Impact of mutualistic root fungi on crop quality and pest defense

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List of publications and respective contributions

The present thesis is a cumulative work of four publications selected from my list of literature (already published or submitted for publication) and highlighted below by Roman numerals according to their respective chapters.

II. Cosme M, Stout M, Wurst S (2011) Effect of arbuscular mycorrhizal fungi (*Glomus intraradices*) on the oviposition of rice water weevil (*Lissorhoptrus oryzophilus*). Mycorrhiza 21: 651-658. **MC** designed and conducted the experiment, analyzed and interpreted the data, wrote the manuscript, and submitted it to Mycorrhiza. SW and MS mentored the design and reviewed the manuscript.

III. Cosme M, Lu J, Erb M, Stout MJ, Franken P, Wurst S (201X) A fungal endophyte helps plants to tolerate root herbivory through changes in gibberellin and jasmonate signaling. Submitted to New Phytologist NPH-MS-2015-20036. **MC** designed and conducted the experiments, analyzed and interpreted the data, wrote the manuscript, and submitted it to New Phytologist. JL mentored laboratory analyses. ME, PF and SW mentored the design and reviewed the manuscript.

IV. Cosme M, Franken P, Mewis I, Baldermann S, Wurst S (2014) Arbuscular mycorrhizal fungi affect glucosinolate and mineral element composition in leaves of *Moringa oleifera*. Mycorrhiza 24: 565-570. **MC** designed and conducted the experiment, analyzed and interpreted the data, wrote the manuscript, and submitted it to Mycorrhiza. IM and SB mentored laboratory analyses and reviewed the manuscript. PF and SW mentored the design and reviewed the manuscript.

V. Cosme M, Ramireddy E, Franken P, Schmülling T, Wurst S (201X) The plant cytokinin status regulates the arbuscular mycorrhizal symbiosis between *Nicotiana tabacum* and *Rhizophagus irregularis*. Submitted to Mycorrhiza MCOR-S-15-00129. **MC** designed and conducted the experiment, analyzed and interpreted the data, wrote the manuscript, and submitted it to Mycorrhiza. ER conducted gene expression analyses and reviewed the manuscript. TS, PF and SW mentored the design and reviewed the manuscript.

Summary

The increasing global population brings major challenges to the human food supply, whereas modern agriculture is accompanied by several environmental problems and still leaves many people hungry or malnourished. A more sustainable production and a higher nutritional value of plant foods are therefore new agricultural paradigms. Part of the solution to enhance yield and nutritional value of crop foods in a more sustainable manner might be found in the overlooked rhizosphere, where ancient plant-microbe mutualisms are known to provide important ecological functions. These microbes interact actively with plant intrinsic regulators, which in turn have the potential not only to mediate plant-microbe interactions, but also to influence plant growth, health and quality. The present thesis had two main objectives: 1) to test novel effects of beneficial microbes on crop plants related with the new agricultural paradigms; and 2) to investigate the role of intrinsic plant regulators involved in microbial effects on crop plants.

In **chapter II,** I tested whether the beneficial arbuscular mycorrhizal (AM) fungi can affect the aboveground oviposition of *Lissorhoptrus oryzophilus* (rice water weevil; RWW), a root-feeding insect of rice plants. Rice is one of the major staple foods worldwide. RWW is an important global pest of rice, whose adults feed aboveground and the larvae feed belowground. I found that AM fungi enhanced the aboveground oviposition by RWW, which is a novel aspect of agro-ecological interactions. This suggests that AM fungi can reduce rice resistance against RWW. Therefore, soil fungi that are generally considered beneficial in terms of nutrient uptake may not be beneficial in respect to protection against particular herbivores.

In **chapter III**, I tested whether an endophyte can protect root against herbivory though gibberellic acid (GA) and jasmonic acid (JA) signaling in rice. In contrast to AM fungi, the Sebacinalean root endophyte *Piriformospora indica* attenuated the negative effects of RWW on growth through induced root tolerance, without affecting root resistance. This induced tolerance was mediated by induction of GA signaling and suppression of JA signaling. Thus, belowground plant-microbe mutualisms can enhance the tolerance of a globally important crop plant in response to the attack by an insect pest. These effects were at least partially mediated by plant intrinsic regulators and should be considered in future management practices.

To explore the potential of AM fungi in improving the nutraceutical value of plant foods, in **chapter IV** I tested whether AM fungi can affect the bioactive compounds and mineral elements in edible leaves of *Moringa oleifera*, a high nutritional vegetable crop cultivated in the tropics and sub-tropics. AM fungi enhanced non-specifically the levels of glucosinolates, reduced species-specifically the levels of carotenoids, and increased the levels of two microelements in *M. oleifera* leaves. These results encourage research on other AM fungal species and their combinations to achieve general benefits on the nutraceutical value of *M. oleifera*.

The role of cytokinin (CK) levels in roots and shoots in AM symbiosis is yet unclear. In **chapter V**, I tested whether plant CK status regulates the AM symbiosis between tobacco plants and the AM fungus *Rhizophagus irregularis*. The organ-specific CK status affected profoundly the performance of tobacco in response to AM symbiosis, and suggested that CK in roots and shoots contribute to balance the nutrient exchange between symbionts.

Overall, the functions of plant-microbe mutualisms can vary considerably, while phytohormones can play a defining role in these mutualistic functions. These findings provide significant contributions to the field of plant-microbe interactions with potential for application in crop production.

Zusammenfassung

Das globale Bevölkerungswachstum führt zu erhöhten Anforderungen an die Nahrungsversorgung, während die moderne Landwirtschaft durch verschiedene umweltbedingten Problemen beeinträchtigt wird. Hunger und Unterernährung sind bleibende globale Probleme. Daher stellen nachhaltige Produktion und eine höherer Nährwert von pflanzlicher Nahrung die neuen Paradigmen der Landwirtschaft dar. Ein Teil der Lösung zur Verbesserung der Erträge und der Erhöhung des Nährwerts von Nutzpflanzen auf nachhaltige Weise könnte in der bisher vernachlässigten Rhizosphäre liegen, in der bekanntermaßen evolutionär ursprüngliche mutualisticshe Pflanzen-Mikroorganismen-Wechselwirkungen wichtige Funktionen erfüllen. Diese Mikroorganismen interagieren aktiv mit Pflanzen-intrinsischen Regulatoren, welche wiederum das Potenzial haben, nicht nur die mutualistischen Wechselwirkungen zu vermitteln, sondern auch die Pflanzenwachstum, -gesundheit und –qulaität zu beeiflussen. Die vorliegende Doktorarbeithat zwei zentrale Ziele: 1.) Die Überprüfung bisher unbekannter Effekte von mutualistischer Pilzen auf Nutzpflanzen in Verbindung mit den neuen landwirtschaftlichen Paradigmen und 2.) Die Untersuchung der Rolle intrinsischen Pflanzenregulatoren bei den Effekten der Mikroorganismen auf Nutzpflanzen.

In **Kapitel II**, überprüfte ich, ob die mutualistischen arbuskulären Mykorrhizapilze (AM) die überirdische Eiablage von *Lissorhoptrus oryzophilus* (Rice Water Weevil), ein sich von Reiswurzeln ernährendes Insekt, beeinflussen. Reis ist eines der wichtigsten Grundnahrungsmittel weltweit und der Rice Water Weevil (RWW) ist eine globale Plage, bei der sich die erwachsenen Tiere überirdisch und die Larven unterirdisch ernähren. Ich habe herausgefunden, dass die überirdische Eiablage des RWW durch die AM-Pilze erhöht werden kann, was einen neuen Aspekt agroökologischer Interaktionen darstellt. Daraus lässt sich vermuten, dass die AM-Pilze die Resistenz von Reis gegen den RWW reduzieren. Während diese Bodenpilze für die Nährstoffaufnahme generell als nützlich verstanden werden, trifft dies anscheinend nicht bei der Abwehr von Herbivoren zu.

In **Kapitel III**, habe ich getestet ob ein Endophyt über die Gibberellinsäure (GA) und die Jasmonsäure (JA) Signaltransduktionswege die Wurzel vor Herbivoren schützen kann. Im gegensatz zu AM-Pilzen kann der Wachstums-fördernde Wurzelendophyt *Piriformospora indica*

die negativen Auswirkung des RWW auf das Wachstum von Reis durch induzierte Wurzeltoleranz abmildern, ohne die Resistenz der Wurzel zu beeinflussen. Diese induzierte Toleranz wurde durch die Induktion des GA Signaltransdruktion und durch die Unterdrückung von JA Signaltransduktion vermittelt. So können unterirdische Pflanzen-Mikroorganismen Wechselwirkungen die Toleranz einer global sehr wichtigen Kulturpflanze als Reaktion auf die Angriffe durch ein Insektenparasit erhöhen. Diese Effekte wurden zumindest teilweise durch pflanzlich intrinsische Regulatoren vermittelt und sollten in der zukünftigen Managementpraxis berücksichtigt werden.

Um das Potenzial von AM-Pilzen für Nährstoffgehalte von Nahrungspflanzen zu untersuchen, testete ich in **Kapitel IV**, ob AM-Pilze bioaktive Komponenten und mineralische Elemente in essbaren Blättern von *Moringa oleifera* beeinflusst. *M. oleifera* ist eine hochgradig nährstoffhaltige Gemüsepflanze, die in den Tropen und Sub-Tropen kultiviert wird. AM-Pilze verbesserten unspezifisch den Gehalt an Glucosinolaten, reduzierten artspezifisch den Gehalt an Carotinoiden und erhöhten den Gehalt von zwei Mikroelementen in *M. oleifera* Blättern. Diese Ergebnisse regen zu weiteren Untersuchungen mit anderen AM-Pilz-Spezies und deren Kombinationen an, um höhere Nährstoffgehalte bei *M. oleifera* zu erzielen.

Die Rolle von Cytokininen (CK) in Wurzeln und Sprossen von AM Pflanzen ist noch weitgehemnd unbekannt. In Kapitel V habe ich überprüft, ob der Gehalt von CK die AM Symbiose zwischen Tabakpflanzen und dem AM-Pilz *Rhizophagus irregularis* reguliert. Der organ-spezifische CK Gehalt beeinflusste signifikant Wachstum und Phosphataufnahme von Tabak in Reaktion auf die AM Symbiose tiefgreifend. Dies lässt annehmen, dass CK in Wurzel und Spross einen Beitrag zur Regulation des Nährstoffaustausch zwischenden Symbionten leistet.

Insgesamt kann die Funktion von mutualistischen Pflanzen-Mikroorganismen Wechselwirkungen sehr stark variieren, während Phytohormone eine zentrale Rolle in der Regulation der mutualistischen Funktionen spielen. Die hier beschriebenen Forschungsergebnisse stellen neue Erkenntnisse für das Feld der Pflanzen-Mikroorgansimen Wechselwirkungen dar und sind von wesentlicher Bedeutung für die für die Anwendung in der Produktion von Kulturpflanzen.

Chapter I: General introduction

The global human population has increased exponentially from 1.4 billion to 7.2 billion inhabitants in less than two hundred years and is projected to reach 11.2 billion by 2100 (United Nations 2015).This dramatic increase brought major demands and challenges to the human food supply. Crop plants as the primary component of food supply are central to the solutions to these challenges, and despite the global intensification of agriculture and the great progress over the last decades that boosted food production through the breeding of high-yield crop varieties and the use of pesticides, nitrogen (N)-based fertilizers and more water, the goal to reduce the problems associated with food security is far from being reached and still left many hungry or malnourished (Welch & Graham, 1999; Waller *et al.*, 2005; Mayer *et al.*, 2008; Gewin, 2010). Moreover, agricultural intensification was accompanied by an alarming set of environmental problems such as the indiscriminate use of toxic chemicals, waterway pollution, loss of soil fertility by erosion, acidification, salinization and desertification (Welch & Graham, 1999; Gewin, 2010) and left a high dependence on limited resources. For instance, phosphorus (P) is one of the major plant nutrients that is least available in soils (Raghothama, 1999) and is introduced as fertilizer derived from mined phosphate rock. There is a general consensus that the quality and accessibility of remaining reserves of phosphate rock are decreasing and could be exhausted within the next 30 to 300 years (Cordell & White, 2011). In addition, our food supply appears to be failing globally by not providing enough balanced nutrient output to meet all the human nutritional needs, particularly for those living in developing regions, which often leads to severe chronic diseases due to micronutrient malnutrition (Welch & Graham, 1999; Mayer *et al.*, 2008; Sands *et al.*, 2009; White & Broadley, 2009). These problems are, however, indirectly linked to the effectiveness of crop roots to overcome mineral nutrient and water limitations and a part of the solution to enhance yields and nutritional value of crop foods with reduced inputs might be found in the often overlooked rhizosphere (Ryan *et al.*, 2009; Gewin, 2010; Smith, F & Smith, S, 2011; Antunes *et al.*, 2012). This could include for instance a better exploitation of belowground microbial mutualists which are known to perform important ecological functions in nature and were largely unnoticed during crop domestication and breeding. Advancing our understating on this belowground agro-ecological sub-system may potentially contribute to improve our ability to meet the nutritional needs of an increasing global population.

Terrestrial plants have evolved over more than 460 million years in close association with mutualistic microbes that affect the ecological dynamics of plants in nature (Redecker *et al.*, 2000; Rillig, 2004). The mycorrhizal fungi, for instance, developed specialized structures that supply soil-derived nutrients to roots in exchange for plant-delivered photosynthates (Smith & Read, 2008; van der Heijden *et al.*, 2015). Fossil records of fungal hyphae and spores strongly resembling those of the arbuscular mycorrhizal (AM) fungi (Glomeromycota) indicate that these microbes were present at a time when terrestrial flora only consisted of bryophytes, and suggests that they may have been instrumental in facilitating the land colonization by ancient plants (Simon *et al.*, 1993; Redecker *et al.*, 2000). The presence of genes required for AM formation in a broad set of plant lineages, including liverworts and hornworts, suggests that mycorrhizal genes were present in the common ancestor of land plants (Wang *et al.*, 2010), while an extensive literature survey presenting a checklist of mycorrhizal occurrence in 92 % of plant families confirms the ubiquity and ancient origin of these belowground plant-fungus mutualisms (Wang & Qiu, 2006). These are, however, not the only belowground plant mutualisms widely distributed. A recent study using DNA-based detection and electron microscopy on more than one hundred root samples from phylogenetically and ecologically diverse plants, including close to thirty plant families from four continents, suggest that Sebacinalean fungi are almost universally present as root endophytes (Weiß *et al.*, 2011). Endophytes, as opposed to mycorrhizal or endoparasitic microbes, are microbes that colonize the tissues of living plants without forming specialized structures such as interaction apparatus or arbuscules and without causing symptoms of disease on their host plants (Wilson, 1995; Weiß *et al.*, 2011). Some of these endophytes are able to confer protection to and promote the growth of their host plants (Waller *et al.*, 2005; Barazani *et al.*, 2007; Dolatabadi *et al.*, 2011). How old the mutualisms between plants and Sebacinalean endophytes are is unclear, but these microbes can associate with bryophytes and liverworts in nature (Weiß *et al.*, 2011), which could be indicative of a ancient nature for this endophytic life form as well.

If we would resume the history of plant-microbe mutualisms into a timescale of one calendar year with 365 days, crop plants would appear only in the last 12 hours and modern plant breeding in the last 5 minutes. Crop domestication began approximately 10,000 years ago (Doebley *et al.*, 2006; Meyer & Purugganan, 2013). Archaeological evidence suggests that humans initially planted or carried deliberately for wild plants that had favorable nutritional

traits, which then led to the domesticated species. This was followed by a diversification period involving the spread and adaptation of domesticated plants into different cultural environments. Finally, a conscious and deliberate breeding of crops was initiated. Although breeding has been practiced since early domestication (Meyer & Purugganan, 2013), it was the fundamental discoveries of Darwin and Mendel at the turn of the 20th century that established the scientific basis for plant breeding and genetics (Moose & Mumm, 2008). The most economically important crop varieties of today are a result of that intensive development period of modern breeding brought by the "Green Revolution" in the 1940s (Moose & Mumm, 2008; Gewin, 2010; Meyer & Purugganan, 2013). But how breeding has affected plant-microbe mutualisms is still an open question. For example, wheat varieties released before 1950 showed more consistent growth responses to AM fungi than varieties released afterwards, which led to the suggestion that breeding has reduced the mycorrhizal growth response of this crop (Hetrick *et al.*, 1993). However, a more recent meta-analysis on 39 publications working on 320 different crop varieties found no evidence that new crop varieties lost their ability to respond in terms of growth to their mycorrhizal symbionts (Lehmann *et al.*, 2012). Nevertheless, from an evolutionary point of view, crop plants represent a rapid and widespread distribution of novel plant genotypes, whose complex interactions with ancient microbial mutualisms are far from being fully understood (Smith, SE & Smith, FA, 2011; Weiß *et al.*, 2011; Pozo *et al.*, 2015).

The benefits of microbial mutualisms to plants have been documented by many studies comparing inoculated plants with mock-inoculated controls (Wang & Qiu, 2006; Waller *et al.*, 2008; Rodriguez *et al.*, 2009; Pieterse *et al.*, 2014; van der Heijden *et al.*, 2015). These benefits may vary with different factors but generally involve improved mineral nutrition, enhanced primary productivity and fitness as well as greater tolerance and resistance against biotic and abiotic stresses. Mutualistic microbes can also indirectly effect their hosts by altering ecological processes that are important for plant growth, such as the improvement of soil structure (Siddiky *et al.*, 2012) and the reduction of the risk of nutrient loss in soils (Veresoglou *et al.*, 2012; van der Heijden *et al.*, 2015). Beneficial microbes may become, however, parasites under particular conditions (Schulz & Boyle, 2005; Kogel *et al.*, 2006; Johnson, 2010). As their benefits are context-dependent, deciphering how intrinsic plant regulators affect the plant interaction with mutualistic microbes (Jacobs *et al.*, 2011; Pieterse *et al.*, 2014; Pozo *et al.*, 2015) is an important step to understand how crops can optimize their symbiotic strategies to improve performance.

