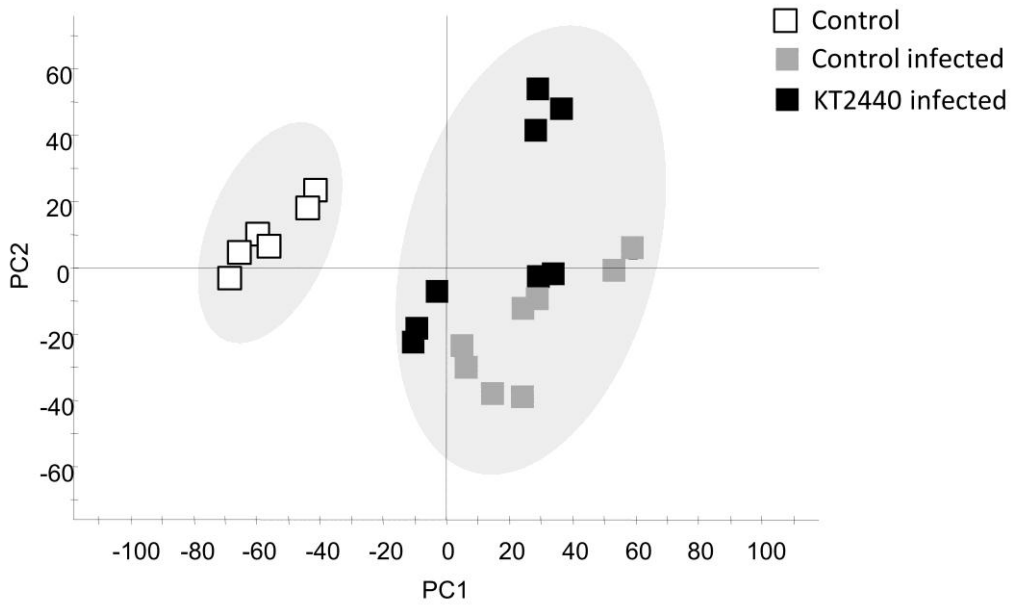

Figure 6. Principal component analysis (PCA) score plot of metabolomic fingerprinting of maize leaves (var. Jubilee) of control plants (white squares), *C. graminicola*-infected plants (grey squares) and bacterized infected plants (black squares) four days after fungal inoculation. n=6 for control plants and n=8 for infected plants (plant material from a single experiment). Analysis was performed on EZInfo.

As a supervised analysis (a PLS-DA) enhanced the differences between the three groups of treatment (data not shown), a comparison between control and KT2440-inoculated plants that were infected with *C. graminicola* was processed. The OPLS-DA (Figure 7) enhanced a difference due to the plant treatments, indicating a distinct metabolomic profile. Two hundred and fourty six significant leaf markers were identified on MarVis between control and KT2440-inoculated plants after infection with *C. graminicola*. Most significantly different metabolites in leaves were up-regulated in control infected plants compared to bacterized infected plants.

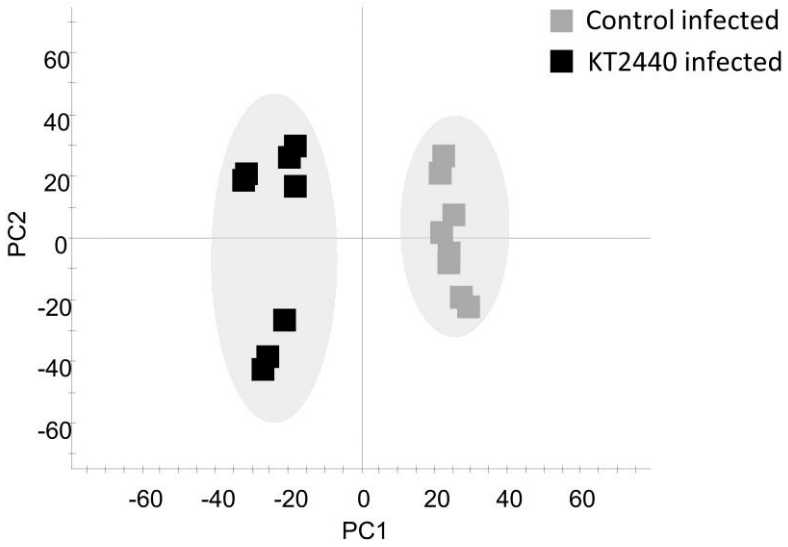

Figure 7. Orthogonal partial least squares discriminant analysis (OPLS-DA) score plot of metabolomic fingerprinting of maize leaves (var. Jubilee) of *C. graminicola*-infected control plants (grey squares) and bacterized infected plants (black squares) four days after fungal inoculation. n=8 (plant material from a single experiment). Analysis was performed on EZInfo.

The most representative changes in metabolite content between treatments concerned HDMBOA-Glc. Benzoxazinones (BXD) are important defense compounds of maize plants against herbivores, pathogenic fungi and aphids (Ahmad et al., 2011; Marti et al., 2013). To identify a role of BXD in ISR triggered by KT2440 against *C. graminicola*, a relative quantification of three different BXD was performed on leaf and root tissues four days after infection (Figure 8). No changes of HMBOA-Glc were observed. Changes of DIMBOA-Glc were only present in leaves and were characterized by a decreased amount of this compound in KT2440-inoculated plants in comparison with control plants. The quantity of HDMBOA-Glc in leaves was similar in control plants and KT2440-inoculated infected plants. However, in roots, HDMBOA-Glc was present in significantly higher amounts in KT2440-inoculated infected plants compared to control plants. Four days of *C. graminicola* infection seemed to induce an increased level of HDMBOA-Glc in leaves and roots but no changes were observed concerning the other BXD.

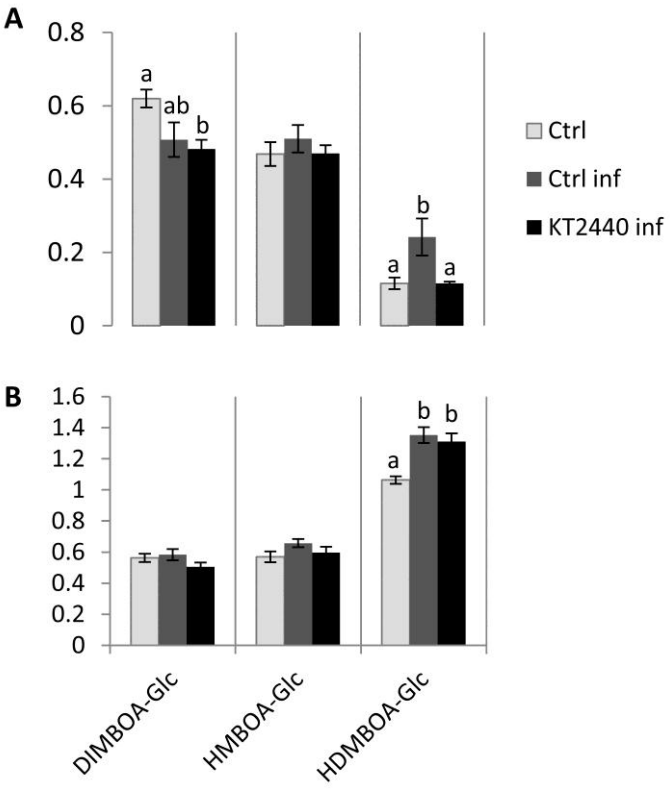

Figure 8. Relative quantification of benzoxazinones in leaves (A) and in roots (B) four days after *C. graminicola* inoculation (maize var. Jubilee). n=6 for control plants and n=8 for infected plants (plant material from a single experiment). Ctrl = control, inf = infected with *C. graminicola*. Differences between treatments within the same compound are presented as different letters.

Based on Balmer et al. (2013), several compounds, mainly hydroxycinnamic acid derivatives that showed an accumulation in maize leaves during *C. graminicola* infection, were identified (Appendix, Table S1). Markers were all more abundant in control infected plants in leaf tissue compared to KT2440-inoculated plants that were infected with *C. graminicola*.

Systemic metabolomic profiling during ISR against *Colletotrichum graminicola*

The systemic effect of ISR on metabolite profiles was analyzed with a principal component (PC) analysis and a PLS-DA of metabolomic fingerprinting of non-infected control plants and KT2440-inoculated plants and mock-treated plants four days after *C. graminicola* inoculation (Figure 9). Neither a PCA, nor a PLS-DA managed to identify groups within the samples. ISR triggered by KT2440 in leaf parts does not seem to induce changes systemically.

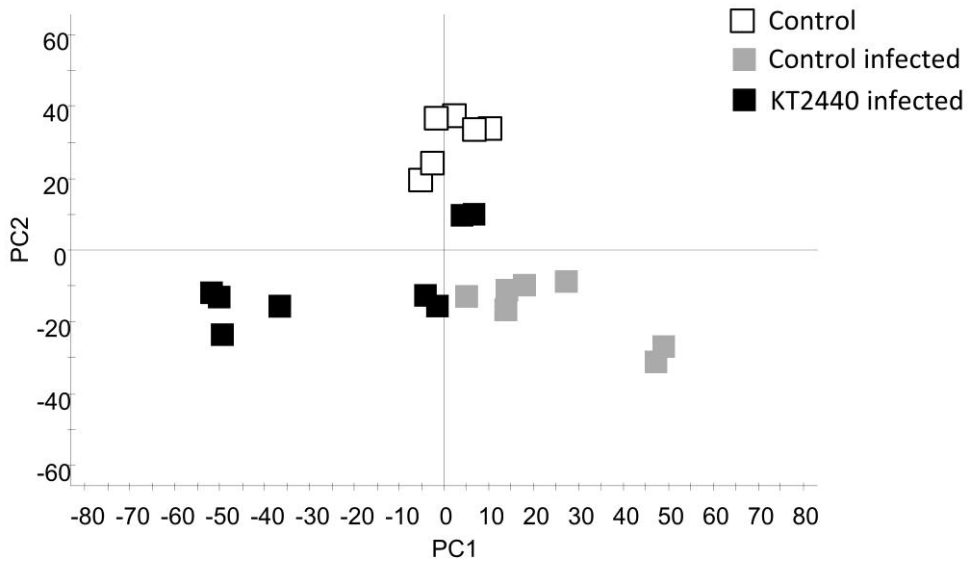

Figure 9. Partial least squares discriminant analysis (PLS-DA) score plot of metabolomic fingerprinting of maize roots (var. Jubilee) of control plants (white squares), *C. graminicola*-infected control plants (grey squares) and bacterized infected plants (black squares) four days after fungal leaf inoculation. n=6 for control plants and n=8 for infected plants (plant material from a single experiment). Analysis was performed on EZInfo.

Discussion

ISR triggered by KT2440 against *C. graminicola*

C. graminicola, the causal agent of maize anthracnose, has a worldwide impact on maize production and leads to important economic losses (Frey et al., 2011). This fungal pathogen has a hemibiotrophic life-style that starts with a biotrophic phase and then switches to a necrotrophic phase around two to three days after fungal leaf inoculation in laboratory conditions (Vargas et al., 2012; Balmer et al., 2013). The latter stage is characterized by cell death resulting in visible leaf necrosis. During the biotrophic phase, hemibiotrophic fungi induce specific plant responses that are different from biotrophic ones. Contrary to the biotrophic fungus *Ustilago maydis*, plants activate defense mechanisms already during the biotrophic phase by inducing an up-regulation of SA-related genes in response to *C. graminicola* or *Magnaporthe oryzae* attack (Vargas et al., 2012). Our results confirm data published previously by Balmer et al. (2013) on the activation of SA- as well as JA-dependent pathways in response to *C. graminicola*.