Phytohormones such as jasmonic acid (JA), gibberellic acid (GA), cytokinin (CK), abscisic acid, auxin, brassinosteroids, ethylene, salicylic acid and strigolactones are small metabolites that regulate intrinsic developmental and physiological pathways in plants but also mediate the response of these pathways to environmental cues (Erb *et al.*, 2012b; Pozo *et al.*, 2015). By modifying the biosynthesis, allocation or signal transduction of these metabolites, plants are able to regulate and coordinate growth, stress tolerance and/or resistance to promote survival, fitness or escape from environmental stress (Colebrook *et al.*, 2014). For instance, JA is a universal regulator of plant induced resistance against a broad spectrum of chewing insect herbivores and necrotrophic pathogens (Howe & Jander, 2008; Pieterse *et al.*, 2014), many of which constitute important agricultural pests and diseases. Induced resistance is a state of resistance in plants triggered by biological or chemical inducers which protects non-exposed plant parts against future attack (Pieterse *et al.*, 2014). Plant growth–promoting bacteria and fungi in the rhizosphere can activate the plant JA signaling which induces systemic resistance in the whole plant body for enhanced defense (Pieterse *et al.*, 2014). JA also regulates plant growth via antagonistic interactions with GA signaling (Yang *et al.*, 2012; Heinrich *et al.*, 2013; Matschi *et al.*, 2015). GA is essential for developmental processes in plants, including seed germination, stem and root elongation, leaf expansion, trichome development, pollen maturation and the induction of flowering, and has been recently linked to tolerance against cold, salt and osmotic stress (Ubeda-Tomás *et al.*, 2009; Davière & Achard, 2013). The Sebacinalean root endophyte *Piriformospora indica* can recruit GA signaling in roots to suppress immunity and establish a mutualistic association with its host plants (Schäfer *et al.*, 2009; Jacobs *et al.*, 2011). Among the phytohormones implicated in growth and development of plants, CK is a major regulator of the shoot to root ratio, shoot and root architectures, photosynthesis and nutrient uptake (Werner & Schmülling, 2009; Kieber & Schaller, 2014), and causes distinct changes in sink and source relation of photosynthetically fixed carbon (C) within the plant (Werner *et al.*, 2008). The plant symbiotic association with AM fungi, which are biotrophs entirely dependent on plant-derived C (Smith & Read, 2008), is regulated by nearly all phytohormones (Pozo *et al.*, 2015). Although relatively less studied, CK is known to accumulate in AM plants and could be functionally important for the symbiotic outcome as well (Drüge & Schonbeck, 1992; Shaul-Keinan *et al.*, 2002; Cosme & Wurst, 2013). Overall, mutualistic microbes are able to interact actively with intrinsic developmental and physiological pathways of their host plants, which in turn has the

potential to mediate not only the plant-microbe mutualism but also the plant interaction with several ecological factors.

Important ecological drivers of plant yield and physiology are the heterotrophic consumers that depend on plant-derived nutrients to complete their life cycles (Wardle *et al.*, 2004; Erb *et al.*, 2012b). For instance, many insect herbivores that feed above- and/or belowground can have dramatic effects on their host plants by removing considerable amounts of plant tissues (Hunter, 2001; Erb *et al.*, 2012a). Ecological linkages between soil organisms and aboveground insect herbivores has gathered increasing recognition (Bezemer *et al.*, 2003; Wurst & Jones, 2003; Wardle *et al.*, 2004; Soler *et al.*, 2007). Although often ignored in studies on plant-herbivore interactions, mycorrhizal symbiosis can influence the performance of insect herbivores, but the magnitude and direction of these effects generally depend on the insect's feeding guild and the fungal identity (Gange, 2001; Koricheva *et al.*, 2009). Therefore, a fungus that may be considered beneficial in terms of nutrient uptake may not be necessarily beneficial in terms of anti-herbivore protection, and in order to generalize about fungal functionality it would be important to collect a deeper understanding of the mechanisms behind these effects. Moreover, fungal endophytes that live within the leaf tissues of grasses are well known for their ability to produce alkaloids that deter herbivory, conferring protection to their host plants (Rodriguez *et al.*, 2009), but the effects of root endophytes on root-herbivore interactions are largely unknown, even though they could have potential effects on plant physiological and yield responses to belowground herbivory.

The enhancement of crop yields has been traditionally the main emphasis of modern agriculture, but at the turn of the new millennium a more sustainable production and a higher nutritional value of plant foods emerged as new agricultural paradigms (Graham *et al.*, 1999; Welch & Graham, 1999; Morris & Sands, 2006; Mayer *et al.*, 2008). Nutritional-related chronic diseases in humans such as coronary heart diseases, stroke and cancers are paramount causes of mortality in high income countries worldwide (World Health Organization, 2013), and the risk of these diseases can be reduced with dietary intake of vegetable crops possessing high amounts of bioactive secondary metabolites such as carotenoids, glucosinolates, flavonoids and others (Steinmetz & Potter, 1996; Kaur & Kapoor, 2001; Liu *et al.*, 2001; Traka & Mithen, 2009). Therefore, the characterization of factors leading to the accumulation of these metabolites in

edible plant tissues has prompted much research attention. Moreover, micronutrient malnutrition, which affects the health of about 2 billion people globally, particularly of those living in developing regions, is primarily caused by nutritional deficits in iron, vitamin A, iodine, zinc, vitamin B_9, and selenium; although deficits in vitamin C, calcium, magnesium and copper are also present in some populations (Welch & Graham, 1999; Mayer *et al.*, 2008; White & Broadley, 2009). To reduce the health burden associated with micronutrient malnutrition, most of these vitamins and mineral elements have been targeted for crop biofortification (Welch & Graham, 1999; Mayer *et al.*, 2008; White & Broadley, 2009). Belowground plant-microbe mutualisms are known to affect the plant accumulation of secondary metabolites and to improve plant nutrient status (He & Nara, 2007; Antunes *et al.*, 2012; Giovannetti *et al.*, 2012; Zeng *et al.*, 2013). Therefore, these microbes could potentially help to improve the nutraceutical value of plant foods while contributing to a more sustainable crop production by reducing the agrochemical inputs.

Thesis outline

The present thesis has two main objectives: 1) to test novel effects of beneficial microbes on crop plants related with new agricultural paradigms; and 2) to investigate the role of intrinsic plant regulators involved in microbial effects on crop plants. To this end, I used the AM fungal species *Rhizophagus irregularis* (former *Glomus intraradices*) and *Funneliformis* mosseae (former *G. mosseae*) and the Sebacinalean root endophyte *P. indica* as the model beneficial microbes. The model crop plants were: rice (*Oryza sativa*), one of the most economically important cereal staples worldwide; *Moringa oleifera*, a vegetable crop with high nutraceutical value cultivated in tropics and sub-tropics; and tobacco (*Nicotiana tabacum*), used here essentially as a model for genetic investigation. As a model herbivore, I used the rice water weevil (RWW; *Lissorhoptrus oryzophilus*), which is an important belowground pest of rice worldwide. By testing several model systems under greenhouse conditions, I intended to provide significant advances to the field of plant-microbe interactions with the following chapters:

Chapter II: Effect of arbuscular mycorrhizal fungi (*Glomus intraradices*) on the oviposition of rice water weevil (*Lissorhoptrus oryzophilus*)

Chapter III: A fungal endophyte helps plants to tolerate root herbivory through changes in gibberellin and jasmonate signaling

Chapter IV: Arbuscular mycorrhizal fungi affect glucosinolate and mineral element composition in leaves of *Moringa oleifera*

Chapter V: The plant cytokinin status regulates the arbuscular mycorrhizal symbiosis between *Nicotiana tabacum* and *Rhizophagus irregularis*

In the study presented in **chapter II**, a choice bioassay on flooded rice plants was used to test whether the root colonization by *R. irregularis* can affect the aboveground oviposition preference by adults of RWW. I hypothesized that AM colonization affects the aboveground oviposition behavior of RWW females in order to potentially optimize the performance of their root-feeding offspring. The AM fungal colonization, the plant biomass, the number of eggs laid by RWW, the consumed leaf area by RWW feeding, the concentrations and contents of N, C, and P in shoots and of N and C in roots were determined.

In the study presented in **chapter III**, two greenhouse experiments were carried out. In experiment I, a fully crossed factorial design was used to test whether the leaf-feeding by RWW adults aboveground influences the subsequent belowground activity of conspecific root-feeding larvae and whether prior root inoculation with *P. indica* can protect the rice plants against the subsequent attacks by RWW. The plant, larval and fungal performances, the root morphology, the consumed leaf area by RWW feeding, the mineral elements concentration in shoots, the accumulation of jasmonates in leaves and in roots, and the expression of GA and JA biosynthetic genes in roots were measured. In experiment II, split-root systems were combined with fully crossed factorial designs, using the same factors as in experiment I, to test whether RWW larvae reduce root growth locally or systemically within roots and whether these affects are affect by RWW leaf-feeding and/or *P. indica* inoculation. Furthermore, the JA-insensitive *coi1-18* rice mutant and the GA-deficient *Eui1-OX* rice mutant were used as host plants in addition to their untransformed wild type to test whether JA signaling mediates the RWW effects on plant growth and whether the endophyte suppression of the effects of RWW on rice requires GA signaling. The plant and larval performances were measured with the root halves of the split-root system analyzed separately.

In the study presented in **chapter IV**, a full factorial pot experiment, initially grown in the greenhouse and later transferred to the outdoor, was conducted using *R. irregularis* and *F. mosseae* inoculated alone or simultaneously in order to determine whether the impacts of AM fungi are species-specific and whether species interact in affecting the nutraceutical value of *M. oleifera* leaves. The root colonization by AM fungi and the biomass of *M. oleifera* root, stem and leaves as well as the concentration of glucosinolates, flavonoids, phenolic acids, carotenoids, and mineral elements in leaves were determined.

In the study presented in **chapter V**, a greenhouse experiment was conducted using single or simultaneous inoculation with two different strains of *R. irregularis* on several *N. tabacum* transgenic lines differing in the CK status as well as on their untransformed wild type. It was tested whether the root CK status influences the AM symbiosis, and eventually the CK status of the shoot has a role as well, and whether AM fungal strains and/or their interaction influences the symbiotic outcome. The shoot and root biomasses, the number of flowers, the AM fungal colonization, the content of P, N and C in shoots, and the root transcript levels of phosphate transporter genes were determined.

Chapter II: Effect of arbuscular mycorrhizal fungi (*Glomus intraradices*) on the oviposition of rice water weevil (*Lissorhoptrus oryzophilus*)

Cosme M, Stout M, Wurst S (2011) Mycorrhiza 21: 651-658.

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Abstract

Root-feeding insects are important drivers in ecosystems, and links between aboveground oviposition preference and belowground larval performance have been suggested. The root-colonizing arbuscular mycorrhizal fungi (AMF) play a central role in plant nutrition and are known to change host quality for root-feeding insects. However, it is not known if and how AMF affect the aboveground oviposition of insects whose offspring feed on roots. According to the preference–performance hypothesis, insect herbivores oviposit on plants that will maximize offspring performance. In a greenhouse experiment with rice (*Oryza sativa*), we investigated the effects of AMF (*Glomus intraradices*) on aboveground oviposition of rice water weevil (*Lissorhoptrus oryzophilus*), the larvae of which feed belowground on the roots. Oviposition (i.e., the numbers of eggs laid by weevil females in leaf sheaths) was enhanced when the plants were colonized by AMF. However, the leaf area consumed by adult weevils was not affected. Although AMF reduced plant biomass, it increased nitrogen (N) and phosphorus concentrations in leaves and N in roots. The results suggest that rice water weevil females are able to discriminate plants for oviposition depending on their mycorrhizal status. The discrimination is probably related to AMF-mediated changes in plant quality, i.e., the females choose to oviposit more on plants with higher nutrient concentrations to potentially optimize offspring performance. AMF-mediated change in plant host choice for chewing insect oviposition is a novel aspect of below- and aboveground interactions.

Chapter III: A fungal endophyte helps plants to tolerate root herbivory through changes in gibberellin and jasmonate signaling

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Summary

- Plant-microbe mutualisms improve plant defense, but the impact of root endophytes on belowground herbivore interactions remains unknown. We investigated effects of the root endophyte *Piriformospora indica* on interactions between rice (*Oryza sativa*) plants and its root herbivore rice water weevil (RWW; *Lissorhoptrus oryzophilus*), and how plant jasmonic acid (JA) and gibberellic acid (GA) regulate this tripartite interaction.
- Glasshouse experiments with wild type rice and *coi1-18* and *Eui1-OX* mutants combined with nutrient, jasmonate and gene expression analyses were used to test: i) whether RWW adult herbivory aboveground influences subsequent larval herbivory belowground; ii) whether *P. indica* protects plants against RWW; and iii) whether GA and JA signaling mediate these interactions.
- The endophyte induced plant tolerance to root herbivory. The RWW adults and larvae acted synergistically via JA signaling to reduce root growth, while endophyte-elicited GA biosynthesis suppressed the herbivore-induced JA in roots and recovered plant growth.
- Our study shows for the first time the impact of a root endophyte on plant defense against belowground herbivores, adds to growing evidence that induced tolerance is an important root defense, and implicates GA as signal component of inducible plant tolerance against biotic stress.

Introduction

Plants require sophisticated defense mechanisms supported by microbial alliances against a broad spectrum of heterotrophic attackers, including insect herbivores (Rodriguez *et al.*, 2009; Erb *et al.*, 2012b; Pieterse *et al.*, 2014). Herbivores attack from above- and belowground, and both shoots and roots deploy resistance mechanisms that reduce herbivore infestation and performance (Howe & Jander, 2008; Lu *et al.*, 2015) as well as tolerance mechanisms that allow regrowth and fitness recovery after tissue damage (Strauss & Agrawal, 1999; Poveda *et al.*, 2010; Robert *et al.*, 2014). Compared to the well-documented role of microbes in induced plant resistance aboveground (Hartley & Gange, 2009; Rodriguez *et al.*, 2009; Pieterse *et al.*, 2014), little is known about microbial mechanisms leading to plant defense against belowground herbivores.

Plants typically allocate more than 50 % of primary production to belowground tissues, where insect herbivores of at least 25 families feed on roots, including many critical agricultural pests (Hunter, 2001; Erb *et al.*, 2012a). Losses of plant productivity caused by root herbivory can be amplified when combined with aboveground herbivory (Zvereva & Kozlov, 2012). Combined shoot and root injury is a scenario common for many insect species whose adults feed on leaves and whose larvae feed on roots (Clark *et al.*, 2011; Cosme *et al.*, 2011; Currie *et al.*, 2011).

The perception of aboveground chewing herbivory by plants triggers a sophisticated defensive machinery with jasmonic acid (JA) as the central signal (Howe & Jander, 2008). JA can also reduce shoot growth via antagonistic interaction with the gibberellic acid (GA) signaling pathway (Yang *et al.*, 2012; Heinrich *et al.*, 2013; Matschi *et al.*, 2015). Regulation of GA biosynthesis and interactions between DELLA and JAZ proteins are central to JA and GA signaling crosstalk which ultimately modulates growth-defense trade-off in shoots. Several lines of evidence suggest that JA may regulate root resistance to belowground herbivores: i) root herbivory induces JA signaling in roots (Lu *et al.*, 2015); ii) exogenous application of jasmonates reduces root herbivore infestation and survival (McConn *et al.*, 1997; Omer *et al.*, 2000; Hamm *et al.*, 2010; Lu *et al.*, 2015); and iii) JA-deficient rice plants may suffer stronger root damage by belowground herbivory (Lu *et al.*, 2015). However, roots commonly display a much weaker herbivore-induced JA burst than leaves and other plant signals might be more important for

induced defenses to belowground herbivores (Erb *et al.*, 2012a; Acosta *et al.*, 2013). JA can also reduce root growth as demonstrated by exogenous application of MeJA (Staswick *et al.*, 1992; Moons *et al.*, 1997; Lu *et al.*, 2015), whereas GA promotes root growth by controlling cell elongation and root meristem size (Ubeda-Tomás *et al.*, 2009). Whether JA and GA signaling crosstalk regulates regrowth as a tolerance mechanism against root herbivores remains to be determined.

Plant signaling pathways can be also modulated by endophytes, i.e. non-pathogenic microbes that often colonize plants in nature without forming detectable interaction structures or producing visible disease symptoms in the plant (Jacobs *et al.*, 2011; Weiß *et al.*, 2011). Among the Sebacinalean root endophytes, *Piriformospora indica* is a model organism with an exceptionally broad host range that significantly enhances plant productivity and protects plants against abiotic stress and pathogens (Varma *et al.*, 1999; Barazani *et al.*, 2005; Waller *et al.*, 2005; Qiang *et al.*, 2012). To suppress the host immunity, *P. indica* requires JA signaling in roots during biotrophic root colonization, while during cell death-associated colonization the endophyte recruits GA signaling to degrade DELLAs and establish cell apoptosis susceptibility (Schäfer *et al.*, 2009; Jacobs *et al.*, 2011). To date, the impact of root endophytes on belowground herbivore interactions remains unknown.

Here, we investigated the effects of *P. indica* on rice (*Oryza sativa*) defense against its major root pest, the rice water weevil (RWW; *Lissorhoptrus oryzophilus*). RWW is native to North America but now present in rice paddies around the globe (Stout *et al.*, 2013). The adults feed on leaves without causing significant damage, but the root-feeding larvae markedly reduce rice productivity. Using this system, we addressed the following questions: i) does aboveground feeding by RWW adults influence the subsequent activity of their conspecific larvae belowground? ii) Does prior root colonization by *P. indica* protect rice plants against RWW attack? And iii) do JA and GA signaling pathways in rice mediate this tripartite interaction?

Materials and methods

Plants, fungi, insects, and soil

Wild type (WT) rice (*Oryza sativa*, cultivar Nipponbare) was used as the background of all plant mutants. In experiment (Exp) I, we used WT seeds kindly provided by Dr. Claus-Peter Witte (FUB, Germany). In Exp II, we used seeds of WT, *coi1-18* and *Eui1-OX* kindly provided by Prof. Dr. Zuhua He (Chinese Academy of Sciences, China). Plants were germinated on Murashige and Skoog medium in Petri dishes during 3 d and planted according to the experimental designs.

The fungal root endophyte *Piriformospora indica* (Sebacinales, Basidiomycota) was propagated at the Leibniz Institute of Vegetable and Ornamental Crops (Großbeeren, Germany) by routine procedures on potato dextrose agar (PDA) in Petri dishes for Exp I or in liquid culture containing a complete medium for Exp II (Verma *et al.*, 1998).

Adults of rice water weevil (RWW; *Lissorhoptrus oryzophilus*, Coleoptera: Curculionidae) were collected from flooded rice fields at the Louisiana State University (Louisiana, USA) and maintained in a laboratory as described (Cosme *et al.*, 2011). RWW adults were captured *in copula* and used in the leaf infestation bioassays. RWW neonates were reared *in vivo* using freshly germinated rice seedlings and were used in the root infestation bioassays (Zhang *et al.*, 2004).

A sandy loam soil from Berlin (52° 28'N, 13° 18'E) was sieved and mixed with peat (Floragard Vertriebs GmbH, Oldendurg, Germany) and sand (CEMEX GmbH, Kraatz, Germany) to produce the soil substrate (2 : 1 : 1, v : v). The soil substrate was fertilized in the pots with 125 mL of 0.05 % solution of GABI Plus 12-8-11 N-P-K fertilizer (Detia Freyberg GmbH, Laudenbach, Germany) per L of soil substrate.

Exp I: design and growth conditions

To test whether leaf-feeding by RWW adults aboveground influences the subsequent belowground activity of conspecific root-feeding larvae, we conducted a full factorial experiment in a glasshouse (16 h light and 22°/28°C night/day temperatures). The roots of 3-d-old WT rice

seedlings were dipped overnight in sterile 0.05 % Tween-20 aqueous solution to establish the control for *P. indica* inoculation (described below) (n = 32). Each seedling was then planted into 16 cm x 16 cm round Teku pots filled with 2 L of soil substrate. To confine the roots and larvae within the pot, a Plantex DuPont mesh had been previously glued with silicone onto the bottom of each pot. The soil substrate was routinely moistened with tap water. Fifteen d after germination, a 7 cm x 5 cm round clip cage was attached to the first leaf with a mating pair of RWW adults inside (Fig. III.1a) (n = 16). An identical clip cage without adults (n = 16) was clipped to uninfested control plants. All plants infested with RWW adults showed leaf feeding scars 2 d after infestation. The adults were then recollected, the clip cage was removed, and a photo of the injured leaf was recorded using a standardized focal distance. Control plants were photographed under the same conditions. The photos were analyzed using WinDIAS 3.1 software (Delta-T Devices, Cambridge, UK) to determine the leaf area consumed by RWW adults. The pots were placed into 24 cm x 20 cm round plastic buckets and flooded with tap water 23 d after germination. In the field, the presence of standing water triggers RWW oviposition and females lay eggs in submerged rice shoots (Stout *et al.*, 2002). To establish the root infestation, 36 d after germination the plants with adult feeding scars (n = 8) and their corresponding controls without scars (n = 8) received 8 neonates per plant over 4 d. Infestation of 8 or more neonates per plants is common in the field (Stout *et al.*, 2013). The remaining plants were not infested with larvae as controls (n = 8 + 8). To test whether *P. indica* can defend the rice plants against RWW attacks, we simultaneously conducted the same RWW treatments described above on rice plants inoculated with *P. indica* (n = 32). The inoculation was established by dipping the roots of 3-d-old WT rice seedlings overnight in 0.05 % Tween-20 aqueous solution containing 4.9 x 10^7 ml^{-1} chlamydospores of *P. indica*. The RWW larvae develop through four instars in 21–27 d before forming pupae (Hamm *et al.*, 2010). To provide the utmost exposure of larvae to roots and avoid eclosion, rice plants were harvested 22 d after neonate infestation (58 d after germination). All experimental plants (n = 64) were treated and distributed in randomized fashion on a glasshouse table.

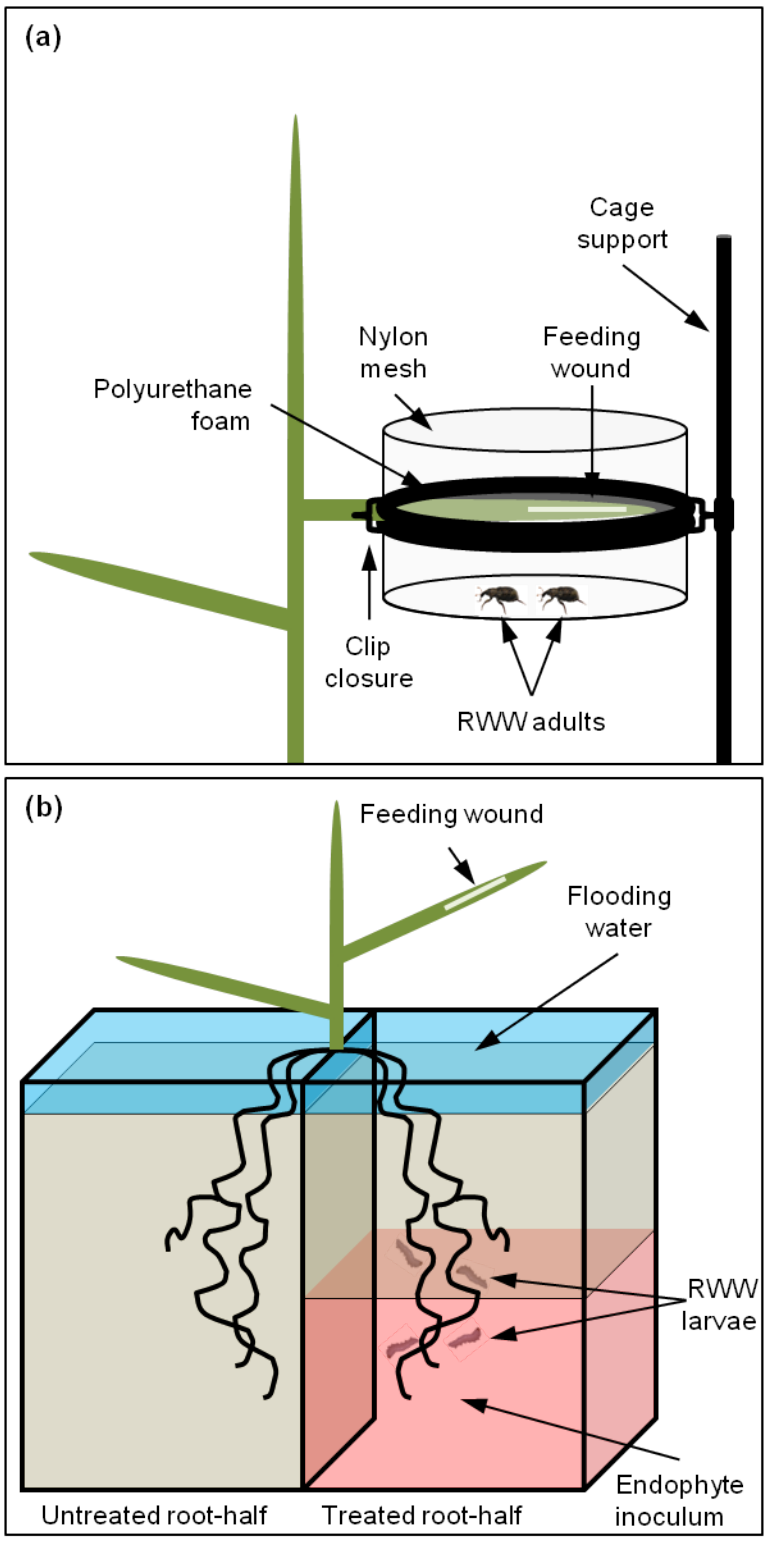

Fig. III.1 For figure legend, see next page.

Figure III.1. *Schematic representation of a clip cage and a split-root system*. (a) Clip cage attached to the first leaf of a rice plant infested with a mating couple of RWW adults. The clip cage consisted of two plastic transparent cups with a joint made of polyurethane foam to avoid injuring the leaf, a top made in nylon mesh on the upper cup to allow transpiration, and a wood support suspending the cage weight to minimize pressure on the plant. Uninfested plants received a similar clip cage without adults inside. (b) Split-root system used in experiment II to test within roots systemic effects. The split-root system consisted of two hydroponic squared pots paired side-by-side, with the root system equally divided in two halves, where one root-half received soil treatments (endophyte and/or RWW larvae) and the other root-half was left untreated. The endophyte inoculation was added to one side by filling half of the hydroponic pot with a soil previously mixed with endophyte mycelium. Uninoculated plants had a similarly pot side half filled with soil previously mixed with autoclaved mycelium.

Exp I: plant, larval and fungal performance

To determine the performance of plants from Exp I described above, the shoots and roots were excised separately 58 d after germination, the number of tillers was counted and the soil substrate was carefully washed from roots. Subsamples of the youngest leaf and of intact root tissue without visible symptoms of wounding were immediately frozen in liquid nitrogen and stored at -80 °C to analyze jasmonates and gene expression as described below. To recover larvae 22 d after neonate infestation, the soil substrate and roots were screened methodically in buckets filled with water. The larvae and pupae were counted as they floated to the surface (Zou *et al.*, 2004). Fresh weights of insects and plants were measured. The total root length and average root diameter was determined using WinRhizo software (Regent Instruments Inc, Québec, Canada) as described (Cosme & Wurst, 2013). To determine endophyte performance 55 d after inoculation, subsamples of root fragments were stained using trypan blue solution and then destained prior to observation at the microscope (Phillips & Hayman, 1970). The root colonization by the endophyte was quantified as the percentage of microscope fields of view containing root segments with chlamydospores (McGonigle *et al.*, 1990).

Exp I: mineral elements in shoot

After measuring plant biomass, the shoots of the 58-d-old rice plants were dried in an oven (60 °C for 1 wk) and homogenized into a fine powder using sintered corundum alumina jars and balls in a Planetary Micro Mill Pulverisette 7 (Fritsch, Idar-Oberstein, Germany). To assess the mineral nutrition status of rice plants, the concentration of nitrogen (N), phosphorus (P),

potassium (K), calcium (Ca), magnesium (Mg), sulfur (S), manganese (Mn), iron (Fe), zinc (Zn), boron (B), copper (Cu) and molybdenum (Mo) in shoots were measured using a CN Elemental Analyzer (Euro EA, HEKAtech GmbH, Wegberg, Germany) or a Inductively Coupled Plasma-Optical Emission Spectrometer (iCAP ICP-OES Duo, Thermo Fisher Scientific Inc, Massaschusetts, USA) as described (Cosme *et al.*, 2014).

Exp I: jasmonates in leaves and roots

To quantify the jasmonate homeostasis of 58-d-old rice plants, i.e. 43 d after leaf infestation with RWW adults and 22 d after root infestation with RWW neonates, 12-oxophytodienoic acid (OPDA), JA and jasmonoyl-isoleucine (JA-Ile) were extracted from frozen leaf and root subsamples (n = 8) following Lu et al (2015). The extracts were analyzed by liquid chromatography coupled with mass spectrometry using an API 3200TM LC/MS/MS system (Applied Biosystems, Framingham, USA) as described (Vadassery et al., 2012).

Exp I: gene expression in roots

Quantitative real-time PCR (qRT-PCR) analyses were conducted following Lu et al (2015). To assess *de novo* biosyntheses in JA and GA signaling pathways, we analyzed the gene expression of JA-Ile synthase *OsJAR1* (Riemann *et al.*, 2008) and *ent*-Kaurene synthase *OsKS1* (Sakamoto et al., 2004) (Table III.S1), respectively. To normalize cDNA concentrations, we used the rice actin *OsACT* as housekeeping gene (Table III.S1). qRT-PCR was performed with Mx3000P qPCR System (Stratagene, La Jolla, USA) using Brilliant III Ultra-Fast SYBR® Green QPCR Master Mix (Agilent technologies, Santa Clara, USA). The relative expression levels of genes were calculated by using the double standard curve method.

Exp II: design and growth conditions

To test whether RWW larvae reduce root growth locally or systemically within roots, we repeated the RWW infestation treatments applied in Exp I (described above) with minor alterations adapted to a split-root system (Fig. III.1b), in which only one half of the root system was treated with RWW larvae. Hydroponic 1L square pots were paired by gluing two pots side-by-side. Each pot was filled with 500 mL of soil substrate before transplanting. One side of the

split-root systems assigned to RWW larval infestation (n = 16) and to uninfested controls (n = 16) received a mock inoculum of autoclaved (121 °C for 20 min) endophyte mycelium (4 mg) to establish the control for endophyte inoculation (described below) (n = 32). To allow enough root growth before transplanting, 3-d-old WT rice seedlings were planted in nursery trays filled with 60 mL of soil substrate per vessel. Rice roots were carefully washed 20 d after germination and transplanted into the split-root system by dividing the roots into two halves. Each pot side of the split-root systems was then filled with 500 mL additional soil substrate to completely cover the root system, resulting in 2 L of soil subtract for each plant. 26 d after germination, a clip cage was attached to the first leaf with a couple of RWW adults inside (n = 16). An identical clip cage without adults was attached to control plants (n = 16). The split-root systems were flooded with tap water 28 d after germination. The plants with adult feeding scares (n = 8) and their controls (n = 8) received 16 RWW neonates in one half of the root system 30 d after germination. The remaining plants were kept uninfested as control (n = 8 + 8). To test whether the endophyte inhibits the systemic effects of RWW larvae on root growth, we conducted simultaneously the same RWW treatments in the split-root systems (described above) using pots previously inoculated with endophyte (n = 32). The inoculation was established by mixing 4 mg of endophyte mycelium into the pot-side assigned to RWW larvae (n = 16) or one pot-side in the uninfested larval control (n = 16) before transplanting the rice plants. To test whether JA signaling mediates the RWW effects on plant growth, we conducted the same experimental treatments simultaneously in split-root systems using JA-insensitive *coi1-18* rice mutant as the host plant (Yang *et al.*, 2012). To test whether the endophyte suppression of the effects of RWW on rice requires GA signaling, we simultaneously conducted the same experimental treatments in split-root system using the GA-deficient *Eui1-OX* rice mutant as host plant (Zhu *et al.*, 2006). All experimental plants (n = 192) were treated and distributed in randomized fashion in glasshouse (14 h light, 24/28 °C night/day temperatures) and were harvested 28 d after neonate infestation (58 d after germination).

Exp II: plant, larval and fungal performance

We analyzed the same performance parameters described in Exp I with the exception of root morphology. The root halves from the split-root system were excised and analyzed separately.

Exp II: chlorophyll content in leaves

To determine whether the changes in plant growth following RWW attacks and endophyte inoculation could be explained by changes in chlorophyll, we determined chlorophyll content in leaves of WT, *coi1-18* and *Eui1-OX* rice plants. The chlorophyll was quantified non-destructively 56 d after germination using a portable chlorophyll meter (SPAD 502; Konica Minolta, Tokyo, Japan) that provides an index value positively correlated with chlorophyll. The measurements were conducted in all treatments (n = 8) on 3 different young leaves per plant with 3 repetitions per leaf.

Data analysis

Statistical analyses were performed in R Studio Desktop software (http://www.rstudio.com/). All data on plant responses were analyzed by factorial three-way analyses of variance (ANOVA) with the two-level factors “Endophyte” (-, +), “RWW adults” (-, +) and “RWW larvae” (-, +). Consumed leaf area by RWW adults was analyzed by one-way ANOVA with the two-level factor “Endophyte”. Endophyte root colonization was analyzed by two-way ANOVA with the two-level factors “RWW adults” and “RWW larvae”. Data on larval performance were analyzed by two-way ANOVA with the two-level factors “Endophyte” and “RWW adults”. We checked the assumptions of ANOVA (using Shapiro and Levene test), and data were transformed if necessary. When transformation did not meet assumptions, or when a sample size differed, we performed ANOVA using general linear model (GLM) with best fitted family errors.

Results

Experiment I

Root herbivory decreases endophyte fitness

Plant infestation with RWW larvae (Fig. III.2g) reduced the endophytic chlamydospore (Fig. III.2e) colonization (two-way ANOVA, df = 28, F = 4.368, P = 0.046) from 43.19 ± 5.99 % in uninfested plants to 26.69 ± 5.21 % in plants infested for 22 d (Mean ± SE; n = 16). The 2 d infestation with RWW adults (Fig. III.2f) in 15-d-old rice plants did not change the chlamydospore colonization at the end of the experiment (n = 16; two-way ANOVA, df = 28, F = 2.359, P = 0.136). Neither did the interaction between adult and larval infestations (n = 8; two-way ANOVA, df = 28, F = 0.001, P = 0.975).

P. indica does not affect plant resistance against RWW

The consumed leaf area by RWW adults was not altered by the endophyte (n = 16; one-way ANOVA, df = 30, F = 0.206, P = 0.653) and was on average 0.244 ± 0.019 cm^2 (Mean ± SE, n = 32). Furthermore, the survival of RWW larvae was not altered by the endophyte (n = 16; two-way ANOVA, df = 28, F = 0.173, P = 0.681), RWW adult feeding (n = 16; two-way ANOVA, df = 28, F = 1.556, P = 0.223), or their interaction (n = 8; two-way ANOVA, df = 28, F = 0.000, P = 1.000). Survival of RWW larvae averaged 46.88 ± 3.68 % (n = 32). Likewise, the weight of RWW larvae and pupae was on average 17.16 ± 1.31 mg (n = 32) and was not affected by the endophyte (n = 16; two-way ANOVA, df = 28, F = 0.240, P = 0.628), RWW adult feeding (n = 16; two-way ANOVA, df = 28, F = 0.142, P = 0.709), or their interaction (n = 8; two-way ANOVA, df = 28, F = 0.080, P = 0.779). Taken together, these results indicate that the fungal endophyte did not affect the leaf and root resistance against RWW in this experiment.

P. indica restores growth of herbivore attacked plants

RWW adults alone did not affect plant biomass, whereas considerable plant damage was found in endophyte-free plants infested with RWW larvae, i.e. endophyte-free plants infested with RWW larvae produced 32 % less shoot biomass (n = 16; three-way ANOVA, F = 5.371, P = 0.024; Fig. III.2a), had 18 % fewer tillers (n = 16; three-way ANOVA, F = 9.534, P = 0.003; Fig.

III.2b), and produced 32 % less root biomass (n = 16; three-way ANOVA, F = 7.001, P = 0.011; Fig. III.2c) compared with endophyte-free uninfested plants. The most remarkable result was the impact of the endophyte on the growth of plants infested with RWW larvae. Endophyte-inoculated plants infested with RWW larvae gained 26 % more shoot biomass (n = 16; three-way ANOVA, F = 5.371, P = 0.024; Fig. III.2a), and produced 27 % more tillers (n = 16; three-way ANOVA, F = 9.534, P = 0.003; Fig. III.2b) and 36 % more root biomass (n = 16; three-way ANOVA, F = 7.001, P = 0.011; Fig. III.2c) compared with endophyte-free plants infested with RWW larvae. In addition, we found that RWW adults enhanced the negative effect of their conspecific larvae on total root length in endophyte-free plants (n = 8; three-way ANOVA, F = 6.176, P = 0.016; Fig. III.2d), causing together a 74 % decrease in total root length compared with that of endophyte-free uninfested plants. However, the endophyte suppressed this additive negative effect by RWW adults and larvae (n = 8; three-way ANOVA, F = 6.176, P = 0.016; Fig. III.2d). By contrast, the average root diameter was 7 % larger (n = 32; three-way ANOVA, F = 12.295, P < 0.001) in plants infested with RWW larvae (0.281 ± 0.003 mm) compared with the average root diameter (0.263 ± 0.004 mm) of uninfested plants, and was not affected by the endophyte, the RWW adults or the interactions between factors (Table III.S2).