ISR is a plant defense that acts against a wide variety of attacks and in various plant varieties. Necrotrophic but also hemibiotrophic pathogens are negatively affected by plant

defenses induced by beneficial microbes in dicotyledonous and monocotyledonous plants. Trichoderma-induced systemic resistance in Arabidopsis reduced necrotic symptoms caused by *Pseudomonas syringae* pv. *tomato* (*Pst*; Brotman et al., 2012). Rice resistance triggered by mycorrhiza or rhizobacteria was phenotypically associated with a reduction of lesions caused by *M. oryzae* (De Vleesschauwer et al., 2008; De Vleesschauwer et al., 2009; Campos-Soriano et al., 2011). In our study, plants pre-treated with KT2440 did not exhibit (or only to a small extend) leaf necrosis caused by *C. graminicola* inoculation in contrast to non-inoculated plants. In control infected plants, necroses were observed already after two to three days (data not shown) as described in Balmer et al. (2013). Thus, the necrotrophic phase of *C. graminicola* was blocked (or delayed) in KT2440-inoculated plants. This suggests a bioprotective effect of KT2440 that tends to inhibit the switch of the fungal life-style from biotroph to necrotroph by *C. graminicola* or block the fungal developpment very early during the biotrophic infection process.

KT2440 does not produce volatile antimicrobial compounds as no fungal growth differences were observed between Petri dishes containing KT2440 and/or M9 in our *in vitro* assays. However, KT2440 is able to produce pyoverdin, an antibiotic compound. Since no KT2440 were found in leaves, we rule out the possibility of a direct effect against *C. graminicola* via a direct antagonism. Pyoverdin is an important ISR-triggering substance (De Vleesschauwer et al., 2008). It would be interesting to test if pyoverdin or another bacterial determinant plays a role in KT2440-triggered ISR. Our previous study on direct effects induced by KT2440 inoculation of maize plants showed that no metabolite changes are induced in KT2440-inoculated plants at 10 days after KT2440 inoculation (Chapter 3). This shows that prior to infection with *C. graminicola*, there were no important metabolite differences between bacterized and non-bacterized plants. The metabolite profiling four days after *C. graminicola* infection indicated no fundamental changes in defense reaction in presence or absence of KT2440. Most metabolite differences between controls, infected plants and bacterized infected plants were due to the infection. Similar metabolites were more abundant in infected plants (bacterized or not). However, these metabolites were more abundant in infected plants than in KT2440-inoculated infected plants, illustrating the most severe disease development in non-bacterized plants in comparison to bacterized plants. Coupling these results and phenotypic observations points to a possible priming of plant defenses instead of a direct induction of different defense mechanisms by KT2440.

In order to establish which hormone-related defense pathway is activated during ISR triggered by KT2440, a transcriptional analysis was performed four days after inoculation (representing two days after the necrotrophic stage starts). ISR was previously demonstrated as acting against hemibiotrophic fungi via several mechanisms. In mycorrhizal rice plants that were challenged with the hemibiotrophic *M. oryzae*, priming of genes of the SA-dependent pathway was associated with ISR mechanisms (Campos-Soriano et al., 2011). In rice, *P. fluorescens* WCS374r induced a resistance against *M. oryzae* by enhancing JA- and ET-dependent responses (De Vleesschauwer et al., 2008). *Trichoderma virens* inoculation of

maize roots induced decreased lesion sizes via the induction of JA- and green leaf volatile-associated genes (Djonović et al., 2007). In Arabidopsis, genes related to pathogenesis related proteins and ET/JA responsive genes were up-regulated in *Trichoderma*-colonized plants in response to *Pst* challenge (Brotman et al., 2012). In our study, an ET-responsive gene was differently regulated in presence of KT2440 indicating an involvement of the ET-signaling pathway in the plant defense state four days after *C. graminicola* infection. It is noteworthy that the SA-dependent pathway was activated in both treatment but the JA-dependent pathway was only extensively up-regulated in mock-treated infected plants. This KT2440-associated regulation pattern was similar to the one in a leaf-*C. graminicola* interaction around 48 hours after inoculation (Balmer et al., 2013), when the fungus is switching from the biotrophic to the necrotrophic phase. It is in agreement with the absence of necrosis observed in bacteria-inoculated plants. Several JA-related genes were up-regulated in control plants infected with *C. graminicola* such as *WIP*, *AOS* or *LOX3*. *LOX3* up-regulation is known to be involved in disease severity caused by *C. graminicola* in maize. *LOX3* products such as oxylipins play a positive role in fungal pathogenicity. Thus, inactivation of *LOX3* in maize plants resulted in a decreased disease severity caused by *C. graminicola* (Gao et al., 2007). Interestingly, whereas *LOX3* is one of the earlier genes up-regulated in control plants infected with *C. graminicola* and remains up-regulated (Balmer et al., 2013), infected KT2440-inoculated plants showed no up-regulation of this gene. It remains to be determined if *LOX3* is not activated earlier or later in bacterized plants and which mechanisms could be related to this non-regulation. A recent study showed that KT2440 is able to enhance volatile emission and to prime a JA-related gene in response to JA and wounding treatment (Neal and Ton, 2013). Therefore, further studies would be needed at earlier time point during the biotrophic and the necrotrophic phases to investigate a potential priming of SA- and/or JA/ET-dependent responses.

To extend our comprehension of ISR mechanisms triggered by KT2440, a metabolomic profiling was undertaken. Only few differences in metabolite profiles were found between control plants and KT2440-inoculated plants four days after *C. graminicola* infection. Similar metabolites were found to be present in infected plants whether bacterized or not. However, the intensity of the response was generally lower in KT2440-inoculated plants, probably due to the reduced disease progression in these plants. In infected plants, compounds involved in defense mechanisms were mostly present, such as chlorogenic acid and lysophosphatidic acid. These compounds are known to be more abundant in response to pathogen attack or herbivory (Marti et al., 2012; Balmer et al., 2013). In non-infected plants, conjugates of potential defense-related compounds were more abundant. Hence, 2-*O*-caffeoyl hydroxycitric acid is a potential precursor of chlorogenic acid that is synthesized from caffeic acid. The kaempferol conjugate could be a source for kaempferol, a flavonoid that has an antibiotic effect against *C. graminicola* (Balmer et al., 2013) and the linolenic acid attached to the MGDG is an important compound for the JA-signaling pathway. As observed for the defense response to herbivory, attacked maize plants had a decreased amount of maysin (Marti et al., 2012).

The two-by-two comparison of metabolite profiles between control and bacterized plants four days after infection revealed that a benzoxazinone, HDMBOA-Glc, was the most different compound between treatments. HDMBOA-Glc was more abundant in control infected plants in comparison with bacterized infected plants. HDMBOA-Glc is known to be formed from DIMBOA-Glc in response of tissue damages caused by a fungal or a herbivore attack (Ahmad et al., 2011; Marti et al., 2012). The differences of DIMBOA-Glc and HDMBOA-Glc caused by *C. graminicola* infection in non-bacterized plants was similar as leaf BXD production during maize infection with the necrotrophic fungus *Setosphaeria turcica* (Ahmad et al., 2011). That is probably due to the leaf damages caused during the necrotrophic life-style of *C. graminicola* at that time. In *C. graminicola*-infected KT2440-inoculated plants, where almost no necrosis was present, there was no HDMBOA-Glc accumulation and DIMBOA-Glc was less abundant in comparison to non-infected plants. This difference of DIMBOA-Glc content could be explained by the formation of DIMBOA from DIMBOA-Glc. DIMBOA is known as an antifungal and antiherbivore compound (Niemeyer, 2009). However, it has been shown that *C. graminicola* is not affected by DIMBOA content in maize plants (Lyons and Nicholson, 1989). Thus, benzoxazinones seem not to be involved in ISR triggered by KT2440. Balmer et al. (2013) supported the hypothesis that early down-regulation of *BX1*, a key enzyme in BXD biosynthesis, could benefit to anthracnose development. Further investigations earlier in *C. graminicola* infection stages would be necessary to verify the involvement of BXD in ISR induced by KT2440.

In addition to BXD, other secondary metabolites could have been primed. Chlorogenic acid priming was, for example, an important mechanism in systemic resistance induction against *S. littoralis* (Erb et al., 2009). Phenolic compound accumulation and callose deposition could be important defense mechanisms in ISR triggered by KT2440. Hence, in cucumber plants, inoculation with PGPR and challenged by *C. orbiculare*, ISR was associated with phenolic compound accumulation at the penetration site and callose deposition 3 days after infection (Jeun et al., 2003). In addition, hydroxycinnamic acid accumulation was found as a maize response to *C. graminicola* infection (Vargas et al., 2012). Further investigations in metabolic leaf profiles through a time-point experiment would give an enhanced comprehension of the defense process.

Systemic response during ISR triggered by KT2440 against *C. graminicola*

It is interesting to note that there were also some transcript and metabolite changes at the root level four days after fungal leaf inoculation. ISR triggered by KT2440 against *C. graminicola* in leaf tissue induced regulation of ET-, JA- and volatile-related genes. Recently, Carvalhais et al. (2013) showed that the activation of the JA signaling pathway influenced the rhizosphere bacterial community: from a bacterial composition related to plant growth (before treatment) into a bacterial composition of bacteria involved in ISR and in plant growth. Thus, it would be interesting to test for a potential fluctuation in KT2440 root

colonization during ISR. Metabolite profiling of roots did not show any specific response within the three different treatments. It is noteworthy that HDMBOA-Glc was similarly present in roots of infected plants (bacterized or not), whereas HDMBOA-Glc content in leaves was significantly lower in KT2440-inoculated infected plants compared to infected plants. This reveals a different above- and belowground HDMBOA-Glc ratio that is different depending on the presence of the bacteria. Oikawa and colleagues (2001) demonstrated that treating wheat leaves with jasmonic acid induced an accumulation of HDMBOA-Glc in roots. Thus, HDMBOA-Glc accumulation in roots could be a response of the JA-dependent pathway activation in aboveground parts that was induced by *C. graminicola* infection. As presence of BXD in roots is important for KT2440 conspecific recruitment, KT2440-inoculated plants could maybe also undergo BXD ratio changes in roots as a result of KT2440 presence in roots (Neal et al., 2012).

In summary, this study demonstrates the ability of KT2440 to induce maize systemic resistance against a biotic stress: in KT2440-inoculated plants, anthracnose symptoms were almost absent. Whereas the specific mechanisms underlying ISR triggered by KT2440 remain to be elucidated, our transcript and metabolite analyses revealed some important elements for further investigations. Defense mechanisms that are linked to phenolics production and cell wall reinforcement seem to be of major interests in ISR against pathogens.

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Appendix

1. Metabolomic changes in maize plants during ISR against *Colletotrichum graminicola*

Table S1. Identified compounds in maize leaves during ISR against *C. graminicola* (4 days after infection) that were more abundant (>2 fold) in control infected plants in comparison with bacterized infected plants (median values).

Name	Molecular formula	Mass	Fold	P value
Coumaroyl-tyramine	$C_{17}H_{16}NO_3$	282.11	2.69***	<0.001
Coumaroyl-feruloyl-glycerol	$C_{22}H_{21}O_8$	413.12	2.5***	0.001
Feruloyl-tyramine	$C_{18}H_{18}NO_4$	312.12	3.6***	0.001
Genkwanin	$C_{16}H_{11}O_5$	283.06	2.5**	0.01
N-feruloyl-tryptamine	$C_{20}H_{19}N_2O_3$	335.13	2.26***	0.001
N-*p*-coumaroyl tryptamine	$C_{19}H_{17}N_2O_2$	305.12	2.79**	0.005
Feruloyl-feruloyl-glycerol	$C_{23}H_{23}O_9$	443.13	2.37*	0.02

CHAPTER 5

Systemic resistance of maize plants induced by *Pseudomonas putida* KT2440 against leaf herbivores

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Abstract

Colonization of plant roots by beneficial microbes can indirectly reduce colonization by pathogens through the induction of systemic resistance that is essentially effective against necrotrophic pathogens and chewing herbivores. However, this defense capacity depends on combination of plant and pathogen or pest involved. The rhizobacteria *Pseudomonas putida* KT2440 are well-known colonizers of maize seeds and roots. However, only little is known on their capacity to induce systemic defense. In the present study, the ability of KT2440 to trigger ISR in maize plants against leaf chewing herbivors is reported. Interestingly, the presence of KT2440 had a variable effect on plant defense depending on the feeding specialization level of insects. Whereas bacterization of plants led to a reduction of larval growth and leaf consumption of the generalist *Spodoptera littoralis*, no effect was found against the specialist *S. frugiperda*. These differences in plant resistance capacity that are linked to the host plant specialization of the herbivores were reflected at the plant metabolomic level.