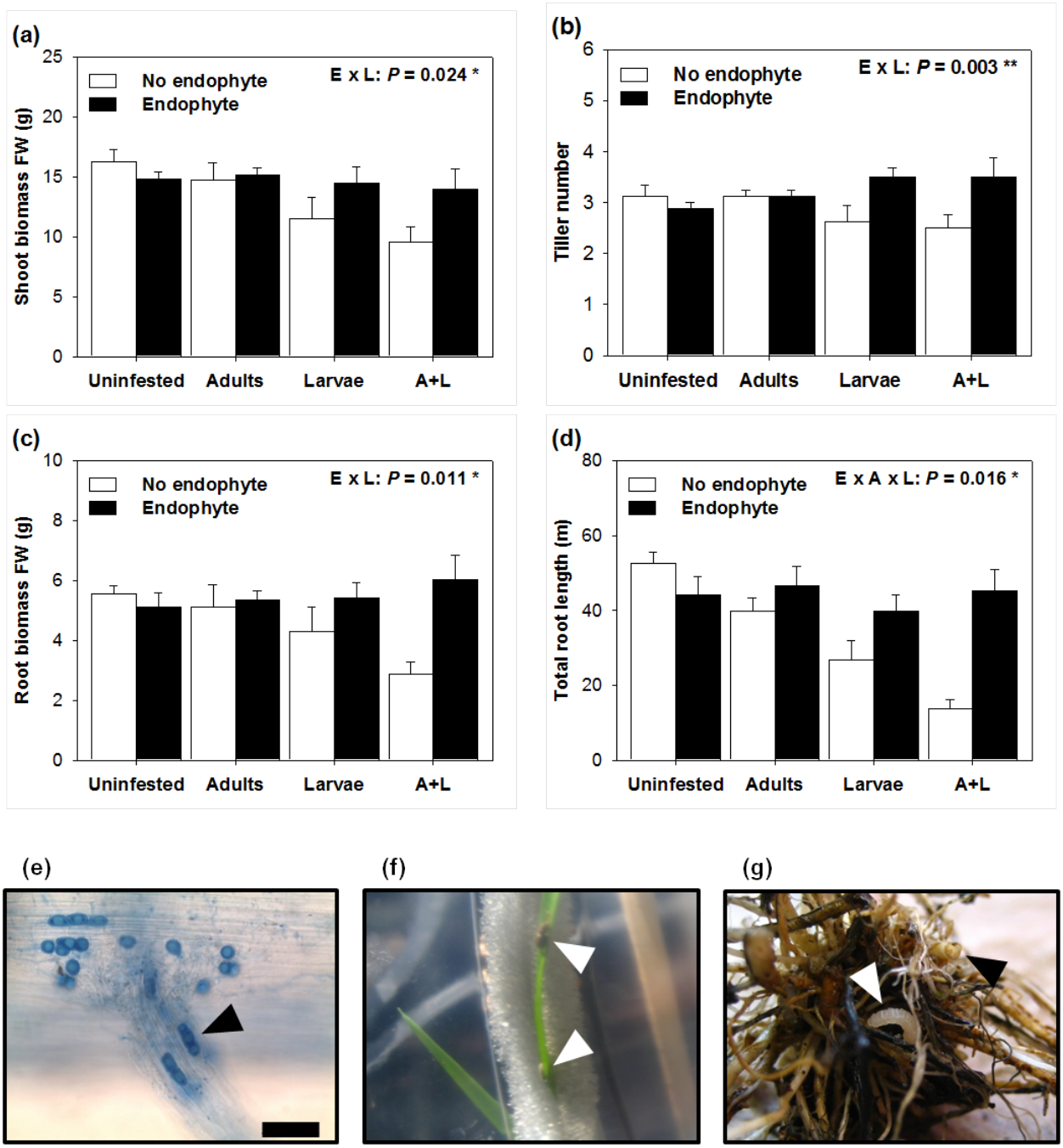

Figure III.2. *P. indica restores growth of herbivore attacked plants.* Rice plants (variety Nipponbare) were inoculated with the fungal root 'endophyte' *Piriformospora indica* 3 days after germination (DAG), then infested aboveground on the first leaf with rice water weevil (RWW) 'adults' 15 DAG, and finally infested belowground with RWW 'larvae' 36 DAG, in a fully crossed experiment. (a) shoot biomass, (b) tiller number, (c) root biomass, and (d) total root length were measured 58 DAG and their values were analyzed by three-way ANOVA. Mean ± SE, n = 8. Significant P values of the higher level interaction effects are shown (E = endophyte; A = adults; L = larvae). For other P values see Table III.S2. (e) Trypan blue-stained root segment of rice colonized by endophytic chlamydospores (black arrow) 55 d after inoculation (bars = 50 µm). (f) Couple of RWW adults (white arrows) inside a clip cage chewing on the first leaf of a 15-d-old rice plant. (c) RWW larva (white arrow) dwelling in rice roots and visible symptoms of larval pruning (black arrow) 22 d after root infestation with RWW neonates.

Rice nutritional deficit caused by root herbivory is not affected by P. indica

The RWW larvae were found to reduce the mass fraction of P (n = 32; three-way GLM, F = 64.938, P < 0.001; Table III.S3), K (n = 32; three-way GLM, F = 7.353, P = 0.009; Table III.S3) and Mn (n = 32; three-way GLM, F = 12.659, P < 0.001; Table III.S3) in shoots by 16, 6 and 12 % compared with that of uninfested plants, respectively. Despite the root pruning by herbivores, we discovered that plants infested with RWW larvae accumulated 17 % more Ca (n = 32; three-way GLM, F = 39.657, P < 0.001; Table III.S3) and B (n = 32; three-way GLM, F = 23.289, P < 0.001; Table III.S3) and tended to accumulate more S (Table III.S3) in shoots compared with uninfested plants. Furthermore, the endophyte led to a 7 % reduction of Ca in shoots (n = 32; three-way GLM, F = 6.743, P = 0.012; Table III.S3) and a small but significant 5 % increase of P in shoots (n = 32; three-way GLM, F = 5.205, P = 0.026; Table III.S3) compared with that of endophyte-free plants, and interacted with RWW larvae to suppress the larvae-mediated increase of Mo in shoots (n = 16; three-way ANOVA, F = 4.313, P = 0.042; Table III.S3). The RWW adults and the other interactions between factors did not affect nutrient accumulation in shoots and the levels of N, Mg, Zn, Fe, and Cu in shoots persisted unaltered in this experiment (Table III.S3).

P. indica induces GA biosynthesis and suppresses herbivore-induced JA in roots

To profile plant JA signaling response to above- and belowground herbivory under endophyte colonization, we quantified the amounts of OPDA, JA and JA-Ile in leaves and in roots of rice. Plants infested 15 d after germination for 2 d with RWW adults produced 99 % more OPDA (n = 32; three-way GLM, F = 4.732, P = 0.034; Fig. III.3a), 130 % more JA (n = 32; three-way GLM, F = 6.420, P = 0.014; Fig. III.3a) and 68 % more JA-Ile (n = 32; three-way GLM, F = 5.340, P = 0.025; Fig. III.3a) in leaves at the end of the experiment compared with uninfested plants. Furthermore, we found that plants infested with RWW larvae had 56 % less JA (n = 32; three-way GLM, F = 4.554, P = 0.037; Fig. III.3a) and tended to have less OPDA and JA-Ile in leaves compared with uninfested plants (Table III.S4; Fig. III.3a). Moreover, OPDA, JA and JA-Ile in leaves were not affected by the endophyte or by the interactions between factors (Table III.S4, Fig. III.3a). Endophyte-free plants infested with RWW larvae accumulated 52 % more JA in roots compared with uninfested plants without endophyte, but when plants were inoculated with the endophyte this JA accumulation was suppressed (n = 15-16; three-way GLM, F = 4.113, P = 0.047; Fig. III.3b). Moreover, the levels of JA-Ile in roots were induced by RWW larvae (n = 30-31; three-way GLM, F = 14.864, P < 0.001; Fig. III.3b) and tended to be reduced by the endophyte (n = 30-31; Table III.S4, Fig. III.3b). Although the RWW adults did not significantly affect levels of JA and JA-Ile in roots they apparently enhanced the induction by their conspecific larvae (Fig. III.3b). The root level of OPDA was not induced by RWW larvae, neither was it affected by the endophyte, RWW adults, or the interactions between factors (Table III.S4, Fig. III.3b).

We found no significant effects on the transcription level of *OsJAR1* and observed a trend for an interaction between endophyte and larvae suggesting that the endophyte suppressed the larvae negative effect (Table III.S5, Fig. III.3c). The endophyte elicited 20 % more transcription of *OsKS1* in roots compared with that of endophyte-free plants (n = 30-31; three-way GLM, F = 6.037, P = 0.017; Fig. III.3d). In contrast, the transcription level of *OsKS1* was found to be 54 % lower in roots infested with RWW larvae compared with uninfested roots (n = 30-31; three-way GLM, F = 48.198, P < 0.001; Fig. III.3d). Furthermore, we observed a trend for an interaction between the endophyte, the RWW adults, and the RWW larvae on the transcription level of *OsKS1* in roots (n = 7-8; Table III.S5, Fig. III.3d), i.e. endophyte-inoculated plants infested with

RWW adults, larvae or both tended to have higher transcription level of *OsKS1* in roots compared with their respective endophyte-free control plants, but this difference was stronger in plants infested only with RWW adults.

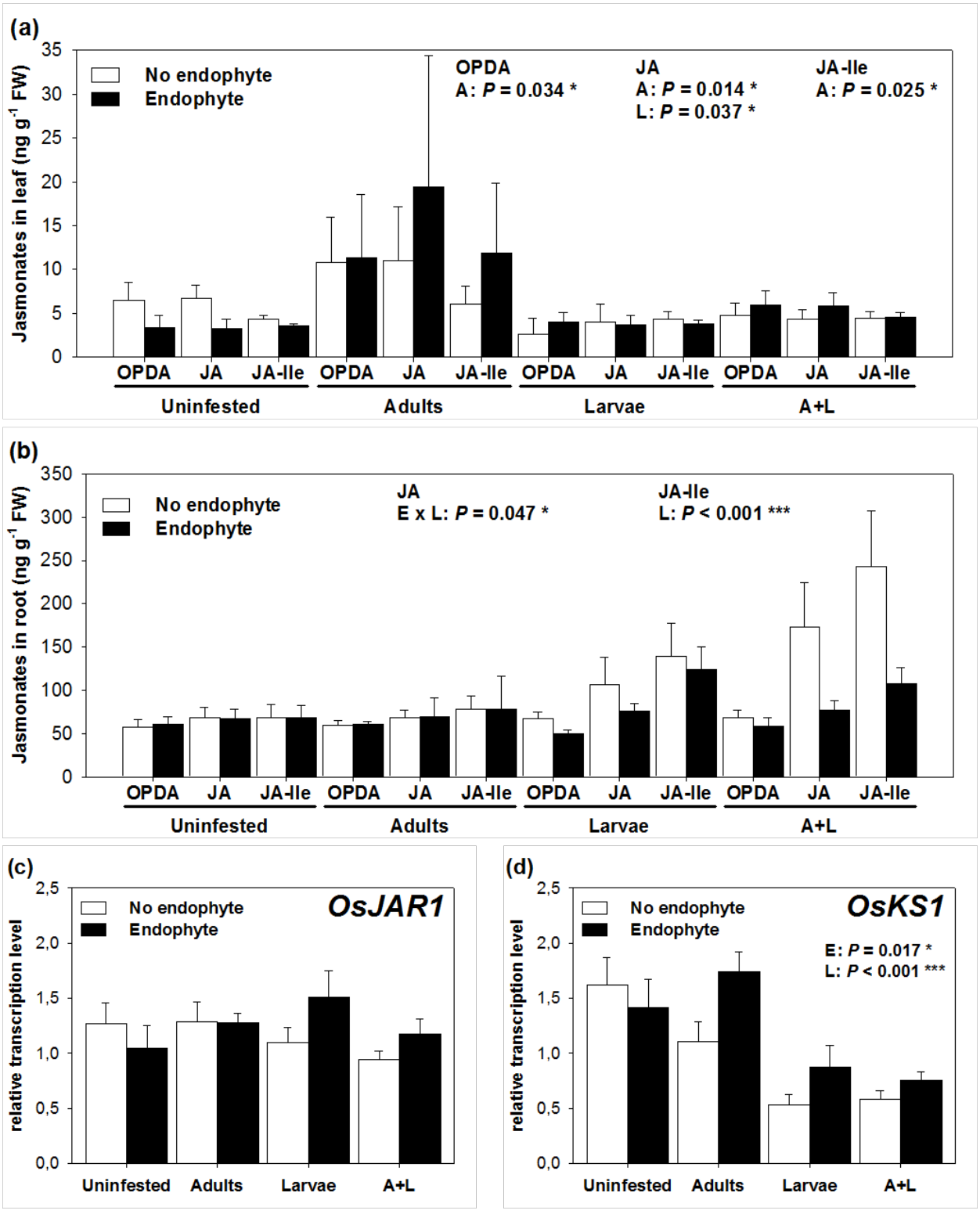

Fig. III.3. For figure legend, see next page.

Figure III.3. *Piriformospora indica induces a synthase gene of gibberellic acid pathway and suppresses herbivore-induced jasmonic acid in roots.* Rice plants (variety Nipponbare) were inoculated with the fungal root 'endophyte' *Piriformospora indica* 3 days after germination (DAG), then infested aboveground on the first leaf with rice water weevil (RWW) 'adults' 15 DAG, and finally infested belowground with RWW 'larvae' 36 DAG, in a fully crossed experiment. The level of 12-oxophytodienoic acid (OPDA), jasmonic acid (JA), and jasmonoyl-isoleucine (JA-Ile) in leaves (a) and in roots (b) were measured. The relative transcription levels in roots of JA-Ile synthase *OsJAR1* (c) gene and the *ent*-Kaurene synthase *OsKS1* (d) gene of gibberellic acid pathway were determined. All values were analyzed by three-way ANOVA using GLM. Mean ± SE, n = 8 for (a), and n = 7-8 for (b,c,d). Significant P values of the higher level interaction or the main factors effects are shown (E = endophyte; A = adults; L = larvae). For other P values see Table S4 for (a,b) and S5 for (c,d).

Experiment II

Endophyte and herbivore have dissimilar effects on systemic plant growth responses

Adding RWW larvae to only one half of the root system disabled their negative effect on WT shoot biomass, while no systemic effects within WT roots were found on the uninfested root-half biomass (Table III.S6; Fig. III.4a). However, RWW larvae reduced (n = 32; three-way GLM, F = 4.030, P = 0.049, Fig. III.4a) and tended to interact with RWW adults to reduce (n = 16; Table III.S6, Fig. III.4a) the infested root-half biomass, suggesting that prior adult herbivory worsened the negative effect by their conspecific larvae on WT root biomass. By contrast, the endophyte increased the biomass of the inoculated WT root-half (n = 32; three-way GLM, F = 5.223, P = 0.026; Fig. III.4a), increased systemically the WT shoot biomass (n = 32; three-way GLM, F = 4.738, P = 0.034; Fig. III.4a) and tended to increase systemically within roots the biomass of the uninoculated WT root-half (n = 32; Table III.S6; Fig. III.4a) compared with that of endophyte-free WT plants. Taken together, the root herbivory damage in WT rice was primarily local but was systemically worsened by the prior leaf herbivory, whereas the endophyte had wider and positive systemic effects on WT plant biomass.

JA and GA antagonize systemic effects on plant growth

In contrast to the observed effects on WT plants, no effects of RWW adults or larvae were detected on any of the measured plant biomass components of the JA-insensitive *coi1-18* rice

mutant (Table III.S6, Fig. III.4b), suggesting that JA signaling is involved in mediating the reduction in growth of WT plants in response to RWW attack. By contrast, *coi1-18* plants inoculated with the endophyte had larger biomasses of shoots (n = 31-32; three-way GLM, F = 16.305, $P < 0.001$; Fig. III.4b), of uninoculated root-half (n = 31-32; GLM, F = 19.430, $P < 0.001$; Fig. III.4b) and inoculated root-half (n = 31-32; three-way GLM, F = 17.243, $P < 0.001$; Fig. III.4b) compared with endophyte-free *coi1-18* plants. As these effects were stronger than those observed on WT plants, this confirms that JA signaling is a negative regulator of *P. indica* plant-growth-promoting effects (Barazani *et al.*, 2005). By contrast, when the same fully crossed experiment using a split-root system was conducted on GA-deficient *Eui1-OX* rice mutant, the endophyte had no detectable effects on any of the measured plant biomass components (Table III.S6, Fig. III.4c). However, *Eui1-OX* plants infested with RWW larvae had reduced biomasses of shoots (n = 29-31; three-way GLM, F = 5.612, P = 0.022; Fig. III.4c) and of infested root-half (n = 29-31; three-way GLM, F = 5.789, P = 0.020; Fig. III.4c) compared with uninfested *Eui1-OX* plants. This suggests that shoot growth inhibition by RWW larvae is counteracted by GA in WT plants. Finally, no RWW adult or interaction effects were detected on any of the measured biomass component of *coi1-18* and *Eui1-OX* plants (Table III.S6, Fig. III.4b,c).

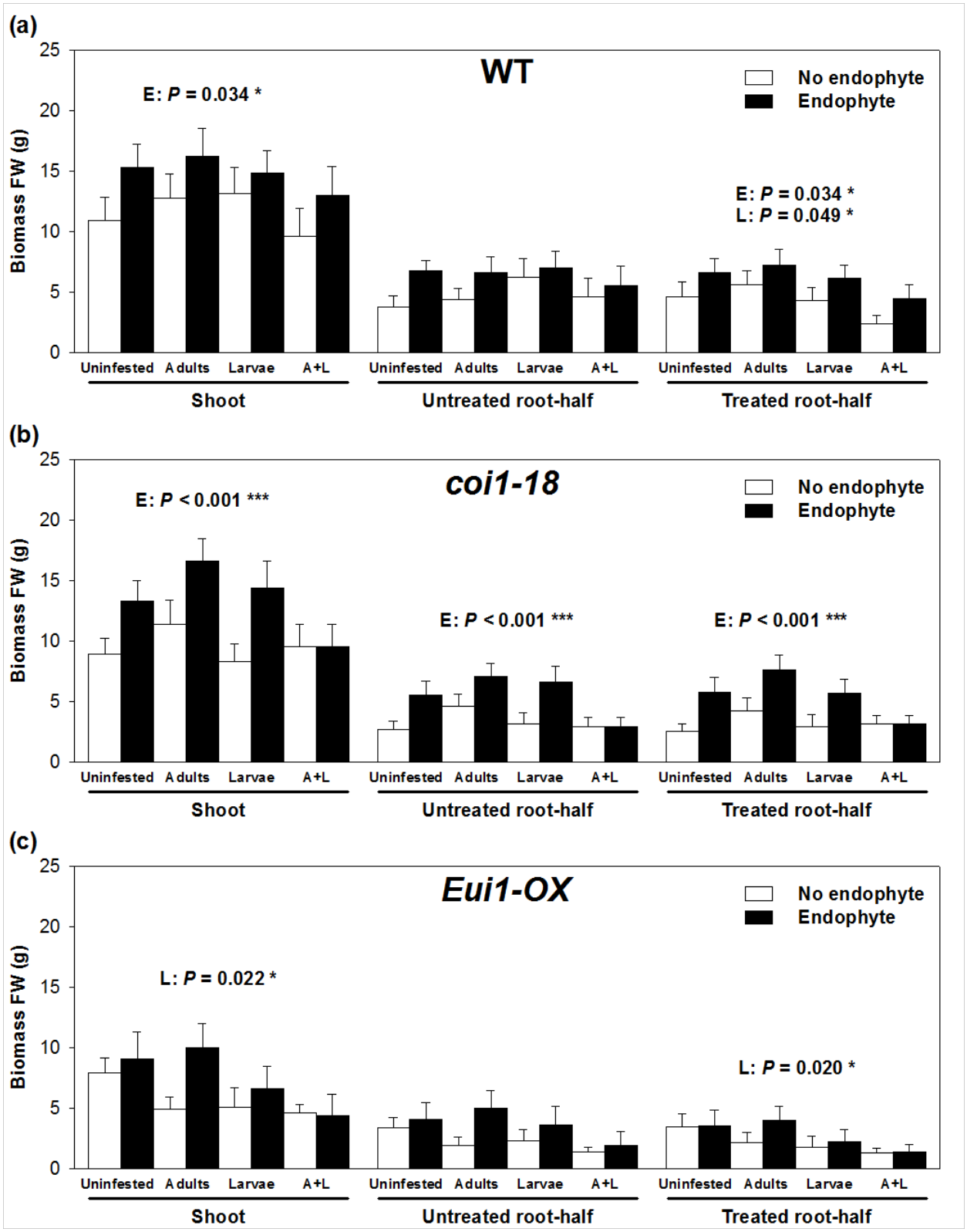

Fig. III.3. For figure legend, see next page.

Figure III.4. *Piriformospora indica and rice water weevil have dissimilar effects on systemic plant growth responses and jasmonic acid and gibberellic acid antagonize respectively these effects.* The rice (variety Nipponbare) wild-type (WT), the JA-insensitive *coi1-18*, or the GA-deficient *Eui1-OX* mutant were planted 20 days after germination (DAG) in a split-root system where only one half of the root was treated with the fungal root 'endophyte' *Piriformospora indica*, then infested aboveground on the first leaf with rice water weevil (RWW) 'adults' 26 DAG, and finally infested in the same root-half with RWW 'larvae' 30 DAG, leaving the other root-half untreated, in fully crossed experiments. The biomasses of shoots, of untreated root-half and of treated root-half of the WT (a), *coi1-18* (b), and *Eui1-OX* (c) plants were measured and their values were analyzed by three-way ANOVA or GLM. Mean ± SE, n = 8 for (a), n = 7-8 for (b,c). Significant P values of main factors effects are shown (E = endophyte; A = adults; L = larvae). For other P values see Tables III.S6.

GA restores chlorophyll content of herbivore attacked plants

The chlorophyll concentrations in WT leaves were not affected by any factor or their interactions (Table III.S6) and was on average 30.34 ± 0.77 SPDA units (Mean ± SE, n = 64). The endophyte reduced the chlorophyll concentration in *coi1-18* leaves (n = 32; three-way GLM, F = 5.386, P = 0.024), from 37.34 ± 0.92 in endophyte-free to 34.40 ± 0.89 SPDA units in endophyte-inoculated *coi1-18* plants. No other effects were detected on chlorophyll in *coi1-18* leaves (Table III.S6). The RWW larvae reduced the chlorophyll concentration in *Eui1-OX* leaves (n = 30-31; three-way GLM, F = 7.281, P = 0.009), from 40.03 ± 0.88 in uninfested to 35.09 ± 1.42 SPDA units in *Eui1-OX* plants infested with RWW larvae. No other effects were detected on chlorophyll in *Eui1-OX* leaves (Table III.S6).

Susceptibility against RWW larvae in P. indica-colonized plants is reduced by JA signaling

The survival and weight of RWW larvae in WT plants was not affected by the endophyte, the RWW adults or their interaction (Table III.S7) and was on average 33.79 ± 3.60 % (Mean ± SE; n = 32) and 7.73 ± 0.22 mg (n = 29), respectively. The endophyte increased the survival of RWW larvae in *coi1-18* plants (n = 16; three-way ANOVA, F = 9.048, P = 0.006), from 24.22 ± 4.03 to 42.58 ± 4.61 % compared endophyte-free *coi1-18* plants. Neither RWW adults nor the interaction between adults and endophyte affected the larval survival in *coi1-18* plants (Table III.S7). The weight of RWW larvae on *coi1-18* plants was not affected by the endophyte, the RWW adults or their interaction (Table III.S7) and was on average 7.07 ± 0.24 mg (n = 31). In

Eui1-OX plants, the survival of RWW larvae was not affected by the endophyte, the RWW adults or their interaction (Table III.S7) and was on average 22.07 ± 3.68 % (*n* = 32), while *Eui1-OX* plants infested with RWW adults tended to increase the growth of RWW larvae from 7.52 ± 0.32 to 8.97 ± 0.67 mg compared with control *Eui1-OX* plants uninfested with adults (*n* = 12; Table III.S7).

Discussion

Mutualistic interactions between higher plants and microbes are increasingly recognized as important factors in terrestrial ecosystems. Positive effects of Sebacinalean root endophytes on plant growth, fitness, defense against pathogens, tolerance to salt stress, as well as negative effects on resistance to leaf herbivory have been documented (Varma *et al.*, 1999; Barazani *et al.*, 2005; Waller *et al.*, 2005; Camehl *et al.*, 2010), but their impact on root-herbivore interactions is unknown. Barazani et al (2005) reported that *S. vermifera* decreased the activity of proteinase inhibitors (PIs) in leaves of *Nicotiana attenuata* and consequently reduced leaf resistance to herbivory by *Manduca sexta*. This reduction resulted from endophyte-inhibited ET signaling independent of JA signaling (Barazani *et al.*, 2007). Our study demonstrates that plant-herbivore interactions are also affected by *P. indica*, a model endophyte with agronomic potential (Qiang *et al.*, 2012). Contrary to Barazani et al (2005), we observed an endophyte-enhanced defense to herbivory mediated by induced root tolerance, i.e. *P. indica*-colonized plants infested with RWW larvae gained more shoot biomass, tillers, root biomass and total root length compared with plants infested with larvae without *P. indica*, but the root resistance measured as larval survival and growth was not affected by the endophyte. Therefore, Sebacinalean root endophytes, in addition to protecting plants against root and shoot pathogens and salt stress (Waller *et al.*, 2005), can improve plant defense against root herbivores.

Although *P. indica* can affect ET signaling in roots (Camehl *et al.*, 2010; Khatabi *et al.*, 2012), ET does not regulate rice resistance or tolerance to root herbivory (Lu *et al.*, 2015). Furthermore, we found no activity of PIs in submerged roots of rice (data not shown), confirming recent results (Lu *et al.*, 2015). Thus, different signaling pathways for defense in above and belowground tissues might explain why Sebacinalean-mediated plant response to herbivory in our study contrasts with previous studies (Barazani *et al.*, 2005; Barazani *et al.*, 2007). Consistent with the current literature (Lu *et al.*, 2015), we found only an attenuated 2-fold induction of JA in roots following root herbivory. This induction, however, was suppressed by *P. indica* without obvious effects on root resistance, i.e. the survival and growth of the larvae was unchanged. JA is perceived as master regulator of induced resistance to chewing herbivores (Howe & Jander, 2008), but the role of JA in roots has been considered elusive (Erb *et al.*, 2012a). For instance, the application of MeJA to rice roots reduced the survival of RWW larvae,

but *hebiba* roots which have a constitutive reduction of JA content did not affect the larval survival or growth, whereas *as*LOX roots impaired in OPDA biosynthesis reduced larval growth due to lower nutritional quality of herbivore-attacked roots, suggesting that 13-lipoxygenase specifically improves root herbivore growth (Lu *et al.*, 2015). Interestingly, in our study the OPDA levels were not induced by herbivory in roots, in contrast to leaves. Furthermore, we found that larval performance in WT roots was similar to that in *coi1-18* roots in absence of *P. indica*, suggesting that herbivore-induced JA signaling in roots is decoupled from root resistance. However, the enhanced larval survival in *P. indica*-colonized *coi1-18* roots compared to *P. indica*-colonized WT roots suggests that JA signaling prevents the endophyte from increasing root susceptibility. Moreover, the RWW larvae also induced JA-Ile in roots, while *OsJAR1* expression was not affected. Even though JA signaling can regulate root resistance, plants may benefit from attenuating positive feedback loops of JA biosynthesis in roots to avoid nutritional enrichment that favors root herbivore growth and therefore could lead to greater injury. Taken together, an attenuated induction of JA in roots seems insufficient to affect the performance of RWW larvae, which could explain why *P. indica*-mediated suppression of herbivore-induced JA in roots did not affect root resistance.

A fundamental aspect that needs to be considered when studying JA signaling is that in addition to plant defense JA also regulates plant growth and development. In rice, root growth and elongation are reduced by exogenous application of MeJA (Staswick *et al.*, 1992; Moons *et al.*, 1997; Lu *et al.*, 2015). In our study, we observed an apparent synergistic positive effect of RWW adults and larvae on induced JA in roots which was associated with a significant additive negative effect on total root length accompanied by similar negative effects on root biomass, shoot biomass and number of tillers. Negative effects of combined above- and belowground herbivory on plant growth are often observed in nature (Zvereva & Kozlov, 2012). In our study, the root colonization by *P. indica* suppressed the herbivore-induced JA in roots and enhanced plant tolerance to RWW attack. Furthermore, we detected only a local negative effect of RWW larvae on WT roots independent of *P. indica* colonization, which tended to be worsened by leaf herbivory and was not detected in *coi1-18* roots. Therefore, our results suggest that the negative effects of RWW herbivory on plant growth were mediated by induced JA signaling in roots. Intriguingly, the unaffected expression of *OsJAR1* in herbivore-attacked roots suggests that *de novo* biosynthesis of JA pathway was inactive, while the reduced levels of JA in leaves following

root herbivory suggests that herbivore-induced JA in roots may be transported from the leaves. Zhang & Baldwin (1997) used [2-^{14}C]JA to demonstrate that direct transport of wound-induced JA from leaves to roots accounts for the systemic increase of JA in roots of *N. sylvestris*. Therefore, a putative transport of JA from leaves to roots in our study could explain why prior herbivore-induced JA in leaves contributed to greater JA accumulation in roots and therefore to stronger reduction of root growth in response to root herbivory.

Mechanisms of JA-mediated growth inhibition in aboveground plant organs have been demonstrated for Arabidopsis, *N. attenuata* and rice (Yang *et al.*, 2012; Heinrich *et al.*, 2013; Matschi *et al.*, 2015). The current conception is that modulation of GA biosynthesis and JAZ interference with the interaction between DELLAs and growth-promoting PIF transcription factors are two key mechanisms leading to JA-mediated growth inhibition. In rice, overexpression of *EUI1* reduces drastically the levels of bioactive GAs (Zhu *et al.*, 2006) and enhances the accumulation of DELLA (Luo *et al.*, 2006), while the crossing of *Eui1-OX* with *coi1-18 plants* showed that growth enhancement in *coi1-18* plants depends on GA signaling (Yang *et al.*, 2012). In our study, the stronger plant growth inhibition of *Eui1-OX* plants due to root herbivory suggests that negative effects of RWW larvae on plant growth are counteracted by GA signaling in WT and *coi1-18* plants. Consistent with the current literature (Schäfer *et al.*, 2009; Jacobs *et al.*, 2011), our study shows that *P. indica* requires GA signaling to establish a mutualistic association with rice, as evidenced by the up-regulation of *OsKS1* expression, stronger growth promotion of *coi1-18* plants and failure to promote growth of *Eui1-OX* plants. Although not as well characterized, GA signaling can also attenuate JA signaling as reported for Arabidopsis (Hou *et al.*, 2010). It is therefore plausible that *P. indica*-elicited GA signaling also mediates the suppression of herbivore-induced JA in rice roots. To explore alternative explanations for the endophyte-induced tolerance, we evaluated possible resource limitations by measuring chlorophyll and mineral nutrients in rice plants. The endophyte did not outweigh the nutritional deficits caused by root herbivory, and while promoting plant growth, it did not affect chlorophyll concentrations in WT plants and even reduced it in *coi1-18* plants, which suggests that *P. indica* was unable to alter the resource paucity following root herbivory. Taken together, our results suggest that enhanced GA signaling and consequent suppression of JA signaling belowground is one mechanism by which *P. indica* induces plant tolerance to RWW (Fig. III.5).

The present tripartite interaction has larger implications. First, while the ecological function of fungal leaf endophytes in improving plant defense against aboveground herbivory is well documented (Rodriguez *et al.*, 2009; Estrada *et al.*, 2013), our study appears to be the first reporting the impact of a root endophyte on plant defense to belowground herbivory. Second, we show that endophyte-enhanced plant defense to root herbivory results from induced plant tolerance, which adds to the growing evidence that induced compensatory regrowth is an important defense strategy for roots to cope with the attack by herbivores (Poveda *et al.*, 2010; Erb *et al.*, 2012a; Robert *et al.*, 2014). Finally, we demonstrate that GA signaling is one mechanism by which an endophyte induces plant tolerance to root herbivory. This implies that GA is a putative signaling component of inducible compensatory regrowth against biotic stress. By showing how a fungal endophyte induces plant tolerance to root herbivory, our study illustrates a novel molecular mechanism underlying the integration of a beneficial microbe in the defense system of a higher plant.

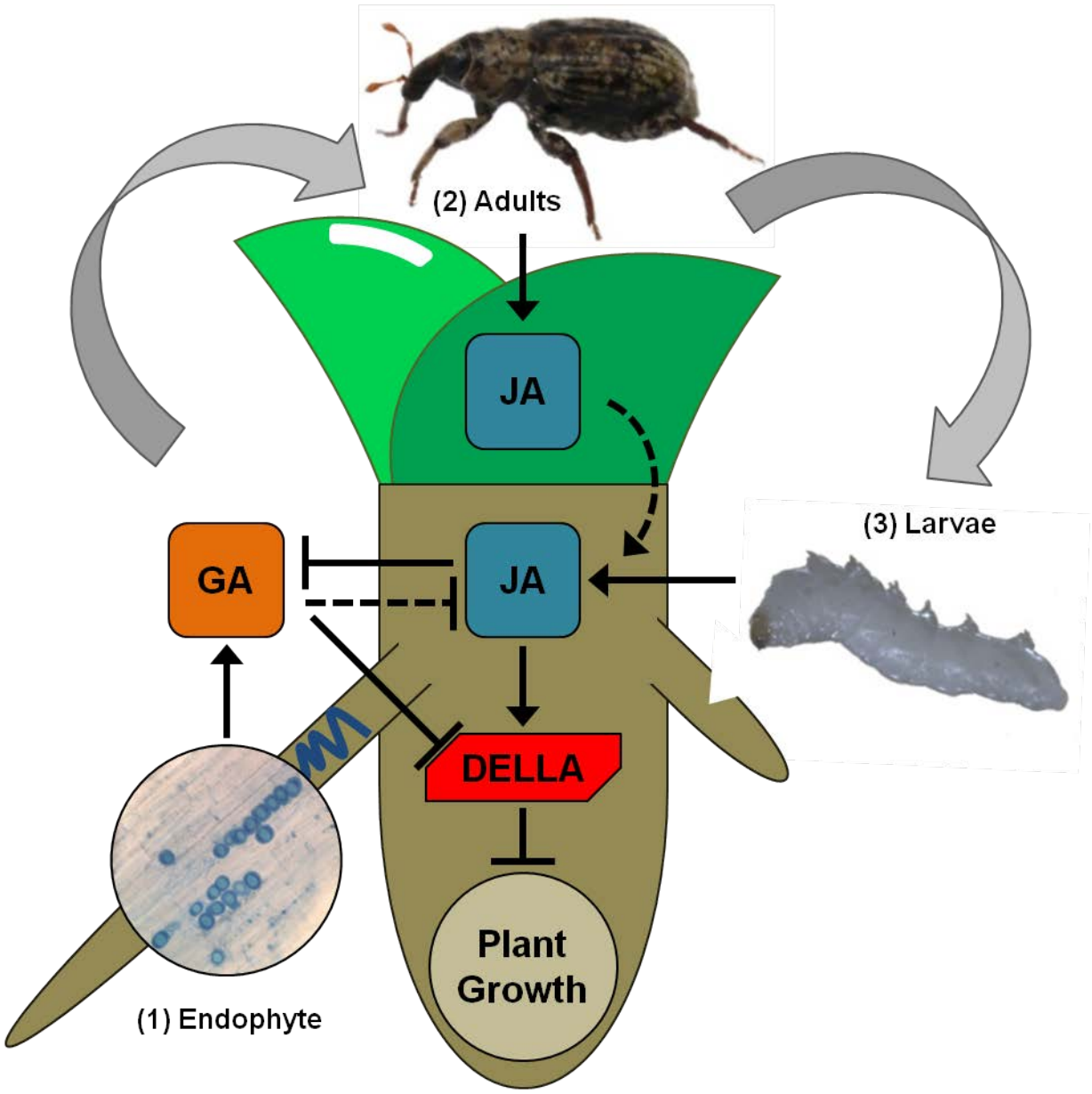

Figure III.5 *Schematic representation of endophyte-induced plant tolerance to root herbivory*. Rice plants were first inoculated with the root endophyte *Piriformospora indica* (1), then infested aboveground with rice water weevil (RWW) adults (2), and finally infested belowground with RWW larvae (3). The larvae induced jasmonic acid (JA) in roots, which was apparently enhanced by prior adult leaf herbivory, possibly through JA transport from leaves to roots. This contributes to both the suppression of gibberellic acid (GA) biosynthesis and the accumulation of DELLA and leads to plant growth inhibition. However, the prior endophyte colonization activates the GA biosynthetic pathway in roots to degrade DELLA and possibly to suppress JA accumulation. By disabling the JA mechanism for herbivore-mediated plant growth inhibition, the endophyte induces plant tolerance to root herbivory. Arrows and blunt-ended bars illustrate well established positive and negative effects, respectively, and dashed lines indicate putative effects.

Acknowledgements

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Supplementary information to Chapter III

Table III.S1 Primers used for Quantitative real-time PCR analyses.

Gene	RGAP LOCUS	Description	F-Primers (5'...3')	R-Primers (5'...3')
JAR1	LOC_Os05g50890	JA synthesis	AAGGTTTGTGAACCCATCAAACAGC	AATAATACTTTGCAGCACTTGTTACG
OsKS1	LOC_Os04g52230	GA synthesis	GACAAGGGACCAGCTCCAGACATTGGAG	CAGGAGCAGCAATCTGCTCATCCATGGC
OsACT	LOC_Os03g50885	Housekeeping	TGGACAGGTTATCACCATTGGT	CCGCAGCTTCCATTCCTATG

Table III.S2 Results of three-way ANOVA on the effects of the root endophyte *Piriformospora indica*, rice water weevil (RWW) adults, RWW larvae and their interactions on shoot biomass fresh weight (FW), tiller number, root biomass FW, total root length and average root diameter of 58 d-old rice plants of experiment I.