Introduction

The presence of certain microbes in the rhizosphere can exert a beneficial effect on plant growth by improving plant nutrition or suppressing plant attacks. Mycorrhizal fungi and rhizobia are well-studied examples of beneficial microorganisms (Van der Ent et al., 2009; Gutjahr and Paszkowski, 2009). Their activity in nitrogen fixation or phosphorous uptake stimulates plant growth. Plant growth-promoting rhizobacteria (PGPR) and fungi (PGPF), form additional classes of beneficial soil-borne microbess that can directly or indirectly stimulate plant growth (Lugtenberg and Kamilova, 2009). In general, the most commonly occurring genera for PGPR are *Pseudomonas* spp. and *Bacillus* spp. and *Trichoderma* spp. for PGPF.

Plant growth can be directly promoted by PGPR or PGPF through different mechanisms such as an enhanced nutrition or the production of plant hormones or indirectly by acting against biotic attacks through nutrient competition or production of antibiotics (Van Loon, 2007; Lugtenberg and Kamilova, 2009). Some PGPR, as well as some PGPF such as *Piriformospora indica* (Waller et al., 2005) and *Trichoderma* spp. (Hermosa et al., 2012), can reduce an attack in above- or belowground plant parts through an enhanced defense capacity, characterized as priming. Induced systemic resistance (ISR), the resulting defense state, is effective against a broad range of different attacks, essentially necrotrophic pathogens and chewing herbivores (see De Vleesschauwer and Höfte, 2009, Van Hulten et al, 2010 and Pineda et al., 2010 for reviews). Interestingly, similar plant defense mechanisms play a role during ISR triggered by rhizobacteria and mycorrhiza (Pineda et al., 2012).

Following microbe perception by the plant through microbe-associated molecular patterns (MAMPs) such as siderophores, flagellin or lipopolysaccharides (LPS), the MAMP-triggered immunity is suppressed by the rhizobacteria (Zamioudis and Pieterse, 2012). A signal is then generated in the plant systemic tissue without inducing direct extensive metabolite or transcript changes. This signal is at the origin of the systemic enhanced defense capability of the plant. ISR is associated with priming, a stronger and/or faster induced response, for an enhanced expression of genes involved in jasmonic acid (JA)-, ethylene (ET)- and abscisic acid (ABA)-dependent pathways after an attack (Van Wees et al., 2008). Recent studies showed that, in some cases, ISR mechanisms were associated with other transduction pathways. Hence, ISR depends on the combination of host plant, the PGPR strains and the attacker that are involved. For example, the rhizobacterium *Pseudomonas fluorescens* SS101 induced a resistance against the hemibiotrophic pathogen *Pseudomonas syringae* pv. *tomato* DC3000 in Arabidopsis via SA-signaling (Van de Mortel et al., 2012), whereas *Bacillus cereus* AR156 activated a SA-, JA-, and ET-dependent signaling defense in the same plant-pathogen system (Niu et al., 2011). *Serratia plymuthica* ICI270-ISR was efficient against the hemibiotrophic fungus *Magnaporthe oryzae* but not against necrotrophs (De Vleesschauwer et al., 2009). The defense mechanisms involved in ISR induced by PGPR against herbivores are still much unknown (Pineda et al., 2010). Differences

in ISR effects were observed depending on the host plant specialization level of herbivores and the feeding method they use (chewing or sucking; Pineda et al., 2010). Thus, generalist leaf chewing herbivores that fed on bacterized Arabidopsis plants seemed to be affected by ISR, whereas specialists, such as *Pieris rapae*, were not affected (Van Oosten et al., 2008). Interestingly, ISR seems to have positive effects on specialist phloem-feeding aphids and no effects against generalists (Pineda et al., 2012). In this latter study a systemic susceptibility induced by rhizobacteria was reported for the first time.

Phenolic compounds and indole derivatives are important defense metabolites linked to ISR mechanisms. For example, phenolic compounds accumulated rapidly during resistance against *M. oryzae* triggered by *P. fluorescens* WCS374r (De Vleesschauwer et al., 2008). The phenylpropanoid pathway is responsible for the biosynthesis of a wide variety of compounds that are important for plant defense against herbivores such as lignin, flavonoids or phenolics (such as coumaric and ferulic acids; McMullen et al., 2009; Cesarino et al., 2012). Benzoxazinones (BXD) are important indole derivatives of the *Poaceae* family. The various forms of BXD are major defense compounds against herbivory and pathogenic attack (Ahmad et al., 2011; Marti et al., 2012). However, Dafoe et al. (2011) showed that changes in BXD content do not necessarily influence the capacity of the plant to be more efficient against herbivory.

Although monocotyledons represent an economically important plant group, studies about wheat, maize or other cereals are less common in comparison to studies involving Arabidopsis or tomatoes. Even though, the regulation of induced resistance pathways and interaction with other plant pathways may be quite divergent between monocots and dicots (Kogel and Langen, 2005; Balmer et al., 2012), ISR mechanisms seem to be similar. ISR in monocot is effective against pathogens (Djonovic et al., 2007) as well as against herbivores (Saravanakumar et al., 2007). While indirectly induced defenses of maize plants and herbivore-induced defenses are well documented (Turlings et al., 1995; Rasmann et al., 2005; Ton et al., 2006; Chen, 2008; Erb et al., 2009), only few studies report on direct systemic induced resistance such as ISR (Balint-Kurit and Johal, 2009; Balmer et al., 2012). ISR has been documented in maize for only a few cases but for a wide variety of beneficial microbes: in response to the root colonization with filamentous fungi from the genera *Trichoderma* ssp. (Harman et al., 2004; Djonovic et al., 2007), with the mycorrhizal fungus *Glomus mosseae* (Song et al., 2011) and the rhizobacteria *Bacillus cereus* C1L (Huang et al., 2012) and *Pseudomonas aurantiaca* JD37 (Fang et al., 2012). In these cases ISR was effective against hemibiotrphic or necrotrophic fungi such as *Colletotrichum graminicola* or *Rhizoctonia solani*. For studies in which ISR mechanisms were analyzed, the involvement of a JA-regulated defense pathway but also an up-regulation of green leaf volatiles or DIMBOA- and SA-related genes were shown.

The rhizobacteria *Pseudomonas putida* KT2440 (KT2440) are well-known root colonizers of a wide variety of crop plants and particularly of maize (Espinosa-Urgel and

Ramos, 2004). KT2440 show a specific ability to live in the maize rhizosphere as they are tolerant to DIMBOA, a biocidal compound released by maize roots, and they use this secondary metabolite as a chemotaxis signal (Neal et al., 2012). Matilla and colleagues (2010) showed the ability of KT2440 to induce plant defenses in Arabidopsis plant against *P. syringae* pathovar *tomato* DC3000. A recent study showed the ability of KT2440 to prime maize defense response to a herbivore-mimicking treatment (wounding and jasmonic acid treatment; Neal and Ton, 2013). However, there is no biological evidence for their capacity to induce maize resistance against herbivores. The aim of our study was to test the capacity of KT2440 to induce maize resistance against leaf chewing herbivores that are differently specialized to maize plants. Here, we report on the differential effect of KT2440 depending on the biotic stress. The mechanisms involved in ISR priming triggered by KT2440 were analysed at the transcriptional and metabolomic level

Material and Methods

Biological material

The maize variety Delprim (DSP Delley, Switzerland) was used for its well-studied interaction with insects (Erb et al., 2011). Maize seeds were rinsed 3-5 times in 70% ethanol and then immerged for 5 minutes in bleach 10%. After sterilization, seeds were rinsed a few times with sterile distilled water and placed for two days of germination in humid rolled Filterkrepp filter paper (E. Weber & Cie AG, Switzerland) in a growth chamber (16:8 Light:Dark 26°C:22°C, 400 µmol m^{-2} s^{-1}). Seedlings were then grown in a standard pot system filled with a mix of one-time autoclaved sand:soil, as described in Planchamp et al. (2012). Seedlings were placed back for 9 days in the same growth chambers.

The rifampicin-resistant strain *Pseudomonas putida* KT2440 was cultivated in the dark at 25°C on Luria-Bertani (LB; Difco LB, Becton, Dickinson and Company, France) agar supplemented with 100 µg mL^{-1} of rifampicin. After two to five days, a single colony of KT2440 was transferred to 100 mL of LB liquid medium containing 100 µg mL^{-1} of rifampicin for an overnight culture (28°C, shaking at 150rpm). Pre-germinated seeds were inoculated with a bacterial suspension, for bacterized plants, or a sterile M9 minimal medium, for control plants, for 30 minutes as described (Planchamp et al., 2012).

Spodoptera littoralis and *Spodoptera frugiperda* were reared on an artificial diet under laboratory conditions as previously described (Turlings et al., 2004). *S. littoralis* eggs were provided by Syngenta. Second-instar larvae of both herbivores were used for plant infestation assays.

Induced resistance assays

Eleven-day old maize plants were infested by five second-instar larvae of either *S. littoralis* or *S. frugiperda* or remained herbivore-free (n=16 for larval weight measurement; n=9 for metabolite analysis). In order to measure induced resistance, herbivores were weighed before applying them to plants and four days later. The weight gain of larvae was then calculated and six plants per treatment were kept for leaf scanning. Plant material was collected and flash-frozen for further analyses. Leaf consumption by herbivores was established by measuring holes in scanned leaves using Adobe Photoshop CS5. Before plant infestation, above-ground parts of plants were measured with the aim to detect a possible promotion of plant growth induced by the presence of KT2440.

Bacterial quantification in roots and shoots

Roots and shoots from treated and control plants were collected at the end of the experiment to test for the presence of KT2440. Hundred mg of fresh root or 50 mg of fresh leaf tissues were ground in 600 µL of sterile $MgSO_4$ 10mM. Serial dilutions of these extracts were plated on King's medium B containing 100 µg mL^{-1} of rifampicin and incubated at 25°C in the dark. One day later, colonies were counted and the colony-forming units (CFU) per gram of fresh root or shoot were determined.

Metabolomic analysis

Plant material from 3 or 4 biological replicates (one replicate representing a pool of three plants) was collected and directly flash-frozen. Samples were ground into a fine powder while keeping the material frozen. Metabolites were extracted from 100 mg of tissue in a solvent mixture composed of methanol, water and formic acid (80:19.5:0.5). Metabolomic analyses were performed in technical duplicates on an UHPLC-QTOF-MS system equipped with an electrospray (ESI) source (Waters, Milford, USA). Separation of compounds was done with an Acquity BEH C18 column (2.1 x 50 mm, 1.7 µm particle size) at a flow rate of 400 µL min^{-1}. The column temperature was kept at 30°C and the injection volume was of 5 µL. The following gradient program was used: solvent A (water containing 0.05% of formic acid) and solvent B (acetonitrile containing 0.05% of formic acid); 0-6 min 5-100% B, 6-8 min holding at 100% B, 8-10 min reequilibration at 2% B. Concerning the QTOF-MS, positive and negative modes were employed over a mass range of 85-1200 Da . The MS^E mode without precursor ion selection was applied using scan times of 0.2 sec at both low (4eV) and high (10-30eV ramp) collision energies. QTOF-MS settings were: capillary voltage at +2800V or -2500V, cone voltage at +25V or -25V, source temperature at 120°C, cone gas flow at 20 L h^{-1}, desolvation gas flow at 800 L h^{-1} and desolvation temperature at 350°C. Calibration was

provided by infusing a peptide leucine enkephalin solution through the ESI probe. MassLyn™ V.4.1. was used to control all the system and to convert raw data into CDF format.

For fingerprint analysis, data were processed using R and the packages "xcms" and "multtest". As an exploratory step, the resulting peak lists were subjected to principal component analysis on MarVis or on EZinfo (Umetrics, Umea, Sweden) after a Pareto scaling. A supervised datra mining approach was applied by proceeding orthogonal partial least squares discriminant analysis (OPLS-DA or PLS-DA). After filtering and ranking, markers were clustered on MarVis colormaps in order to visualize differences between treatments. Discriminant markers were tried to be identified first via the molecular formulae. They were computed according to mass and spectral accuracies using the elemental composition calculator provided by MassLynx™. Proposed molecular were verified into different metabolite databases (KNapSAcK Core system and Dictionary of Natural poducts). Structures were then matched with the MS/MS fragments obtained at high collision energy using MassFragment™ (Waters).