Factors	Shoot FW		Tiller No		Root FW		Root length		Root diameter	
	F	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*
Endophyte (E)	2,984	0,090	**5,906**	**0,018**	**5,177**	**0,027**	**9,056**	**0,004**	0,005	0,947
Adult (A)	0,970	0,329	0,024	0,877	0,238	0,628	1,555	0,218	0,046	0,831
Larva (L)	**9,954**	**0,003**	0,287	0,594	**1,966**	**0,167**	**16,060**	**<0,001**	**12,295**	**<0,001**
E x A	0,845	0,362	0,179	0,674	2,330	0,133	**7,882**	**0,007**	0,774	0,383
E x L	**5,371**	**0,024**	**9,534**	**0,003**	**7,001**	**0,011**	**21,696**	**<0,001**	0,194	0,661
A x L	0,097	0,756	0,389	0,535	0,126	0,724	1,128	0,293	0,389	0,535
E x A x L	0,017	0,897	0,055	0,816	0,621	0,434	**6,176**	**0,016**	1,315	0,256

Significant *P* values (< 0.050) are given in bold, marginally significant *P* values (< 0.100) are given in italic.

Table III.S3 Mean values of macronutrients (mg g^{-1} DW) and of micronutrients (µg g^{-1} DW) in shoot of 58-d-old rice plants as affected by the root endophyte *Piriformospora indica*, rice water weevil (RWW) adults, RWW larvae and their fully crossed combinations in comparison to the untreated control as well as the respective results of the three-way factorial ANOVA or GLM.

Macronutrients	Nitrogen (N)		Phosphorus (P)		Potassium (K)		Calcium (Ca)		Magnesium (Mg)		Sulfur (S)	
mg g^{-1} DW	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Control	29,05 ±	1,75	5,447 ±	0,082	51,22 ±	0,80	4,863 ±	0,141	2,543 ±	0,082	2,775 ±	0,079
Adult (A)	28,92 ±	1,88	5,529 ±	0,119	52,56 ±	0,93	4,897 ±	0,193	2,591 ±	0,063	2,899 ±	0,097
Larva (L)	28,67 ±	3,00	4,459 ±	0,281	46,08 ±	3,36	6,078 ±	0,552	2,394 ±	0,085	2,980 ±	0,198
AL	31,08 ±	1,82	4,510 ±	0,212	50,76 ±	1,57	6,231 ±	0,193	2,492 ±	0,104	3,272 ±	0,162
Endophyte (E)	29,89 ±	1,73	5,514 ±	0,098	52,79 ±	1,53	4,786 ±	0,094	2,439 ±	0,072	2,781 ±	0,115
EA	26,69 ±	1,24	5,773 ±	0,079	53,12 ±	1,20	4,689 ±	0,122	2,552 ±	0,071	2,953 ±	0,129
EL	27,90 ±	1,29	4,856 ±	0,066	50,54 ±	0,77	5,641 ±	0,208	2,545 ±	0,056	2,966 ±	0,066
EAL	28,51 ±	1,45	4,827 ±	0,058	49,28 ±	1,15	5,297 ±	0,109	2,396 ±	0,066	2,863 ±	0,127
Factors	GLM		GLM		GLM		GLM		ANOVA		ANOVA	
	F	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*
Endophyte	0,752	0,390	**5,205**	**0,026**	1,128	0,293	**6,743**	**0,012**	0,169	0,682	1,006	0,320
Adult	0,010	0,919	0,651	0,423	1,116	0,295	0,159	0,692	0,254	0,616	1,795	0,186
Larva	0,074	0,787	**64,938**	**<0,001**	**7,353**	**0,009**	**39,657**	**<0,001**	1,928	0,171	*3,452*	*0,068*
E x A	0,834	0,365	0,031	0,862	2,175	0,146	1,023	0,316	0,720	0,400	0,915	0,343
E x L	0,121	0,730	1,730	0,194	0,060	0,807	1,527	0,222	0,850	0,360	1,769	0,189
A x L	1,461	0,232	0,337	0,564	0,195	0,661	0,029	0,866	0,975	0,328	0,087	0,769
E x A x L	0,092	0,763	0,280	0,599	1,301	0,259	0,182	0,672	2,094	0,153	1,490	0,227

(Continue next page)

Continuation of **Table III.S3**

Micronutrients	Zinc (Zn)			Manganese (Mn)			Iron (Fe)			Copper (Cu)			Boron (B)			Molybdenum (Mo)		
µg g^{-1} DW	Mean		SE	Mean		SE	Mean		SE	Mean		SE	Mean		SE	Mean		SE
Control	117,2	±	4,45	382,1	±	10,47	286,9	±	38,26	13,78	±	0,670	9,626	±	0,287	1,953	±	0,082
Adult (A)	120,7	±	6,19	403,7	±	14,50	257,1	±	48,01	13,51	±	0,559	9,306	±	0,213	2,040	±	0,112
Larva (L)	130,5	±	12,04	316,8	±	40,49	261,3	±	20,36	17,96	±	4,871	11,633	±	0,876	2,249	±	0,080
AL	116,0	±	7,67	345,6	±	27,26	316,1	±	60,82	11,75	±	0,676	12,262	±	0,835	2,370	±	0,126
Endophyte (E)	118,6	±	3,91	401,7	±	6,75	243,2	±	18,89	13,14	±	0,800	9,678	±	0,272	2,072	±	0,097
EA	120,1	±	4,15	396,2	±	6,11	242,3	±	28,76	13,74	±	0,817	9,625	±	0,195	2,115	±	0,105
EL	118,7	±	4,38	365,5	±	10,12	264,0	±	28,18	13,57	±	0,257	11,306	±	0,576	2,154	±	0,057
EAL	111,5	±	4,96	360,8	±	10,68	317,7	±	41,16	12,98	±	0,567	11,055	±	0,853	2,105	±	0,077
Factors	ANOVA			GLM			ANOVA			GLM			GLM			ANOVA		
	F		*P*	*F*		*P*	*F*		*P*	*F*		*P*	*F*		*P*	*F*		*P*
Endophyte	0,399		0,530	1,927		0,171	0,036		0,850	0,467		0,497	0,489		0,487	0,383		0,538
Adult	0,872		0,354	0,537		0,467	0,055		0,815	1,577		0,215	0,000		0,999	0,564		0,456
Larva	0,058		0,811	**12,659**		**<0,001**	1,889		0,175	0,156		0,695	**23,289**		**<0.001**	**6,829**		**0,012**
E x A	0,044		0,835	1,227		0,273	0,273		0,604	1,583		0,214	0,137		0,713	0,644		0,426
E x L	0,669		0,417	0,892		0,349	0,216		0,644	0,273		0,603	1,313		0,257	**4,313**		**0,042**
A x L	2,041		0,159	0,021		0,884	1,731		0,194	1,928		0,170	0,204		0,654	0,047		0,829
E x A x L	0,149		0,701	0,014		0,907	0,045		0,832	0,841		0,363	0,478		0,492	0,229		0,635

Significant *P* values (< 0.050) are given in bold, marginally significant *P* values (< 0.100) are given in italic.

Table III.S4 Results of three-way GLM on the effects of the root endophyte *Piriformospora indica*, rice water weevil (RWW) adults, RWW larvae and their interactions on 12-oxophytodienoic acid (OPDA), jasmonic acid (JA) and jasmonoyl-isoleucine (JA-Ile) in leaves and in roots of 58-d-old rice plants.

Factors	Leaves						Roots					
	OPDA		JA		JA-Ile		OPDA		JA		JA-Ile	
	F	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*
Endophyte (E)	0,000	0,995	0,441	0,509	0,888	0,350	1,040	0,312	6,048	0,017	*3,155*	*0,081*
Adult (A)	4,732	0,034	6,420	0,014	5,340	0,025	0,336	0,565	1,524	0,222	1,513	0,224
Larva (L)	*3,483*	*0,067*	4,554	0,037	*3,274*	*0,076*	0,032	0,858	7,385	0,009	14,864	<0,001
E x A	0,237	0,628	1,662	0,203	1,424	0,238	0,069	0,794	0,829	0,367	0,984	0,326
E x L	0,594	0,444	0,047	0,829	0,609	0,438	2,079	0,155	4,133	0,047	1,657	0,204
A x L	0,047	0,830	2,015	0,161	1,368	0,247	0,196	0,660	0,588	0,447	0,024	0,877
E x A x L	0,608	0,439	0,533	0,469	0,348	0,558	0,291	0,592	0,674	0,415	0,909	0,345

Significant *P* values (< 0.050) are given in bold, marginally significant *P* values (< 0.100) are given in italic.

Table III.S5 Results of three-way GLM on the effects of the root endophyte *Piriformospora indica*, rice water weevil (RWW) adults, RWW larvae and their interactions on the relative transcription level of *OsJAR1* and *OsKS1* in roots of 58 d-old rice plants.

Factors	*OsJAR1*		*OsKS1*	
	F	*P*	*F*	*P*
Endophyte (E)	0,574	0,452	6,037	0,017
Adult (A)	0,166	0,685	0,000	0,993
Larva (L)	0,138	0,711	48,198	<0,001
E x A	0,013	0,910	1,723	0,195
E x L	*3,475*	*0,068*	0,768	0,385
A x L	2,436	0,125	0,109	0,743
E x A x L	0,678	0,414	*3,420*	*0,070*

Significant *P* values (< 0.050) are given in bold, marginally significant *P* values (< 0.100) are given in italic.

Table III.S6 Results of three-way ANOVA or GLM on the effects of the root endophyte *Piriformospora indica*, rice water weevil (RWW) adults, RWW larvae and their interactions on the biomass of shoots, untreated root-half, and treated root-half and on Chlorophyll in shoots of 58-d-old WT, *coi1-18*, and *Eui1-OX* plants.

WT	Shoot		Untreated root-half		Treated root-half		Chlorophyll	
	ANOVA		GLM		GLM		GLM	
Factors	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*
Endophyte (E)	**4,738**	**0,034**	*3,290*	*0,075*	**5,223**	**0,026**	1,496	0,226
Adult (A)	0,197	0,659	0,486	0,488	0,354	0,554	0,050	0,824
Larva (L)	0,620	0,434	0,217	0,643	**4,030**	**0,049**	0,891	0,349
E x A	0,014	0,906	0,003	0,959	0,020	0,887	1,379	0,245
E x L	0,219	0,642	1,178	0,282	0,720	0,400	0,357	0,553
A x L	1,842	0,180	0,810	0,372	*3,234*	*0,078*	1,016	0,318
E x A x L	0,179	0,674	0,220	0,641	0,950	0,334	0,185	0,669

COI1-18	Shoot		Untreated root-half		Treated root-half		Chlorophyll	
	GLM		GLM		GLM		ANOVA	
Factors	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*
Endophyte (E)	**16,305**	**<0,001**	**19,430**	**<0,001**	**17,243**	**<0,001**	**5,386**	**0,024**
Adult (A)	2,295	0,136	1,870	0,177	1,609	0,210	2,739	0,104
Larva (L)	0,140	0,710	0,028	0,869	0,666	0,418	0,939	0,337
E x A	0,263	0,610	0,352	0,556	0,791	0,378	0,062	0,805
E x L	0,558	0,458	1,121	0,294	0,019	0,892	2,064	0,156
A x L	0,242	0,625	0,524	0,472	0,720	0,400	1,224	0,273
E x A x L	0,000	0,997	0,979	0,327	0,323	0,572	0,003	0,959

EUI1-OX	Shoot		Untreated root-half		Treated root-half		Chlorophyll	
	GLM		GLM		GLM		GLM	
Factors	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*
Endophyte (E)	2,155	0,148	2,599	0,113	0,581	0,449	0,015	0,903
Adult (A)	0,975	0,328	0,861	0,358	0,578	0,451	1,005	0,321
Larva (L)	**5,612**	**0,022**	2,505	0,120	**5,789**	**0,020**	**7,281**	**0,009**
E x A	0,703	0,406	1,018	0,318	0,480	0,492	2,555	0,116
E x L	0,212	0,647	0,000	0,999	0,002	0,963	0,169	0,683
A x L	0,279	0,600	1,267	0,266	0,567	0,455	0,712	0,403
E x A x L	1,203	0,278	0,226	0,637	0,252	0,618	1,050	0,310

Significant *P* values (< 0.050) are given in bold, marginally significant *P* values (< 0.100) are given in italic.

Table III.S7 Results of two-way ANOVA or GLM on the effects of the root endophyte *Piriformospora indica* and rice water weevil (RWW) adults and their interaction on survival and growth of RWW larvae 28 d after neonate infestation in roots of 58-d-old WT, *coi1-18* or *Eui1-OX* plants.

	WT				*coi1-18*				*Eui1-OX*			
	Survival		Growth		Survival		Growth		Survival		Growth	
	GLM		GLM		ANOVA		GLM		ANOVA		GLM	
Factors	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*
Endophyte (E)	0,131	0,721	1,085	0,308	**9,048**	**0,006**	0,083	0,775	0,688	0,414	0,161	0,693
Adults (A)	0,066	0,799	0,071	0,792	0,692	0,412	0,072	0,791	0,199	0,659	*4,122*	*0,056*
E x A	0,237	0,630	0,035	0,854	1,479	0,234	0,005	0,944	0,066	0,799	0,016	0,900

Significant *P* values (< 0.050) are given in bold, marginally significant *P* values (< 0.100) are given in italic.

Chapter IV: Arbuscular mycorrhizal fungi affect glucosinolate and mineral element composition in leaves of *Moringa oleifera*

Cosme M, Franken P, Mewis I, Baldermann S, Wurst S (2014) Mycorrhiza 24: 565-570.

http://dx.doi.org/10.1007/s00572-014-0574-7

Abstract

Moringa is a mycorrhizal crop cultivated in the tropics and subtropics and appreciated for its nutritive and health-promoting value. As well as improving plant mineral nutrition, arbuscular mycorrhizal fungi (AMF) can affect plant synthesis of compounds bioactive against chronic diseases in humans. *Rhizophagus intraradices* and *Funneliformis mosseae* were used in a full factorial experiment to investigate the impact of AMF on the accumulation of glucosinolates, flavonoids, phenolic acids, carotenoids, and mineral elements in moringa leaves. Levels of glucosinolates were enhanced, flavonoids and phenolic acids were not affected, levels of carotenoids (including provitamin A) were species specifically reduced, and mineral elements were affected differently, with only Cu and Zn being increased by the AMF. This study presents novel results on AMF effects on glucosinolates in leaves and supports conclusions that the impacts of these fungi on microelement concentrations in edible plants are species dependent. The nonspecific positive effects on glucosinolates and the species-specific negative effects on carotenoids encourage research on other AMF species to achieve general benefits on bioactive compounds in moringa.

Supplementary information to Chapter IV

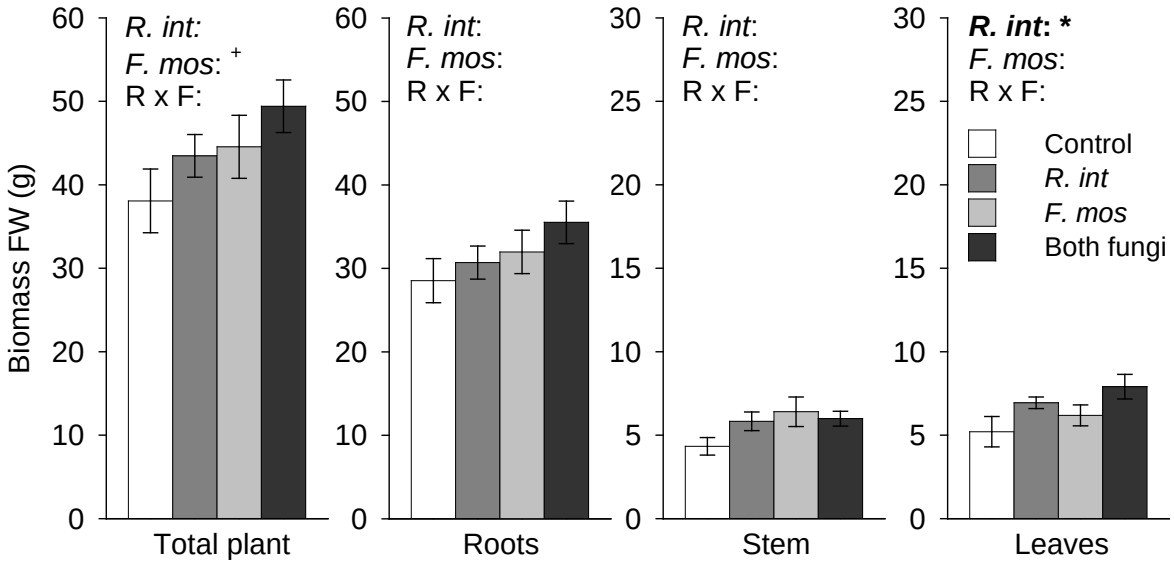

Fig. IV.S1 Effects of *Rhizophagus intraradices* (*R. int*), *Funneliformis mosseae* (*F. mos*) or both fungi combined in comparison to the non-AMF control on total plant, root, stem, and leaves fresh biomass of moringa, with the respective results of the two-way factorial ANOVA. Significance levels of *F* statistics are *, ** and *** corresponding to $P < 0.05$, 0.01 and 0.001, respectively, and are in bold. Marginally significant effects are $^{+}$ corresponding to $P < 0.1$. Mean ± SE, N = 10. For detailed ANOVA summary see Table IV.S2.

Table IV.S1 Mean values of macroelements (mg g^{-1} DW) in leaves of moringa as affected by *Rhizophagus intraradices* (*R. int*), *Funneliformis mosseae* (*F. mos*) or both fungi combined in comparison to the non-AMF control, and the respective results of the two-way factorial ANOVA

	Control		*R. int*		*F. mos*		Both fungi		Two-way ANOVA		
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	*R. int*	*F. mos*	*R. int* x *F. mos*
Macro									*F*	*F*	*F*
N	40.06	±1.88	40.75	±0.88	39.11	±1.31	39.26	±1.05	0.50	0.36	0.31
P	4.95	±0.47	4.63	±0.18	4.33	±0.15	4.69	±0.19	0.07	0.90	1.44
K	20.92	±1.25	20.41	±1.04	20.02	±0.48	20.90	±0.96	0.05	0.04	0.51
Ca	30.58	±1.78	28.43	±1.50	30.56	±1.40	31.10	±1.13	0.31	0.87	0.86
Mg	3.56	±0.23	3.25	±0.21	2.82	±0.22	3.14	±0.16	0.02	*4.07*$^{+}$	2.36

Significance levels of *F* statistics are *, ** and *** corresponding to $P < 0.05$, 0.01 and 0.001, respectively

Values in italics are marginally significant effects ($^{+}$, $P < 0.1$). N = 10

Table IV.S2 ANOVA summary on the effects of *Rhizophagus intraradices* (*R. int*) and *Funneliformis mosseae* (*F. mos*) on plant biomass and on concentration of carotenoids, glucosinolates, flavonoids and phenolic acids in moringa leaves. Significance levels of *F* statistics are *, ** and *** corresponding to $P < 0.05$, 0.01 and 0.001, respectively, and are in bold. Values in italics are marginally significant effects ($^{+}$, $P < 0.1$).