Statistical analysis

Statistical analysis of metabolite and gene expression analysis were performed as described above. The rest of the comparisons were accomplished using SigmaPlot 11.0. After passing the normality test, a Student t-test was used.

Results

Systemic resistance induced by *Pseudomonas putida* KT2440 in maize plants against leaf herbivores

The capacity of KT2440 in inducing resistance against leaf herbivores was tested by using two different herbivores: *Spodoptera littoralis*, a generalist herbivore, and *Spodoptera frugiperda*, a specialist leaf herbivore. Before infestation, shoot length of plants was recorded in order to test for a potential growth promotion induced by the presence of KT2440 in roots. There were no differences between control (43.11 cm) and KT2440-inoculated plants (43.34 cm, $p = 0.732$, t-test). Four days after larval feeding on plants, there were no differences in weight gain of the specialist *S. frugiperda* larvae between treatments (Figure 1; $p = 0.899$, t-test). Interestingly, larvae of *S. littoralis* gained less weight (1.08 mg) on KT2440-inoculated plants in comparison with larvae that fed on control plants (Figure 1; $p = 0.012$, t-test).

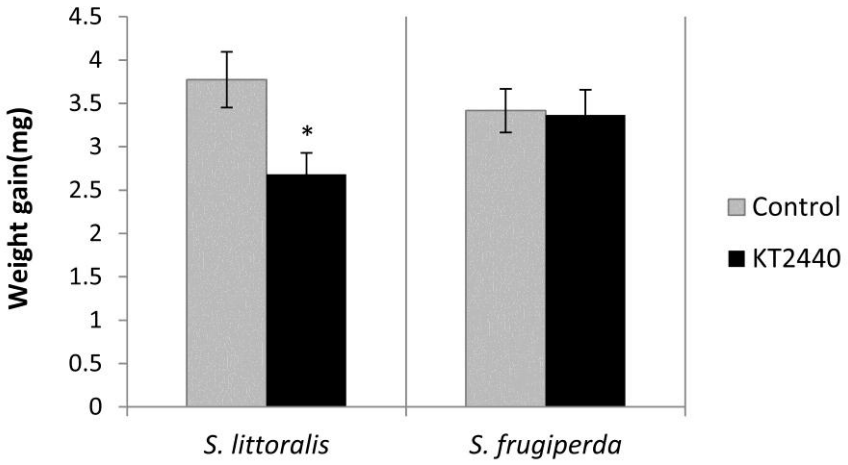

Figure 1. Larval weight gain (mg) of *Spodoptera littoralis* and *Spodoptera frugiperda* larvae on mock-treated and on KT2440-inoculated maize plants var. Delprim, after four days of feeding. n=16 plants per treatment (from a single experiment) Statistical significance * if $p < 0.05$ (t-test).

Since some differences in weight gain by *Spodoptera* larvae feeding on bacterized plants were observed, the quantity of leaf material consumed by the larvae on treated and control plants was measured to test whether these differences in weight gain could be explained by a differences in leaf consumption (Figure 2). There were no differences for *S. frugiperda* leaf consumption between the different treatments. However, there was a tendency of *S. littoralis* larvae to eat less leaf material on bacterized plants in comparison with control plants (3.43 cm^2 of control plants, 2.18 cm^2 of KT2440-inoculated plants, $p = 0.055$, t-test).

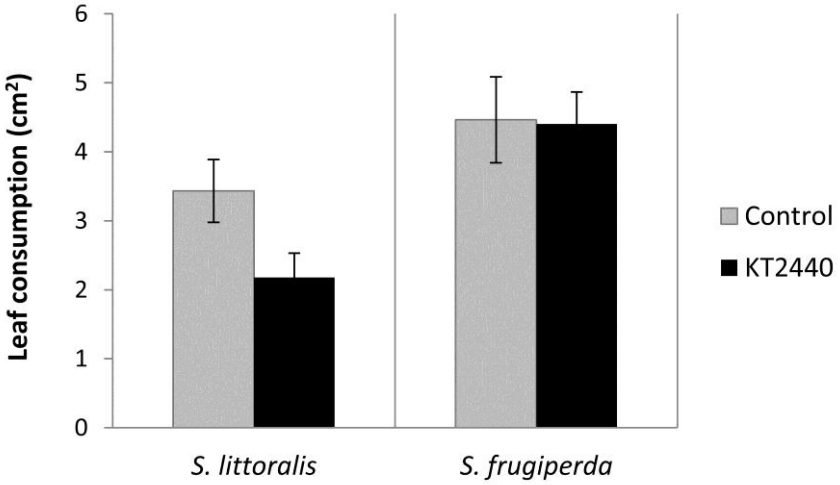

Figure 2. Leaf area (cm^2) consumed by *Spodoptera littoralis* or *Spodoptera frugiperda* larvae on mock-treated and on KT2440-inoculated maize plants var. Delprim, after four days of feeding. n=6 plants per treatment (from a single experiment).

At the end of the experiment, roots and leaves of control and KT2440-inoculated plants were collected and the presence of KT2440 was assessed. KT2440 were found neither in leaves of both treatments, nor in control roots. In KT2440-inoculated roots, 2.25×10^5 CFU g^{-1} of fresh root material were counted.

Metabolomic fingerprinting during ISR against leaf herbivores

To test whether induction of resistance triggered by KT2440 against *Spodoptera littoralis* but not against *Spodoptera frugiperda* can be associated with metabolomic changes, a metabolomic fingerprinting was executed on plant material before and after infestation. A PCA of leaves and roots prior to infestation was able to divide samples between a control group and a KT2440-inoculated group for root material but not for leaves (Figure 3). Most of the up-regulated metabolites of roots were present in control plants. The tentative identification of metabolites present more abundantly in control roots than in bacterized roots revealed the presence of 2-*O*-coumaroyl-hydroxycitric acid (*m/z* 353.051) and another partially identified compound that has the molecular formula $C_{17}H_{28}O_{10}$ (*m/z* 459.14).

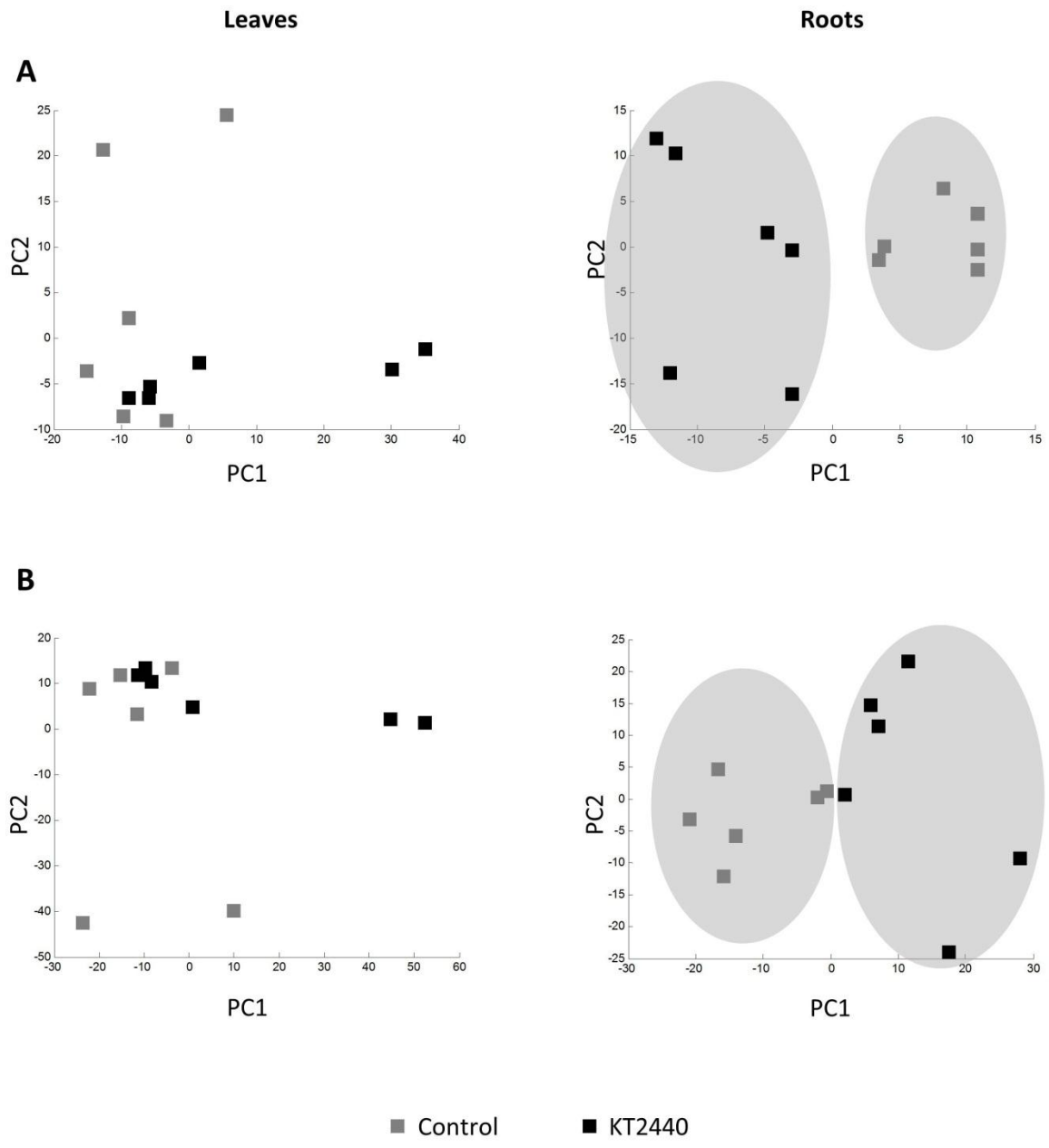

Figure 3. Principal component analysis (PCA) score plot of metabolomic fingerprinting of maize leaves (var. Delprim) of mock-treated and KT2440-inoculated plants before infestation. n=9 plants per treatment (from a single experiment). Metabolites from leaf and root tissues were analyzed by UHPLC-QTOF-MS in negative (A) and positive (B) modes. Analysis was performed on MarVis.

A PCA of metabolites present in leaves 4 days after infestation with *S. frugiperda* did not separate control and KT2440-inoculated plants (Figure 4). PCA of markers found in samples infested with *S. littoralis* was able to divide treatments but the separation was weak. A supervised analysis of markers found after *S. littoralis* infestation managed to separate groups of treatments between KT2440-inoculated and non-bacterized plants (Figure 5). This difference between herbivores was also reflected by the number of significant biomarkers. More significant changes between treatments were found for plants infested with *S. littoralis* (145 markers in negative mode and 346 in positive mode) compared to plants infested with *S. frugiperda* (61 markers in negative mode and 127 in positive mode).

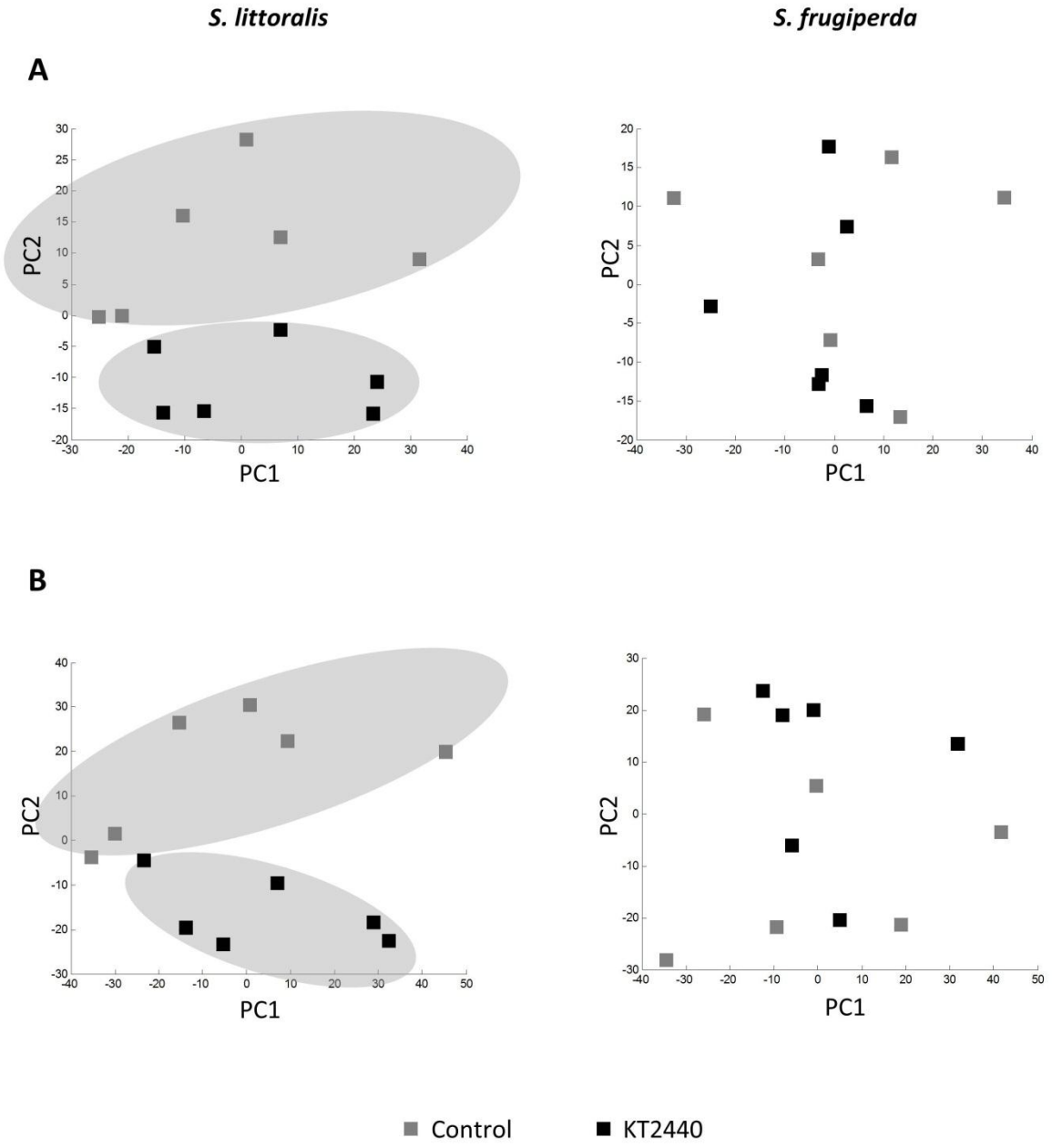

Figure 4. Principal component analysis (PCA) score plot of metabolomic fingerprinting of maize leaves (var. Delprim) of mock-treated and KT2440-inoculated plants four days after herbivore infestation with *Spodoptera littoralis* or *Spodoptera frugiperda*. n=9 plants per treatment (from a single experiment). A = analysis in negative mode, B = analysis in positive mode. Analysis was performed on MarVis.

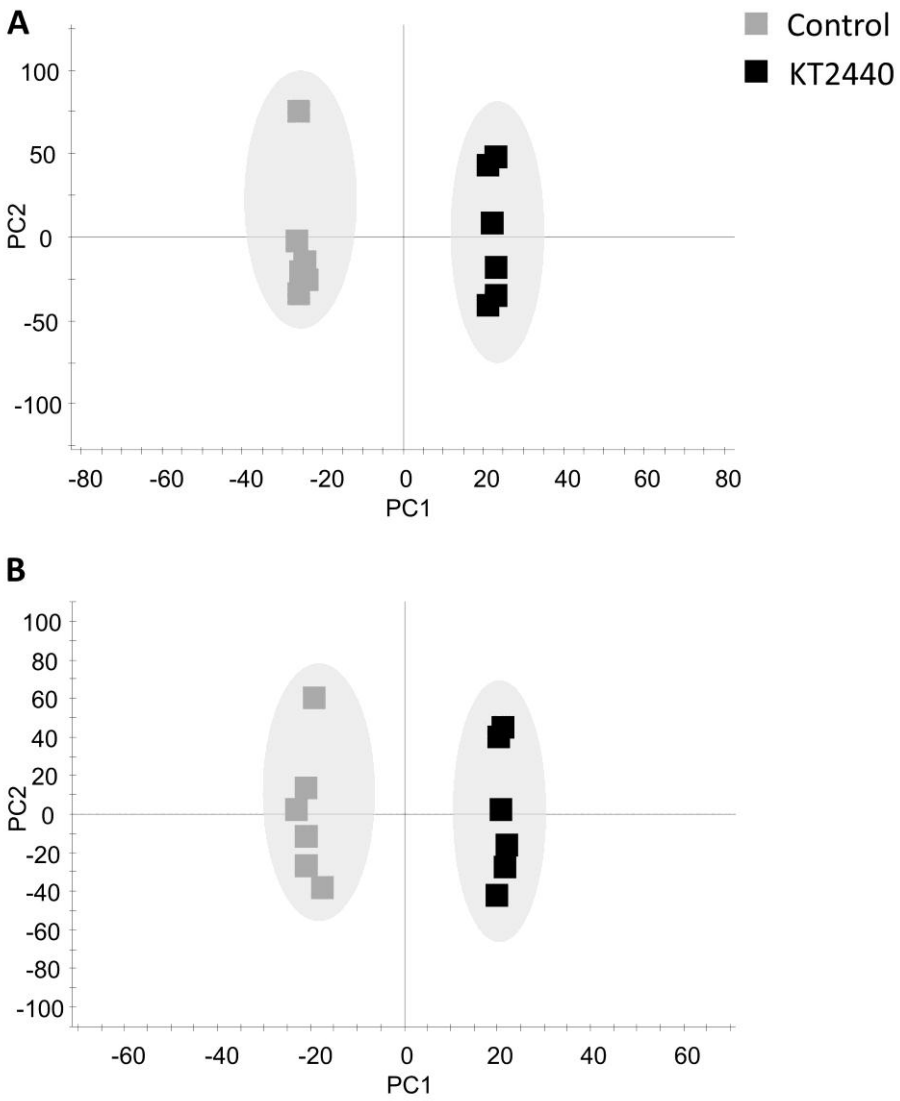

Figure 5. Orthogonal partial least squares discriminant analysis (OPLS-DA) score plot of metabolomic fingerprinting of maize leaves (var. Delprim) of mock-treated and KT2440-inoculated plants four days after herbivore infestation with *Spodoptera littoralis.* n=9 plants per treatment (from a single experiment). A = analysis in negative mode, B = analysis in positive mode. Analysis was performed on EZInfo.

In plants infested with *S. littoralis*, the most abundant metabolites were found in mock-treated infested plants in comparison to KT2440-inoculated infested plants. Several compounds that were more abundant in mock-treated infested plants are known as cinnamate esters or conjugates: 2-*O*-caffeoyl-hydroxycitric acid (*m/z* 369.045), 2-*O*-coumaroyl-hydroxycitric acid (*m/z* 353.051) and coumaroyl-feruloyl-glycerol (*m/z* 413.12). Tryptophan (*m/z* 205.09) and a partially identified compound $C_{26}H_{26}N_2O_{23}$ (*m/z* 575.15) were also more abundant in control plants infested with *S. littoralis*, compared to KT2440-inoculated infested plants. The presence of specific antiherbivory compounds from the

benzoxazinone group was also investigated. There were no differences for the tested BXD (HDMBOA-Glc and DIMBOA-Glc) between treatments for both herbivores (data not shown).

A comparison between metabolomic profile of control plants or KT2440-treated plants that were infested with *S. littoralis* or *S. frugiperda* is available in appendix (Figure S1 for the PCA and Figure S2 for the cluster plot).

Discussion

In a previous study (Chapter 3), we already showed the potential of KT2440 to have beneficial effects on maize plants via plant growth promotion. Here, we examined the influence of the presence of KT2440 in maize roots concerning the enhancement of defense mechanisms against biotic stresses. In our study, we showed that KT2440 was efficient to trigger ISR against a generalist leaf herbivore but failed to help plants against the specialist *Spodoptera frugiperda*. Efficient root colonization by rhizobacteria is necessary to induce a systemic defense in plants (Raajimakers et al. 1995). The assessment of the bacterial presence in maize roots during ISR experiments (Chapter 4 and 5) against different biotic stresses confirmed an efficient KT2440 colonization range between $2x10^5$ and $3x10^6$ CFU g^{-1} of roots.

Interestingly, the measurement of maize shoot length during our experiments concerning ISR (Chapter 4 and 5) indicated no growth promotion induced by KT2440 in this context. Plant hormones regulate plant growth as well as defense mechanisms resulting in a critical hormonal balance by the plant in order to survive and to reproduce. Recently, cytokinin and gibberellic acid signaling cascades have been revealed as important regulators in growth-defense trade-offs (Giron et al., 2012; Yang et al., 2012). These hormonal interconnections enhance the complexity of balanced responses that plants use in presence of beneficial microbes. In mycorrhized plants, where plant quality is improved, phloem feeders can benefit from these nutritious tissues and counterbalance the effect of plant activated defenses (Pineda et al., 2012). A similar phenomenon was described for white clover that interacts with rhizobacteria. This interaction led to a positive effect on the generalist chewing herbivore *Spodoptera exigua* (Kempel et al., 2009). Thus, a growth promotion induced by PGPR in non-infected or non-infested plants could be beneficial for a following attacker. In our study, the improvement of plant growth by the presence of KT2440 was not observed simultaneously with ISR triggered by KT2440. These results could indicate a potential cross-talk between the growth promotion and the induction of resistance.

ISR mechanisms are triggered by various beneficial microbes (mycorrhiza, PGPR and PGPF) and effective against a wide range of attacks: bacteria, fungi, oomycetes, viruses and nematodes. It has been recently highlighted that ISR can negatively affect herbivores (Pineda et al., 2010). However, this defense capacity depends on the mode of feeding and the host-

plant range specialization of herbivores. Specialist chewing herbivores that have a restricted host-plant range are not affected by ISR probably because of their adaptation to specific host-plant defense characteristics. However, defenses induced by beneficial microbes negatively affect generalist chewing insects (Van Oosten et al., 2008). A recent study has shown that in contrast to chewing herbivores, phloem feeding herbivores were differently affected by ISR depending on their host specialization (Pineda et al., 2012). , We demonstrate that plant defense mediated by rhizobacteria can affect chewing herbivores in maize plants. Moreover, as demonstrated in dicotyledonous plants, we showed that ISR against herbivores in monocotyledons depends also on the degree of host specialization. Thus, KT2440 triggered plant defenses that were effective against the generalist *S. littoralis* but not against the specialist *S. frugiperda*. This defense induction was not correlated with substantial metabolite changes in plants inoculated with KT2440.

Plants have evolved different types of defense responses against insect herbivores known as direct and indirect defenses. Direct defenses induced by plants can be categorized as toxic and/or anti-nutritious (Chen, 2008). Antinutritional defense can be effective by limiting food quantity or food quality. In our experiment, we found that the presence of KT2440 in maize roots affected the quantity of leaf material consumed by *S. littoralis*. This result contributed to explain the decreased weight of larvae on these plants. We then hypothesized that maize leaves should contain toxic or repellent compounds or stronger structures that limited food quantity ingestion by larvae but to which *S. frugiperda* was insensitive. A secondary metabolomic profile was established in order to find possible toxic, deterrent or anti-nutritious compounds. The differences of plant reaction between both herbivores were reflected by the number of biomarkers. Generalists are known to elicit stronger responses than specialists. We found that more biomarkers were induced by the presence of *S. littoralis* in comparison to *S. frugiperda*.

Benzoxazinones such as DIMBOA and HDMBOA are well-known defense compounds efficient in maize against herbivores (Glauser et al., 2011). However, glycoside forms (DIMBOA-Glc and HDMBOA-Glc) of these compounds were not differently abundant in control or KT2440-inoculated plants after infestations. This could reflect earlier changes during the interaction, the production of other BXD or an involvement of other compounds in ISR triggered by KT2440 against *S. littoralis*. Dafoe et al. (2011) showed that a first maize attack by corn borers induced changes in BXD content that did not influence the capacity of the plant to be more efficient against a following attack by the same herbivore. Indeed, maize defense mechanisms induced against the corn borer were found to be related to JA pathway components and cell wall reorganization, and not to changes of BXD content (Rodriguez et al., 2012). In this latter study, cell wall reinforcement was linked to changes in ester bound cell wall phenolics and in lignin, indicating the importance of the phenylpropanoid pathway in maize defense against the corn borer. The phenylpropanoid pathway is responsible for the biosynthesis of a wide variety of compounds that are important for plant defense such as lignin, flavonoids or phenolics (such as chlorogenic acid).