Biomass		Leaves	Stem	Roots	Total plant
	df	*F*	*F*	*F*	*F*
R. int	1	**6.32***	1.30	1.35	2,32
F. mos	1	2.00	*3.35*$^{+}$	2.83	*3,41*$^{+}$
R. int x *F. mos*	1	0.00	2.24	0.08	0,01
Error	36				
Glucosinolates[1]		GS	GS I	GS II	GS III
	df	*F*	*F*	*F*	*F*
R. int	1	0,59	**4,66***	**12,18****	0,13
F. mos	1	0,63	**6,10***	**12,29****	2,44
R. int x *F. mos*	1	1,14	0,60	0,99	0,56
Error	36				
Flavonoids[2]		KMG	KG	QMG	QG
	df	*F*	*F*	*F*	*F*
R. int	1	0.10	0.06	1.33	1.69
F. mos	1	0.47	*2.89+*	*4.02+*	0.19
R. int x *F. mos*	1	0.42	1.21	0.27	1.04
Error	36				
Phenolic acids[3]		CGA	NCGA		
	df	*F*	*F*		
R. int	1	0.01	0.98		
F. mos	1	0.28	0.12		
R. int x *F. mos*	1	0.05	0.00		
Error	36				
Carotenoids		β-Carotin	Zeaxanthin	Lutein	Neoxanthin
	df	*F*	*F*	*F*	*F*
R. int	1	**5,43***	**4,73***	*3,82+*	**6,32****
F. mos	1	**26,83*****	**48,16*****	**81,11*****	**27,73*****
R. int x *F. mos*	1	3,07	0,10	2,49	0,22
Error	36				

[1] GS, GS I, GS II and GS III correspond to 4-(α-L-rhamnopyranosyloxy)-benzylglucosinolate, and its monoacetyl-Isomer I, II and III, respectively.

[2] KMG, KG, QMG and QG correspond to kaempferol 3-*O*-(6"-malonylglucoside), kaempferol 3-*O*-glucoside, quercetin 3-*O*-(6"-malonylglucoside), and quercetin 3-*O*-glucoside, respectively.

[3] CGA and NCGA correspond to chlorogenic acid and neochlorogenic acid, respectively.

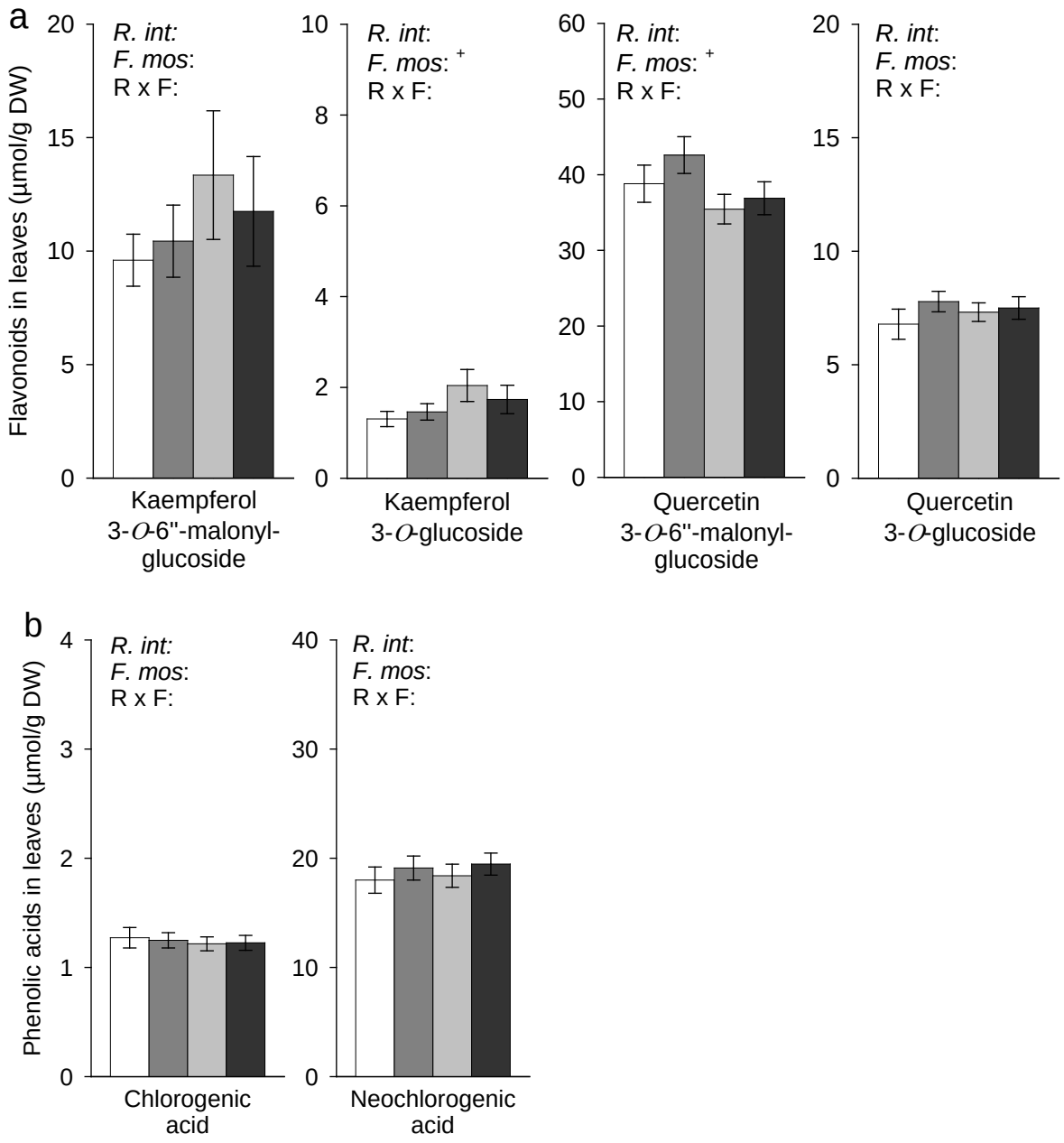

Fig. IV.S2 Effects of *Rhizophagus intraradices* (*R. int*), *Funneliformis mosseae* (*F. mos*) or both fungi combined in comparison to the non-AMF control on levels of flavonoids (a), and phenolic acids (b) in moringa leaves, with the respective results of the two-way factorial ANOVA. Significance levels of *F* statistics are *, ** and *** corresponding to $P < 0.05$, 0.01 and 0.001, respectively, and are in bold. Mean ± SE, N = 10. For detailed ANOVA summary see Table IV.S2.

Chapter V: Plant cytokinin status regulates the arbuscular mycorrhizal symbiosis between *Nicotiana tabacum* and *Rhizophagus irregularis*

Cosme M, Ramireddy E, Franken P, Schmülling T, Wurst S (201X) Submitted to Mycorrhiza MCOR-S-15-00129 (http://link.springer.com/journal/572)

Abstract

The ubiquitous symbiosis between arbuscular mycorrhizal (AM) fungi and the roots of most terrestrial plants plays a key role in nutrient uptake by the plant. In exchange, the plant supplies photosynthetically fixed carbon (C) to the obligate AM fungi. Even though the fungi generally improve plant performance, fungal parasitism may occur. Nearly all phytohormones are involved in the plant regulation of the AM symbiosis. However, only little is known about the role of the phytohormone cytokinin (CK) in this plant-fungus interaction, although CK was shown to accumulate in shoots and roots of AM plants. Here, we used different transgenic lines of tobacco (*Nicotiana tabacum*) and the corresponding wild type to investigate whether a lowered content of endogenous CK in roots or shoots influences the interaction with the AM fungus *Rhizophagus irregularis*. Our data indicates that the shoot CK has a positive impact on the AM symbiotic functioning in roots. A lowered content of CK in roots caused shoot and root growth depression following AM colonization, which was associated with reduced C concentration in shoots, while neither the uptake of phosphorus or nitrogen nor the root transcript levels of an AM-specific phosphate transporter gene were significantly affected. This suggests that the root CK may restrict fungal C sink thus averting parasitism by AM fungi. Taken together, our results clearly demonstrate that organ-specific CK status can affect profoundly the plant performance in response to AM symbiosis.

Introduction

The ubiquitous symbiosis between higher plants and the arbuscular mycorrhizal (AM) fungi is regulated by several phytohormones through yet poorly understood mechanisms (Pozo *et al.*, 2015). Phytohormones are small metabolites that regulate intrinsic developmental and physiological pathways, but also mediate the response of these pathways to environmental cues. For instance, they modify root architecture and the expression of phosphate transporter genes during phosphorous (P) starvation and balance shoot growth according to light intensity (Werner & Schmülling, 2009; Sparks *et al.*, 2013). The AM fungi comprise the fungal phylum Glomeromycota that associates with the roots of 80 % of terrestrial plants in a wide range of environmental settings, including agricultural environments (Smith & Read, 2008). These fungi can confer a variety of benefits to their host plants, such as enhanced mineral nutrition, photosynthesis, growth and seed production. As these benefits are context-dependent, deciphering the role of phytohormones in regulating the plants response to AM symbiosis is crucial to understand how plants can optimize their symbiotic strategies to improve growth and fitness.

AM fungi are obligate symbionts. They obtain photosynthetically fixed carbon (C) from plants, through transport of sugars from root cortex cells to inter- and intracellular hyphae as well as to arbuscules (Smith & Read, 2008; Helber *et al.*, 2011). In exchange, the AM mycelium takes up mineral nutrients from the soil, particularly P, but also nitrogen (N) and others, and delivers them to the plant via the arbuscules in the apoplast of root cortex cells (Marschner & Dell, 1994; Nouri *et al.*, 2014). Despite this reciprocal nutritional benefit, the plant growth response to AM colonization varies markedly along a mutualism-parasitism continuum (Johnson, 2010). Mutualistic benefits of delivered P are predicted to be the greatest in soils that are characterized by high N and low P availability (Johnson, 2010). By contrast, when neither N nor P is limited, fungal growth is primarily limited by C, so the fungal C demand can increase to the point where it may depress plant growth and generate fungal parasitism (Johnson, 2010). Furthermore, other factors such as fungal genotype, competition or complementarity may also influence the AM symbiotic outcome (Maherali & Klironomos, 2007; Jansa *et al.*, 2008; Angelard *et al.*, 2010).

AM symbiosis is regulated by nearly all phytohormones (Gutjahr, 2014; Pozo *et al.*, 2015). The study of mutants and transgenic plants altered in phytohormone metabolism or perception has provided major insights into their role in regulating AM symbiosis. The arbuscule formation vital to the net benefits of AM symbiosis is regulated positively by abscisic acid, via a dual ethylene-dependent/ethylene-independent mechanism (Martín-Rodríguez *et al.*, 2011). In contrast to ABA, gibberellins (GA) negatively regulate arbuscule formation via degradation of DELLA proteins, which are required for arbuscule formation (Floss *et al.*, 2013; Foo *et al.*, 2013; Yu *et al.*, 2014). Jasmonic acid positively regulates arbuscule formation but negatively regulates fungal spread inside the roots via systemic signaling to and from shoots, possibly by controlling the C allocated to colonized roots (Wasternack & Hause, 2014). Salicylic acid slows down fungal growth without changing final colonization (Herrera-Medina *et al.*, 2003). Auxin and brassinosteroids do not appear to affect directly arbuscule formation but increase AM hyphal colonization (Hanlon & Coenen, 2011; Foo, 2013; Bitterlich *et al.*, 2014). In *Pisum sativum* this auxin effect was partially mediated by strigolactones which stimulate the presymbiotic hyphal branching (Foo, 2013).

Much less is known about the role of another class of phytohormones, cytokinin (CK), in the AM plant-fungus interaction. CK are N^6-substituted purine derivatives that regulate many fundamental aspects of plant development, including responses to nutrient starvation (Werner & Schmülling, 2009; Kieber & Schaller, 2014). Generally, AM fungi increase the CK levels in roots and shoots (Allen *et al.*, 1980; Baas & Kuiper, 1989; Barker & Tagu, 2000), which apparently is uncharacteristic to pathogenic fungi (van Rhijn *et al.*, 1997) and seems to be independent from increased P supply in roots (Torelli *et al.*, 2000; Shaul-Keinan *et al.*, 2002). Drüge & Schonbeck (1992) observed a strong correlation between increased CK levels, improved photosynthesis and enhanced growth of AM plants and hypothesized that CK is part of the positive AM effect on plant performance. However, this hypothesis has been contested as the correlation was not always observed (Baas & Kuiper, 1989; Danneberg *et al.*, 1993). Thus, it is as yet unclear why AM plants have generally increased CK levels. Moreover, the scarce knowledge about the role of CK due to a lack of direct evidence elucidating the effects of CK on AM symbiosis may cause an underestimation of its potential importance (Foo *et al.*, 2013; Bucher *et al.*, 2014).

One approach to study the role of CK in AM symbiosis is to use plants with an altered CK content. A useful tool to generate plants with a lower CK content has been the ectopic expression of *CYTOKININ OXIDASE/DEHYDROGENASE* (*CKX*) genes (Werner *et al.*, 2001; Werner *et al.*, 2003). *CKX* genes code for enzymes that irreversible degrade CK in a single enzymatic step to biologically inactive molecules (Schmülling *et al.*, 2003; Werner *et al.*, 2003). Systemic expression of *CKX* genes under the control of the 35S promoter reduces shoot growth (Werner *et al.*, 2001; Werner *et al.*, 2003) as well as leaf chlorophyll and sugar contents (Werner *et al.*, 2008), but increases root growth. Together this leads to an enhanced root-to-shoot ratio and revealed an opposite regulatory function of CK on shoot and root development (Werner *et al.*, 2003). Plants with root-specific CK deficiency have an increased root growth but a normal shoot growth (Werner *et al.*, 2010). The lower CK content in roots altered the expression of nutrient transporter genes and caused the accumulation of more mineral nutrients in shoots (Werner *et al.*, 2010), which indicates that CK negatively regulates typical root responses to nutrient starvation. *35S:CKX2* transgenic tobacco (*Nicotiana tabacum*) plants have been used previously to analyze interactions between arbuscular mycorrhizal fungi, rhizobacteria and soil P (Cosme & Wurst, 2013). It has been found that systemically CK-deficient plants increase AM hyphal colonization without affecting arbuscule formation (Cosme & Wurst, 2013). However, the mechanism for this CK effect remains unknown.

Here, we tested whether a lowered CK content only in the roots has an influence on the interaction with AM fungi and whether the shoot CK status affects AM symbiosis as well. To this end, we compared the plant performance and AM colonization of transgenic tobacco plants with root-specific CK deficiency (*W6:CKX1*) with that of untransformed wild type (WT) and two systemically CK-deficient transgenic plants (*35S:CKX1*, *35S:CKX2*). Furthermore, we tested whether AM fungal strains or their interaction influence the symbiotic outcome by using single and simultaneous inoculation with two different strains of *Rhizophagus irregularis* (formerly *Glomus intraradices*) as symbiotic function may vary with strain genotype (Angelard *et al.*, 2010). Potential functional mechanisms underlying plant and fungal responses were explored by determining the P, N and C content in shoots and the transcript levels of phosphate transporter genes in roots. Our study revealed organ-specific effects of CK on AM symbiosis and suggests that these effects are mediated through the modulation of C availability to the fungus independently in part of P and N supply.

Materials and methods

Plant and fungi

Rhizophagus irregularis (former *Glomus intraradices*) is a widespread AM fungus and the first one that has been used for large-scale transcriptome sequencing (Tisserant *et al.*, 2011). Although it is generally effective in colonizing roots and transferring mineral nutrients to the host plant, *R. irregularis* symbiotic function may vary with strain genotype (Angelard *et al.*, 2010). To unveil eventual strain effects, we used two different *R. irregularis* strains (RI and FM) obtained from INOQ GmbH (Soltau, Germany). Inocula of *R. irregularis* were produced in sand using mixed plant cultures of *Plantago lanceolata*, *Tagetes erecta*, and *Zea mays*, and contained 200 propagules ml^{-1}, consisting of spores, hyphae, and colonized root pieces.

As a host plant we used tobacco (*Nicotiana tabacum* L. cv. Samsun NN). The untransformed control is referred to as wild type (WT). The transgenic lines expressing *W6:CKX1* (line W6-CKX1-24), *35S:CKX1* (line 35S:CKX1-50) and *35S:CKX2* (line 35S:CKX2-38) were described previously (Werner *et al.*, 2001; Werner *et al.*, 2008; Werner *et al.*, 2010). Shortly, the *W6:CKX1* line harbors the *CKX1* genes of *Arabidopsis* under the transcriptional control of the predominantly root-expressed *WRKY6* promoter (Werner *et al.*, 2010). The *35S:CKX1* and *35S:CKX2* plant lines harbor two different *CKX* genes (*CKX1* and *CKX2*, respectively) of *Arabidopsis* under the transcriptional control of the systemically expressed 35S promoter (Werner *et al.*, 2001). Systemic reduction of the CK content in *35S:CKX1* and *35S:CKX2* transgenic tobacco lines causes in addition to root enhancement a reduced shoot growth because CK is a positive regulator of shoot growth. Both lines differ in the expressivity of the phenotypic traits, with *35S:CKX1* expression causing stronger negative effects on shoot growth than *35S:CKX2*, which is reflected by reduced photosynthesis and a lower content of soluble sugar (Werner *et al.*, 2008).

Experimental set up

To test whether the root CK status influences the AM symbiosis, and eventually the CK status of the shoot has a role as well, and whether AM fungal strains and/or their interaction influence the symbiotic outcome, we conducted a factorial experiment in a glasshouse (16 h light and

20°/24°C night/day temperatures) with the factors tobacco line (four levels: WT, *W6:CKX1*, *35S:CKX1*, *35S:CKX2*), *R. irregularis* RI (two levels: -, +), and *R. irregularis* FM (two levels: -, +), distributed over a total of 16 treatments, each treatment with 10 independent replicates. The experimental replicate consisted of a plastic pot (2 L) filled with an autoclaved (121 °C, 20 min) soil:sand mixture (1:1 v:v) described previously (Cosme *et al.*, 2014). We inoculated the pots according to AM treatment by mixing thoroughly 100 ml of inocula on the top layer of the soil:sand mixture. Non-AM (NAM) control pots received sterilized inoculum (autoclaved at 121 °C for 20 min), the *R. irregularis* RI and FM pots received their respective strain inoculum, and the co-inoculated pots received a mixture (1:1 v:v) of both strain inocula. Additionally, the pots received a microbial wash (20 μm sieve) produced from fungal inocula to correct for the potential presence of NAM microbial backgrounds. All tobacco seeds were surface-sterilized in 1.2 % NaClO for 5 min and rinsed with H_2O prior use. Each pot was sown with several seeds of the respective tobacco line and immediately after germination the seedlings were thinned, allowing only one single plant to grow in each pot. Plants were watered regularly and fertilized every week with double strength of a modified Hoagland's solution without P (No. 3 as described by Douds & Schenck, 1990) to facilitate AM colonization. After eight weeks of growth, the number of flowers per plant was counted and all plants were harvested by cutting the shoot at the ground level. The soil was carefully washed away from roots and root sub-samples (0.3 g) were instantly frozen in liquid N_2 and stored in -80 °C for further analyses. The shoots and roots were dried in an oven during one week at 60 °C and subsequently their biomasses were recorded.