Phenylpropanoids such as caffeic and ferulic acids, which are formed from coumaric acid, are the main constituents of lignin and polyphenolic barriers (Paiva, 2000). Coumaric and ferulic acids, the major hydroxycinnamate derivatives in grass species, were found to have a direct toxic effect in maize against herbivores (McMullen et al., 2009; Cesarino et al., 2012). In our study, other metabolites than BXD, mainly hydroxycinnamate esters or conjugates were more present in control plants than in KT2440-inoculated plants during *S. littoralis* infestation. In addition, as shown in Chapter 4, caffeoyl-hydroxycitric acid was more present in non-infected plants in comparison to plants infected with *C. graminicola*. So, on one hand a maize attack by a pathogen decreased the amount of a hydroxycinnamate ester and on the other hand plants that were more resistant to *S. littoralis* presented less quantity of similar compounds. We can hypothesize that instead of having a direct beneficial effect on attacked plants, these compounds could be cleaved in response of the attack. This cleavage could result in the release or the production of toxic compounds or compounds involved in cell wall reinforcement. In accordance with our hypothesis, Parveen et al. (2008) showed that hydroxycinnamate esters such as caffeoyl- and coumaroyl-hydroxycitric acid are substrates for polyphenol oxidase (PPO) in the grass species *Dactylis glomerata*. Oxidation enzymes, such as PPO, have a well-known anti-nutritious action in multiple crops and against multiple insects (Thipyapong et al., 2007). Increased activity of enzymes such as PPO was also found to be an important mechanism in ISR. For example, ISR triggered in rice by *Achromobacter xylosoxidans* against *M. oryzae* (Joe et al., 2012) and ISR in rice against leaf folder by *P. fluorescens* strains (Saravanakumar et al., 2008) were associated with an increased PPO activity. In our study, the presence of defense compounds was more abundant in non-bacterized plants and can be the result of more herbivore-related damages on these plants in comparison with bacterized plants that had less damages due to *S. littoralis* feeding. A time-course experiment, instead of a single collection time, would give an enhanced understanding of the defense processes that are involved in this plant-rhizobacteria-herivore combination.

Here, we focused on secondary metabolites in order to find ISR-related biomarkers however other types of compounds such as proteins or volatiles that we did not analyze could be important for the deterrent effect against *S. littoralis*. In this sense, Neal and Ton (2013) recently showed that KT2440 can prime defense volatile production and the expression of a gene involved in proteinase inhibitors synthesis in maize.

In summary, this study demonstrates the ability of KT2440 to induce maize systemic resistance against generalist leaf chewing herbivores: larvae of *S. littoralis* grew and fed less on bacterized plants. Whereas the specific mechanisms underlying ISR triggered by KT2440 remain to be elucidated, our transcript and metabolite analyses revealed some important elements for further investigations. Defense mechanisms that are linked to phenolics production and cell wall reinforcement seem to be of major interests in ISR against chewing

herbivores. We also showed that ISR against herbivores in monocotyledons is also dependent on the host-plant specialization of herbivores.

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Appendix

1. Metabolomic fingerprinting in leaves infested by two different herbivores

Principal component analysis allowed separation between herbivores for control plants as well as for KT2440-inoculated plants. Interestingly, groups were more distant for bacterized plants than for control plants. This could be due to the presence of a resistance induced in KT2440-inoculated plants against *S. littoralis* but not against *S. frugiperda*.

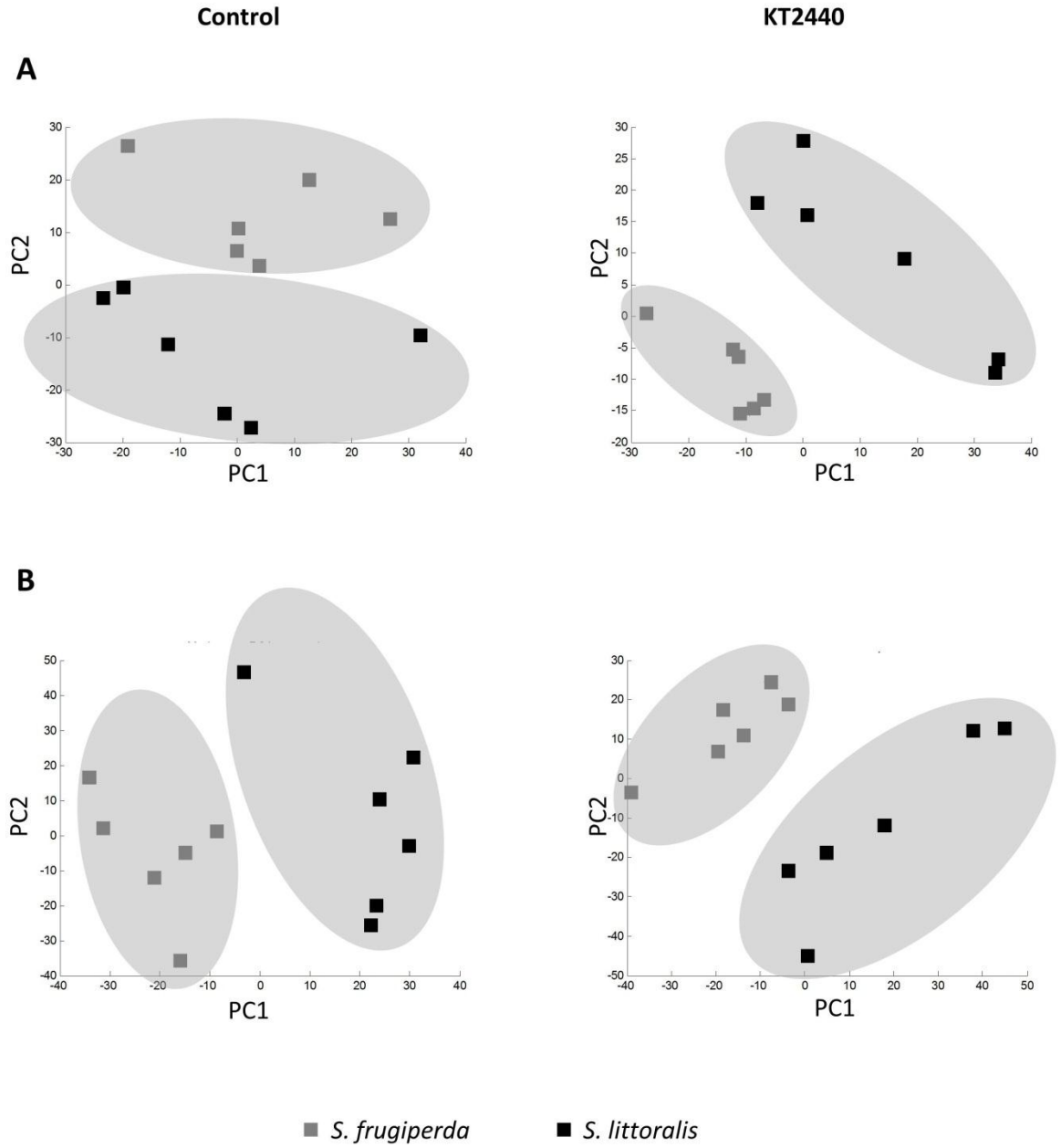

Figure S1. Comparison of plant response to two different herbivores. Principal component analysis (PCA) score plot of metabolite fingerprinting of maize leaves from control or KT2440-inoculated plants that were infested with *S. littoralis* or *S. frugiperda*. UHPLC-QTOF-MS system using negative (A) and positive (B) modes.

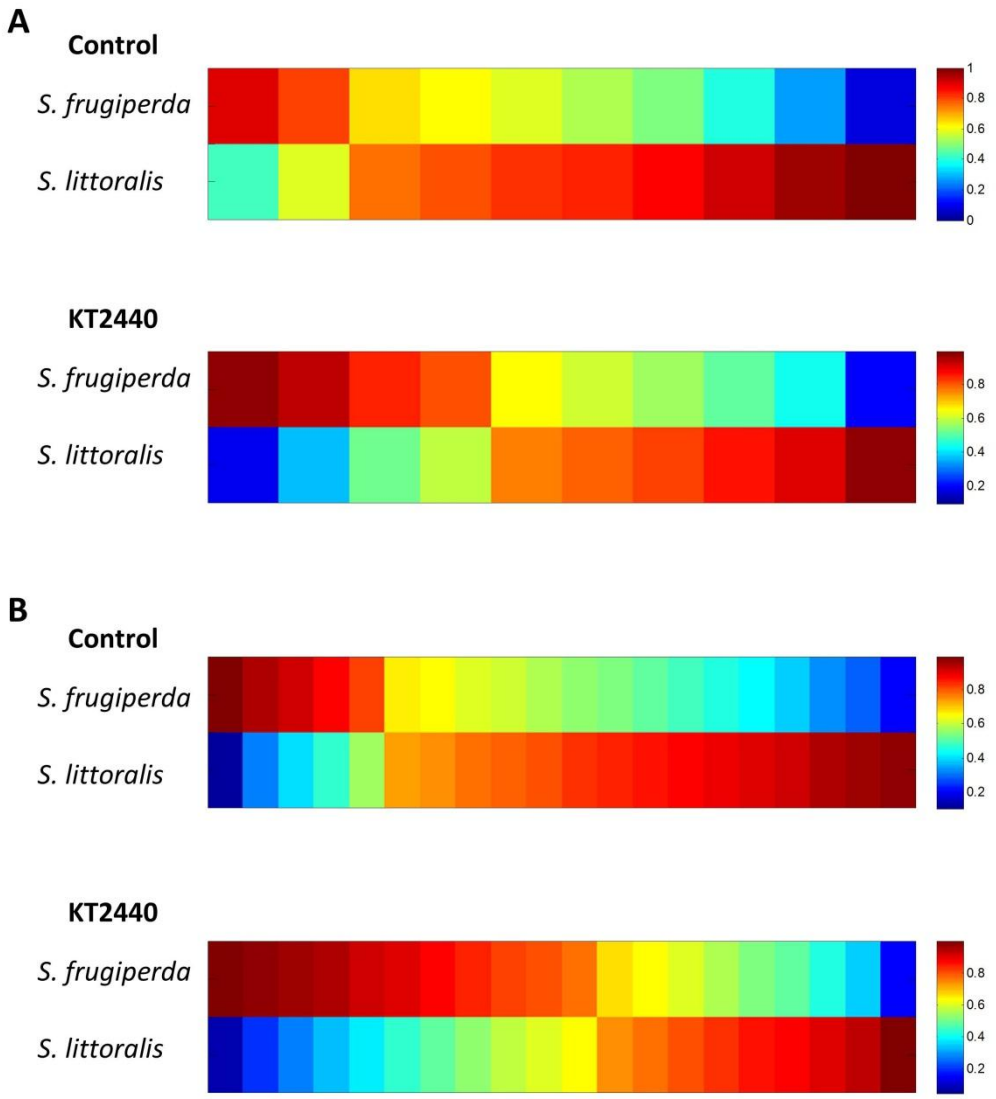

Figure S2. Cluster plot of metabolite fingerprinting of maize leaves from control or KT2440-inoculated plants that were infested with *S. littoralis* or *S. frugiperda*. A = analysis in negative mode, B = analysis in positive mode.

Significantly different markers (between treatments) were clustered on MarVis in order to have an overview of metabolomic profiles. Fewer markers were found in negative mode (271 markers for control, 347 for KT2440) than in positive mode (705 for control, 575 for KT2440). In control plants, more clusters were up-regulated after *S. littoralis* infestation. In KT2440-inoculated plants markers were more uniformly up-regulated for both *S. littoralis* and *S. frugiperda* infestations.

APPENDIX

Several preliminary experiments involving *Pseudomonas putida* KT2440 have been conducted during this project. These assays are of potential interest for further investigations on the relationship between KT2440 and maize plants. For this reason, the related results are briefly presented here.

1. Growth promotion induced by KT2440 on maize plants var. Jubilee

Table S1. Physiological measurements of 14 day-old plants (*i.e.* 10 days after KT2440 inoculation; n=5). Average values were compared by using a t-test and p values are indicated (with a significant level fixed at $p<0.05$).

Parameter	Control	KT2440	p value
Shoot length (cm)	32.3	39.2	0.001
Root length (cm)	37.8	41.1	0.24
Shoot fresh weight (g)	1.17	1.95	0.026
Root fresh weight (g)	0.33	1.16	0.008
Shoot dry weight (g)	0.11	0.17	0.027
Root dry weight (g)	0.05	0.08	0.029
Water content in leaves (%)	90.7	91.2	0.405
Water content in roots (%)	76.1	86.4	0.095

2. Effect of KT2440 inoculation on maize seed germination (var. Jubilee)

Test 1

To test the effect of bacterial inoculation on maize seed germination, non-germinated seeds were inoculated with a bacterial suspension as for pre-germinated seeds. Six replicates (representing a total of 60 plants) per treatment were grown for five days. Plant germination was assessed by counting the number of germinated seeds.

Non-germinated maize seeds were inoculated with KT2440 to verify the effect of bacterial presence on seed germination. Five days after treatment, 88.3% of control plants were germinated, whereas 28.3% (3 fold less compared to control) of bacteria-inoculated seeds were germinated (t-test, $p< 0.001$).

Test 2

Seed germination rate was also tested during the experiment concerning the assessment of growth promotion induced by KT2440 presented in this chapter (7 days after bacterial inoculation, n=3 replicates, corresponding to a total of 35 plants). There were no significant changes in seed germination rate between control plants (73.74%) and KT2440-inoculated plants (60%; p=0.42, t-test).

3. Preliminary work on maize var. Jubilee about ISR triggered by *Pseudomonas putida* KT2440 against herbivory

A first test has been conducted on the maize variety Jubilee to test a potential effect of the presence of KT2440 on the leaf and root herbivory. Plants were grown in a sterilized sand-soil mix as described in Chapter 5. Fifteen-day old plants were infested with L2 larvae of *Spodoptera littoralis*, a generalist herbivore, and *Spodoptera frugiperda*, a specialist herbivore. The larval weight was measured before the infestation and five days later. Before the infestation, shoot length was measured to test the potential growth promotion induced by the bacteria. The shoot length was significantly higher for treated plants (47.74 cm) compared to control plants (43.61 cm; $p < 0.001$, t-test). After five days of feeding there were no differences in larval weight gain for *S. littoralis*, whereas *S. frugiperda* significantly grew better on KT2440-inoculated plants ($p = 0.005$, t-test; Figure S3). In comparison to other experiments on the variety Delprim, the larvae even on untreated plants grew very slowly. We decided to retry the same experiment but with Delprim maize plants.

We also tested in parallel the effect of KT2440 on *C. graminicola* infection as a control in this test. No ISR triggered by KT2440 was observed during this experiment.

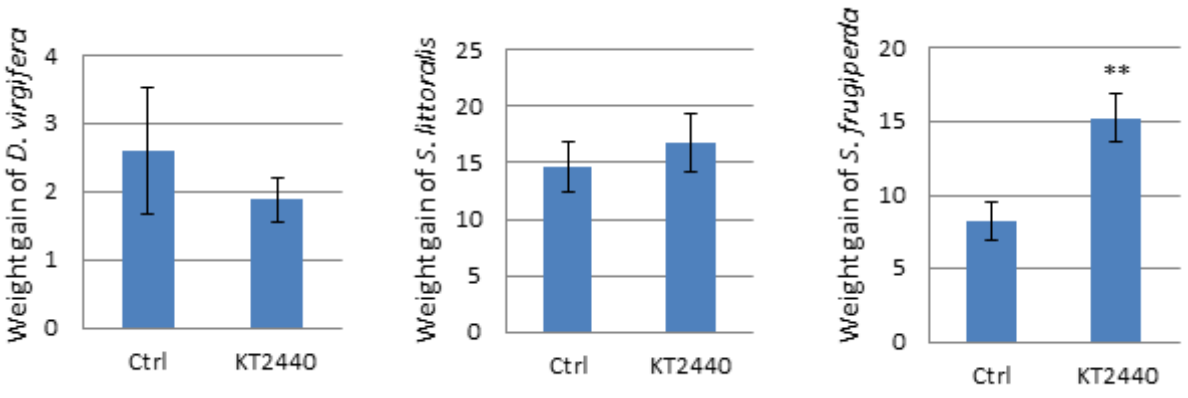

Figure S1. Test on the induction of resistance against herbivores by KT2440. Relative individual weight gain of each herbivore on control (Ctrl) and treated plants with KT2440. The asterisks indicate a significant difference with: ** if $p<0.01$, based on the result of a t-test.

It is noteworthy that a possible trade-off between growth promotion and defense induction effects occurred in this experiment. In contrary to this experiment, in the repetition (see chapter 5), we observed no growth promotion but a defense induction against *S. littoralis*. However, as the maize cultivar is different, it is difficult to really compare between these two experiments.

4. No resistance induction triggered by *Pseudomonas putida* KT2440 against the root herbivore *Diabrotica virgifera virgifera* on maize var. Delprim (in collaboration with Claudia Zwahlen)

An additional experiment using maize var. Delprim has been done to test the potential of KT2440 to induce plant resistance against the root herbivore *Diabrotica virgifera virgifera*. Nine-day old plants (n=10) were infested with four larvae. In order to measure the effect of KT2440 on plant resistance, larval weight was measured before infestation and four days later. A weight ratio was then calculated. Before infestation, no differences of shoot length were measured between control (21.82cm) and KT2440-inoculated plants (20.94cm; p=0.358, t-test). There were no effects on larval weight gain induced by KT2440 in comparison with controls (Figure S2).

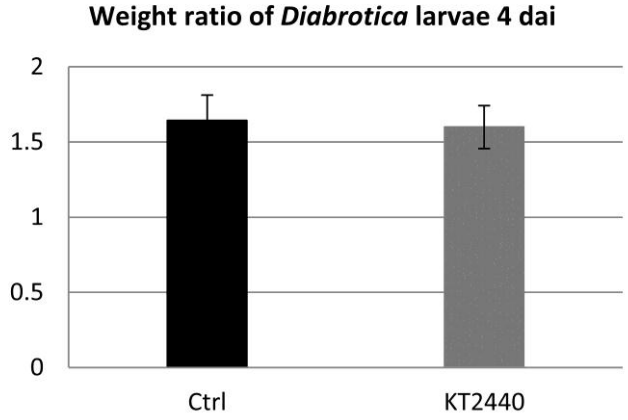

Figure S2. Ratio of larval weight of *Diabrotica* larvae four days after maize var. Delprim infestation.

In the end of the experiment, the number of bacteria present in roots of all varieties and treatments indicated that bacteria were present in KT2440-inoculated plants as well as in control plants that were infested with herbivores. However, the amount of bacteria was systematically higher in KT2440-inoculated plants in comparison with control plants. It was not possible to exclude or to confirm that bacteria recovered from control plants were KT2440.

5. Effect of ISR on the attraction of parasitoids wasps

To test a potential effect of resistance induced by KT2440 on the tritrophic level, the effect of ISR on parasitoid attraction was tested. In order to limit biological fluctuations of herbivory, plant

infestation was chemically mimicked. Nine-day old plants (var. Delprim) have been induced with methyl jasmonate one day before the parasitoid experiment as follows: 2µl of a 95% MeJA solution (Sigma-Aldrich Chemie GmbH, Germany) has been applied onto a 6mm filter. This filter was placed directly on the soil, close to the plant, without touching it. For control plants, 2µl of sterile distilled water instead of MeJA was used. Directly after treatment, plants were placed separately in closed bottles for one night. After induction, plants were placed in a six-arm olfactometer. Two arms were leaving empty. A control plant, a KT2440-inoculated plant, a control plant that was previously induced with MeJA and a KT2440-inoculated plant induced with MeJA were placed in the other arms. Six female wasps were released in the system. After 30 minutes wasps are recollected and their location was noticed (Tables S1). The wasp release was repeated 6 times. Two different percentages were calculated considering or not wasps that did not make any choice (%total and %choices).

Table S1. Attraction test of *Cotesia marginiventris* in a six-arm olfactometer system. Parasitoid wasps had to choose between blank (2 in total), a control plant, a KT2440-inoculated plant, a control plant that was previously induced with methyl jasmonate (MeJA) and a KT2440-inoculated plant induced with MeJA. Insects that did not make any choixe were recorded in the group called “center”.

	Ctrl	KT2440	CtrlMeJA	KT2440MeJA	Blank	Center
Olf 2	4	2	6	6	0	17
Olf 3	1	3	4	9	1	17
Total	5	5	10	15	1	34
% total	7.1	7.1	14.3	21.4	1.4	48.6
% choices	13.9	13.9	27.8	41.7	2.8	-

Results of Table S1 show a higher attraction of wasps for KT2440MeJA in comparison to other treatments. These preliminary results indicate a potential beneficial effect of KT2440 presence in roots for the indirect defense against leaf herbivores.

CHAPTER 6

Conclusions and perspectives

Conclusions

The results presented in this thesis report add to the understanding of the interaction between maize plants and the rhizobacteria *Pseudomonas putida* KT2440. The obtained results are summarized in the following part and the Figure 1 recapitulates our findings on the direct and indirect effects of KT2440 on maize plants. In addition, perspectives of a future research on the topic are discussed.

Growing plants in a soil-free system for a better understanding of belowground interactions?

Belowground interactions between plants and other organisms such as herbivores, fungi or bacteria are less known than similar aboveground interactions. This is mainly due to the difficult accessibility of the plant root system in the soil. To facilitate such studies, we developed a convenient system based on a pre-existing soil-free system. Comparisons between standard soil conditions and our growth system confirmed that a system without soil can be adapted to study belowground beneficial and pathogenic interactions. However, some factors are limiting for the use of such a system. The dimension of the pouches in which plants grow is restrictive for the plant size. For example, maize plants cannot grow longer than twenty days in the system. Furthermore, the nutrient solution was adapted for maize plants. In the case of growing other plant species, it would be probably necessary to adapt it in function of plant requirements. The soil-free system presented here is also inclined to contaminations and necessitates a regular changing of filter papers. In summary, the system we elaborated allows an analysis of belowground interactions and could therefore contribute to a better understanding in this field.

KT2440-maize interactions: manipulating for being accepted

Beneficial interactions between different organisms such as plant and soil-borne microbes are delicate situations that need to be balanced. Mutualistic interactions necessitate first a recognition of organisms that are involved and then an acceptance of this interaction. In this thesis, we demonstrated that, in order to establish a stable interaction, the presence of KT2440 activated maize root defenses early during the interaction is compulsory. Regarding the transcript and metabolite profile of plants colonized by KT2440, we hypothesized that KT2440 manipulate plant defense immune reactions in order to efficiently colonize roots. Whereas ISR triggered by rhizobacteria is a well described phenomenon, the signal that is translocated from roots, where ISR-triggering microbes interact with the plant, to shoots, where the ISR phenomenon is observed, remains unknown. For mycorrhized plants, a lipid signal has been described as a systemic signal (Drissner et al., 2007). The involvement of

phospholipids, as well as ABA, in the interaction between maize and KT2440 is of great interest as it could be the sign of a potential ISR root-to-shoot signal.

KT2440: a friend for maize plants

In this thesis, we demonstrated that root colonization by KT2440 had beneficial effects on maize plants such as a promotion of plant growth and an increased aboveground defense capacity against different types of biotic attacks. KT2440 enhanced the ability of maize to defend itself against the fungus *Colletotrichum graminicola* and the generalist herbivore *Spodoptera littoralis*. We demonstrated for the first time in maize that rhizobacteria can induce ISR against an hemibiotrophic pathogen and a chewing herbivore. The efficacy of KT2440 to induce resistance against chewing insects was correlated with the host-plant specialization of the insect. So, the specialist *S. frugiperda* was not affected by the presence of KT2440. A further understanding of defense mechanisms involved in ISR in maize could be of a great importance for future crop protection methods by exploiting root defense induction via the use of beneficial microbes.

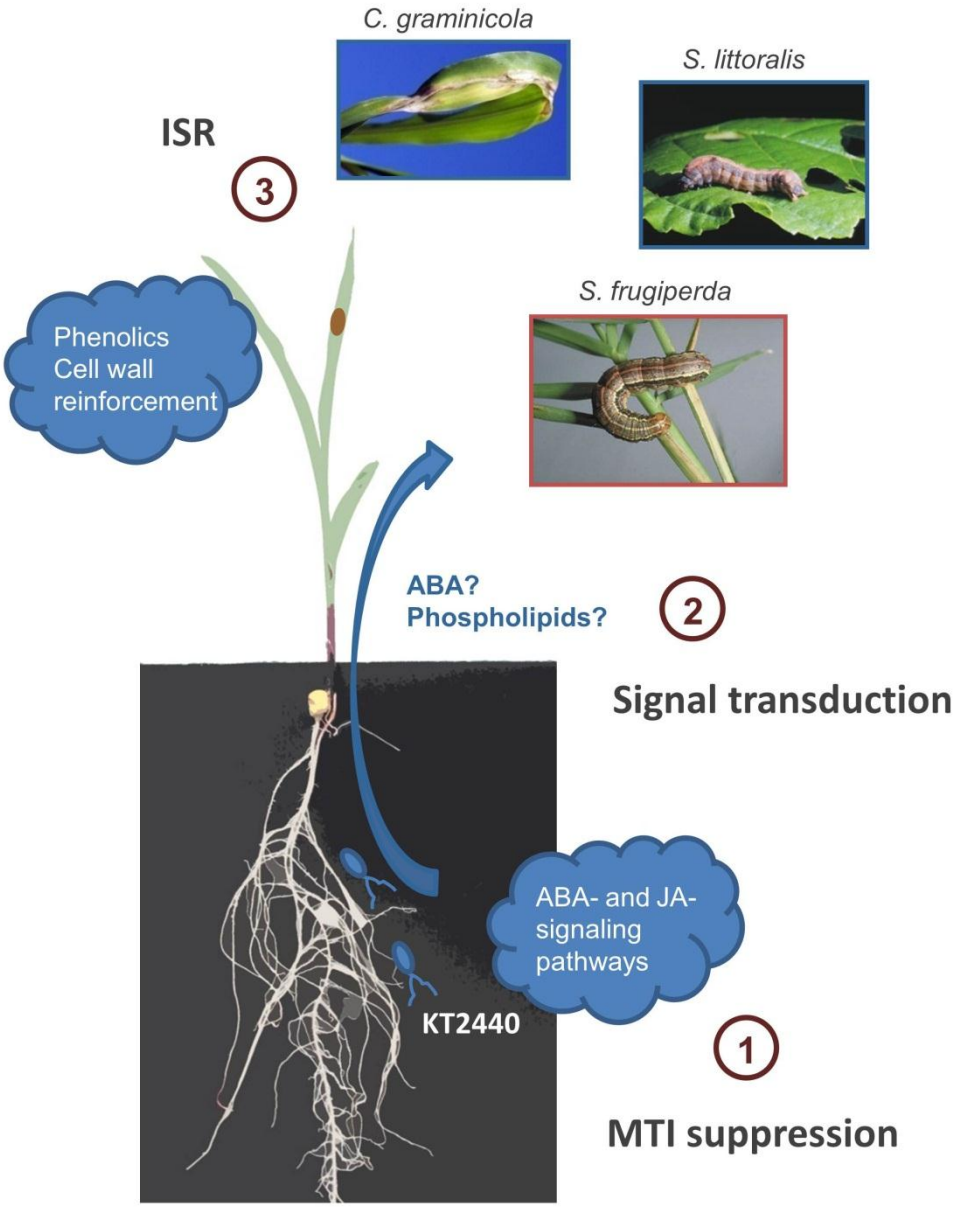

Figure 1. Summary of the direct and indirect effects of *Pseudomonas putida* KT2440 on maize plants. 1. In a first phase, early during root colonization, plant defense reactuibs were manipulated by the presence of KT2440 through the activation of ABA- and JA-signaling pathways. After early changes in metabolites and transcripts, there is the establishment of an accomodation state of the plant. 2. We found ABA and phospholipids as putative root-to-shoot signal transduction messengers. 3. KT2440 was able to induce a resistance against maize anthracnose, that is caused by *Colletotrichum gramincola*, and the generalist leaf chewing herbivore *Spodoptera littoralis*. However, the presence of KT2440 did not induce any resistance against the specialst leaf herbivore *S. frugiperda*. This resistance was mostly linked to a primed state of the plant induced by the presence of KT2440 and associated with the production of phenolic compounds and a cell wall reinforcement.

Importance of benzoxazinones during KT2440-maize interactions

The presence of benzoxazinones (BXD) in the *Poaceae* has been mostly related to their biocidal effects against a wide variety of organisms such as chewing herbivores, aphids or pathogens (see Niemeyer, 2009 for review). However, several reports also showed that BXD are not the most prevalent defense component against fungal pathogens. Recently, Rodriguez et al. (2012) showed that maize defense against the herbivore corn borer was not associated to changes of BXD content in plants. In this thesis, we also showed that BXD are possibly not, or only partially, involved in resistance induced by KT2440 against herbivores and pathogens (Chapter 4). In our study, mainly hydroxycinnamate conjugates were found as potential defense compound precursors. Instead of having a direct biocidal effect these compounds could be cleaved to form toxic compounds or a cell wall reinforcement as found for maize defense against corn borer (Rodriguez et al., 2012).

BXD are not only defense compounds but they are also regulatory signals in plant innate immunity (Ahmad et al., 2011) and chemotaxis signals for KT2440 (Neal et al., 2012). This thesis showed that BXD content in maize roots is influenced by the presence of KT2440 during root colonization establishment (Chapter 3). KT2440 are attracted by BXD but they are also tolerant to these compounds (Neal et al., 2012). Hence, further investigations would be necessary but we hypothesized that KT2440 could exploit BXD content by enhancing DIMBOA release from bacterized roots as a signal for conspecifics and as a toxic metabolite against other rhizobacteria that are less tolerant to DIMBOA.

Perspectives

Several interesting future research perspectives arose from this thesis. These points are presented here.

Use of the soil-free system for other plant species

Testing other plant species, as well as other pathogens or beneficial microbes, would help to enhance our knowledge on belowground interactions. However, testing root herbivory would necessitate some adaptations of the system in order to close it and to avoid herbivore escapes.

Use of the soil-free system for a further understanding of KT2440-maize interactions

Exploiting the ability of the soil-free system to manipulate the nutritional context for the plant would be interesting. For example production of pyoverdin, a possible ISR determinant, by KT2440 could be induced by diminishing the iron content of the nutrient

solution. Mimicking conditions in a soil that is nutrient-poor or nutrient-rich by changing nutrient balance in the nutritive solution would be easy to do.

Growth promotion and defense induction trade-offs

The plant hormone balance regulates two phenomena that are known to be costly for the plant and to have antagonistic effects to each other: growth and defense. As we never observed both effects at the same time, further investigations would be interesting to test which parameters can influence the induction of defense or of growth promotion by KT2440.

Direct effects of KT2440 on maize plants earlier in the interaction

In this thesis we only detected significant changes in maize plants three days after bacterial inoculation but it is still remaining to know what kind of changes occur earlier during bacterial root colonization. The use of the soil-free system would allow an easy bacterial inoculation of older maize plants (that have already well-developed leaf) and the collection of roots as well as leaves a few hours after inoculation (*e.g.*: 6, 12, 24, 36 hours after inoculation). Preliminary investigations showed that bacterial inoculation on older plants than normally (4-6 day-old seedlings) leads to a similar KT2440 root colonization level. A local and a systemic analysis early in the interaction could help to find a potential ISR root-to-shoot signal.

Further experiments for metabolite and transcript analyses for ISR mechanisms triggered by KT2440 against *Colletotrichum graminicola* and *Spodoptera littoralis*

Experiments concerning ISR mechanisms are very variable considering that three partners are involved during the interaction: the plant, the beneficial microbe and the attacker. To minimize this variation between various experiments, challenging the leaves with a fungal elicitor such as chitin (e.g. chitosan) or by mimicking a caterpillar attack via plant wounding and/or treatment with jasmonic acid or oral secretion of *Spodoptera littoralis* larvae.

ISR induced by KT2440 against other plant stresses?

In addition to a further understanding of ISR observed in this thesis, it would be interesting to test if KT2440 can be effective against other stresses, such as biotic root attacks by pathogens or herbivores. Direct, as well as indirect, defenses could be induced by the presence of KT2440. KT2440 is able to produce antibiotic compounds that could directly affect root pathogens. By using a split-root system, it would be also possible to test if the

presence of KT2440 in one part of maize roots could induce a systemic resistance in non-bacterized root parts. To our knowledge, it has never been demonstrated if ISR against root herbivores could be also dependent on the same parameters as for leaf herbivores: the host plant specialization and the feeding mode. For maize plants, it would be possible for example to compare between the specialist *Diabrotica virgifera* and the generalist *D. balteata*. Our preliminary test (Chapter 4, Appendix) using *D. virgifera* seemed to indicate that the presence of KT2440 does not enhance maize resistance against this root herbivore.

Beneficial microbes have been also referred to as potential plant tolerance inducer in the context of abiotic stress conditions in the surrounding plant environment. A master project conducted in our laboratory tended to show the capacity of KT2440 to induce maize tolerance against salt stress. However, repetition of these experiments would be necessary. The up-regulation of the ABA-dependent signaling pathway, important for abiotic stress tolerance, during KT2440 root colonization (Chapter 3) could favor maize tolerance against abiotic stresses.

Preliminary investigations to test the effect of ISR on the third trophic level (Chapter 4, Appendix) indicated a potential beneficial effect of ISR triggered by KT2440. Plant attractiveness to the parasitoid wasp, *Cotesia marginiventris*, was enhanced in bacterized plants that were treated with a herbivore mimicking treatment (methyl jasmonate). Further experiments would be needed to confirm our results.

Effect of leaf attack on KT2440 root colonization

Recently, it has been showed that plant treatment with defense hormones such as SA or JA can affect the rhizosphere colonization. Hence, a leaf attack by a herbivore or a fungal pathogen could have an effect on the presence of KT2440 on roots. The fluctuation of the bacterial concentration could be an important process for ISR induction.

Use of KT2440 as a beneficial microbe in the field

KT2440 are well-known rhizobacteria that have an efficient capacity of root colonization for a wide range of plants of agronomic interest (Molina et al., 2000), even under soil competition (Neal et al., 2012). Moreover, KT2440 are tolerant rhizobacteria to heavy metal presence in polluted soil, possess putative growth promotion traits and have been recognized as a secure microbe that lacks a number of virulence-associated traits (Wu et al., 2010). Despite these encouraging parameters, almost nothing was known on the effect of KT2440 on maize plants and as a potential bioagent for crop production. Our study enhanced the possibility of KT2440 as being an interesting rhizobacteria for an agricultural use. Many microbes have been described as benefic for plant health and an increased

demand for sustainable approaches to control crop disease favored the application of such bioagents in fields. However, exploiting induced resistance elicitors in agriculture is not common. Inconsistency of ISR and a less efficient disease control in comparison with standard pesticide are the main reasons (Walters et al., 2013). Research under laboratory conditions has shown the potential of ISR as an alternative to fungicides or pesticides that are still massively used. Recently, studies also analyzed ISR use as a crop protection practice and further research in this sense remain to be done. In addition to technical and financial challenges linked to the production of bacterial formulation, there is probably also work to do on the general opinion to gain more acceptance for utilizing induced resistance as a crop protection method.

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List of publications and awards

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* these authors contributed equally

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Award for best poster: Planchamp C, Balmer D, Robert C, Zwahlen C, Mauch-Mani B. Induced resistance in maize triggered by the beneficial bacteria *Pseudomonas putida* KT2440. PR IR 2011 meeting, Neuchâtel, Switzerland.