AM fungal colonization

To evaluate whether an altered CK content affects AM fungal development we assessed the percentage of root length colonized by AM hyphae and arbuscules in all experimental plants. To this end, random samples of 10 2-cm-long root fragments were collected from each root and stained using the ink and vinegar method (Vierheilig *et al.*, 1998). The percent of root length colonization was determined at the microscope (×200 magnification) using the gridline intersection method with 100 intersects per sample (McGonigle *et al.*, 1990).

Content of phosphorus, nitrogen and carbon in shoots

To determine whether altered plant growth following *R. irregularis* colonization was correlated with an altered nutrient content, we determined the concentration and total content of N and P as well as the concentration of C in shoots. The dried shoots of each plant were homogenized by grounding to fine particles using a ball mill (MM 400, Retsch, Haan, Germany). Sub-samples (ca. 3 mg) of all ground shoots were placed into individual zinc capsules and the percentages of N and C in shoots were determined by standard procedures using a CN Elemental Analyzer (Euro EA, HEKAtech GmbH, Germany), with acetanilide as standard (HEKAtech M.135.17). The concentration of P was determined from ground shoots of five randomly selected plant replicates per treatment, with two technical repetitions (200 mg each) per plant replicate. Technical repetitions were microwave-digested in 5 mL of 65 % HNO_3 and 3 mL of 30 % H_2O_2 using a MARSXpress (CEM GmbH, Kamp-Lintfort, Germany). The digested solution was filtered and diluted with H_2O in 50 mL volumetric flasks, and P was measured photometrically following the DIN EN ISO 15681-1 norm and using a FIA modula (Medizin- und Labortechnik Engineering GmbH, Dresden, Germany).

Root transcript levels of phosphate transporter genes

Total RNA was extracted from roots of eight-week-old tobacco plants with the TRIzol method as described by Brenner *et al.* (2005). RNA was further purified by using RNeasy mini-columns including the on-column DNase digestion as described in the manufacturer's protocol (appendix D of the Qiagen RNeasy Mini Handbook, QIAGEN GmbH, Hilden, Germany). Equal amounts of starting material (1 µg of RNA) were used for complementary DNA synthesis using SuperScript III Reverse Transcriptase. Real-time PCR using FAST SYBR Green I technology was performed on an ABI PRISM 7500 sequence detection system (Applied Biosystems Inc., California, USA) and universal "FAST" cycling conditions (10 min at 95 °C, 40 cycles of 15 s at 95 °C and 60 s at 60 °C) followed by the generation of a dissociation curve to check for specificity of the amplification. Gene expression data was normalized against two different reference genes (*Nicotiana tabacum Elongation factor 1α* (*EF-1α*), and *L25 ribosomal protein*) according to Vandesompele *et al*. (2002) and are presented relative to the control treatment. Primers used for reference genes and genes of interest are listed in Supplemental Table S1.

Statistical analyses

We analyzed AM fungal colonization by two-way ANOVA using only inoculated plants with the categorical factors tobacco line (four levels: WT, *W6:CKX1*, *35S:CKX1* or *35S:CKX2*) and AM inoculation (three levels: *R. irregularis* RI, *R. irregularis* FM, or both RI and FM). Plant parameters were analyzed by three-way ANOVA with the categorical factors tobacco line (same levels as above), *R. irregularis* RI (two levels: -, +) and *R. irregularis* FM (two levels: -, +). Data were tested for normality of distribution using Kolmogorov-Smirnov tests and for homogeneity of variances using Levene's tests. In the case of non-normality and/or unequal variances, data were log or arcsine transformed prior to ANOVA. Multiple comparisons were analyzed by Duncan's multiple-range test. All data were analyzed in R Studio 0.97.332 (www.rstudio.com).

Results

AM fungal colonization

To assess AM fungal development, we quantified the internal hyphal and arbuscules colonization in stained roots of eight-week-old tobacco plants (Figs. V.1A and V.1B). No evidence was found for AM fungal colonization in plants treated with sterile inocula (NAM control). The AM hyphal colonization in WT plants was remarkably high (close to 96 %) and did not differ among fungal inoculations (Fig. V.1A; Table V1). Lowering the CK content in roots (*W6:CKX1*) did not affect the AM hyphal colonization compared with that of WT (Fig. V.1A). In contrast, plant lines with systemic CK deficiency (*35S:CKX1* and *35S:CKX2*) had reduced AM hyphal colonization compared to the WT. *35S:CKX1* transgenic plants showed a strongly reduced hyphal colonization following all fungal inoculations (from 96 % to 45 %), while *35S:CKX2* plants showed reduced hyphal colonization (64 %) only in the fungal co-inoculation treatment (Fig. V.1A). As both WT and *W6:CKX1* plants showed increased AM hyphal colonization compared with that of *35S:CKX1*, this indicates that the reduced AM hyphal development might have been at least partly dependent on the shoot CK status. Finally, the arbuscule colonization was relatively low across all plant lines and AM fungal inoculations, but followed a similar pattern as the hyphal colonization (Fig. V.1B; Table V1). Differential quantification of the two *R. irregularis* strains following co-inoculation was aimed to be carried out by molecular means. For this purpose, different regions of the rRNA gene cluster and fragments of genes encoding the phosphate transporter or the translation elongation factor EF1-alpha were sequenced. Sequence differences between the two strains were, however, too low for experimental differentiation of the two strains (data not shown).

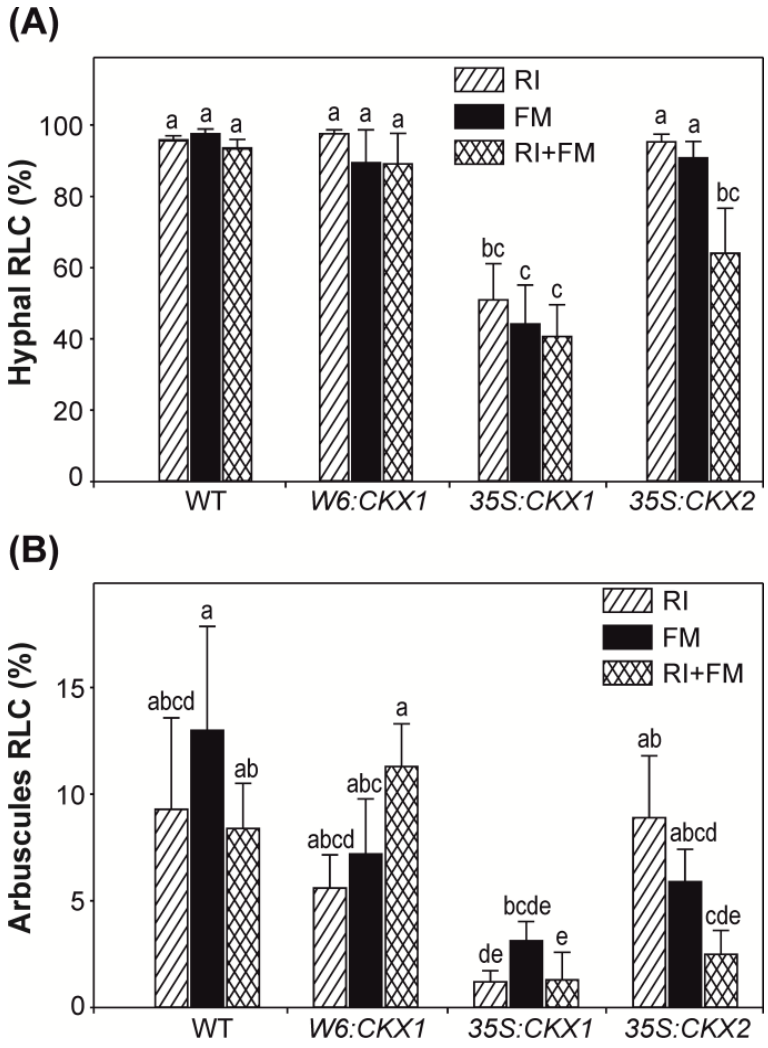

Figure V.1. *Effects of root-specific and systemic cytokinin deficiency on AM colonization*. Tobacco line *W6:CKX1* with root-specific CK-deficiency, the *35S:CKX1* and *35S:CKX2* transgenic lines with systemic CK deficiency and the corresponding wild type (WT) where inoculated with the AM fungus *Rhizophagus irregularis* strain RI, strain FM or both (RI+FM). Plants were grown in a glasshouse for eight weeks and sampled to determine the percentage of root length colonization (RLC) by AM hyphae and arbuscules. Values are means + SE, n = 10. For ANOVA results see Table 1. For each AM fungal parameter, bars with similar letters are not significantly different ($P < 0.05$) according to Duncan's multiple range test.

Table V.1. *Arbuscular mycorrhizal (AM) fungal colonization of cytokinin-deficient plants*. Tobacco line *W6:CKX1* with root-specific CK-deficiency, the *35S:CKX1* and *35S:CKX2* transgenic lines with systemic CK deficiency and the corresponding wild type where inoculated or not with the AM fungus *Rhizophagus irregularis* strain RI or strain FM. Plants were grown in a glasshouse for eight weeks and sampled to determine the percentage of root length colonization (RLC) by AM hyphae and arbuscules. Two-way ANOVAs with the categorical factors tobacco line and AM fungal inoculation were used to determine the significant levels of *F* statistics. For the mean values see Fig. V.1. df, degrees of freedom.

Factors		Hyphal RLC		Arbuscules RLC	
	df	*F*	*P*	*F*	*P*
Tobacco lines (T)	3	**32.7**	***	**9.2**	***
AM fungi (AMF)	2	*2.6*	(*)	0.8	
T x AMF	6	1.1		*2.0*	(*)
Residuals	108				

For $P < 0.05$, 0.01 and 0.001, significance levels of *F* values are presented as *, ** and ***, respectively, and are in bold. *F* values accompanied by (*) are marginally non-significant and are in italic. $n = 10$.

Plant biomass and fitness

Next we analyzed whether AM fungal inoculation had a specific influence on the biomass formation of roots or shoots. It was found that the different plant genotypes showed distinct responses (Figs. V.2A and V.2B; Table V.2). *R. irregularis* did not affect significantly the root biomass of WT plants when compared with NAM WT plants. However, the combined inoculum of *R. irregularis* strains reduced significantly the root biomass in CK-deficient roots when these were not accompanied by a strong CK-deficient shoot phenotype, i.e. in *W6:CKX1* and *35S:CKX2*. The strain RI alone had also a negative influence on the root biomass of *35S:CKX2* plants compared with NAM *35S:CKX2* plants. The shoot biomass of WT plants was reduced significantly following inoculation by single *R. irregularis* strains but was not affected by their co-inoculation as compared with NAM WT plants. A reduction of the shoot biomass was also noted in transgenic plants without or with only moderate CK deficiency in the shoot, i.e. in *W6:CKX1* and *35S:CKX2* (Fig. V.2B and V.2D). In contrast, the low shoot biomass as a

consequence of strong CK deficiency (*35S:CKX1*) was not lowered further following AM inoculation (Fig. V.2B). The co-inoculation with RI and FM neutralized their negative effects on shoot biomass formation of WT. However, it reduced synergistically the shoot biomass of *W6:CKX1*, and maintained the RI negative effects on shoot biomass of *35S:CKX2*. The different growth response to *R. irregularis* in *35S:CKX1* compared to *W6:CKX1* and WT suggests that the effects of the root CK status on AM symbiotic function depends on the shoot CK status. A lowered CK levels in roots appear to increase the plants' susceptibility to growth depression following AM fungal colonization. However, this consequence does not become apparent when the shoot itself is already strongly CK-deficient.

To measure plant fitness we counted the number of flowers in eight-week-old plants as a proxy. To evaluate the data it should be noted that a lowered CK content of the shoots retards the reproductive development of tobacco plants and reduces the overall number of flowers (Werner *et al.*, 2001), which is confirmed by the *35S:CKX1* and *35S:CKX2* NAM plants in Fig. V.2C. We observed that the effects of AM fungal inoculations on the number of flowers depended strongly on the combination of *R. irregularis* strain and plant genotype (Fig. V.2C; Table V.2). *R. irregularis* RI reduced the number of flowers of WT by 75 %, while strain FM and co-inoculation with both strains had no significant effects compared with the NAM counterparts (Fig. V.2C). The single strain inoculations had no significant effects in *W6:CKX1* and *35S:CKX2* (Fig. V.2C), while co-inoculation of RI and FM reduced the number of flowers by 87 % and 58 % in *W6:CKX1* and *35S:CKX2* compared to their NAM control plants, respectively (Fig. V.2C). In contrast, reproductive development of *35S:CKX1* was not affected by AM fungal colonization (Fig. V.2C).

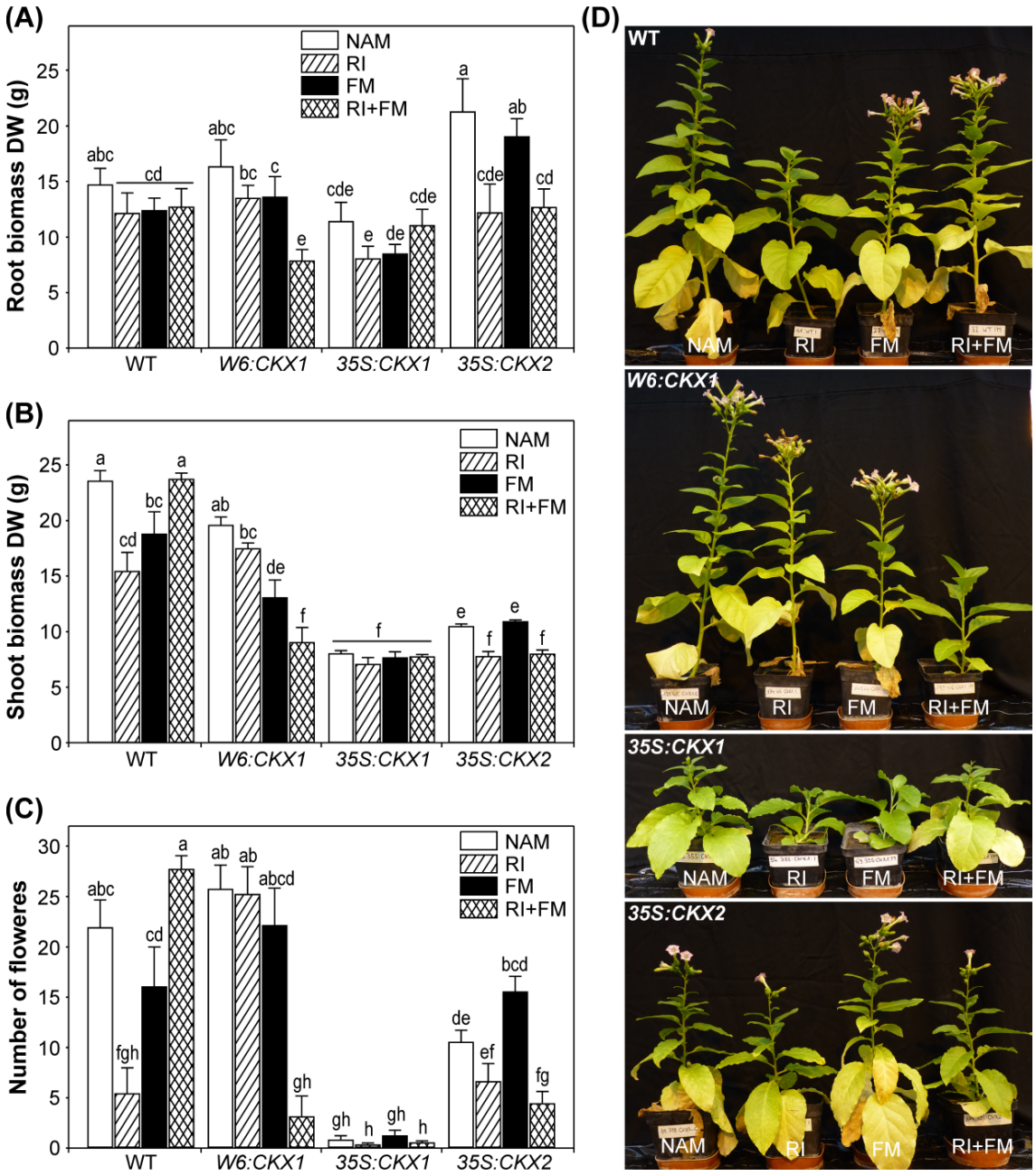

Figure V.2. *Influence of AM inoculation on biomass and reproduction*. The effect of *Rhizophagus irregularis* RI, FM and their co-inoculation (RI+FM) on root (A) and shoot (B) dry weight (DW) and number of flowers (C) of eight-week-old tobacco plants were compared with the respective non-AM (NAM) plants of wild type (WT) and transgenic lines with root-specific (*W6:CKX1*) or systemic (*35S:CKX1* and *35S:CKX2*) cytokinin deficiency. Values are means + SE, $n = 10$. For each plant parameter, bars with similar letters are not significantly different ($P < 0.05$) according to Duncan's multiple range test. (D) The shoot phenotype of eight-week-old plants (WT, *35S:CKX1*, *35S:CKX2* and *W6:CKX1*) inoculated with RI, FM or RI+FM in comparison to NAM plants is shown.

Table V.2. *Biomass and fitness of AM-inoculated cytokinin-deficient plants*. Tobacco line *W6:CKX1* with root-specific CK-deficiency, the *35S:CKX1* and *35S:CKX2* transgenic lines with systemic CK deficiency and the corresponding wild type where inoculated or not with the AM fungus *Rhizophagus irregularis* strain RI or strain FM. Plants were grown in a glasshouse for eight weeks and sampled to determine the root and shoot dry weight (DW) and number of flowers. Three-way ANOVAs with the categorical factors tobacco lines, *R. irregularis* RI, and *R. irregularis* FM were used to determine the significant levels of *F* statistics. For the mean values see Fig. V.2. df, degrees of freedom.

Factors		Root DW (g)		Shoot DW (g)		Number of flowers	
	df	*F*	*P*	*F*	*P*	*F*	*P*
Tobacco line (T)	3	**10.8**	***	**132.2**	***	**59.8**	***
R. irregularis RI	1	**18.1**	***	**23.0**	***	**29.2**	***
R. irregularis FM	1	2.6		**8.7**	**	1.0	
T x RI	3	**3.3**	*	**2.7**	*	**3.6**	*
T x FM	3	*2.6*	(*)	**20.3**	***	**15.7**	***
RI x FM	1	1.7		**5.2**	*	0.1	
T x RI x FM	3	*2.5*	(*)	**8.6**	***	**19.4**	***
Residuals	144						

For $P < 0.05$, 0.01 and 0.001, significance levels of *F* values are presented as *, ** and ***, respectively, and are in bold. *F* values accompanied by (*) are marginally non-significant and are in italic. $n = 10$.

Content of phosphorus, nitrogen and carbon in shoots

The relative availability of P, N and C plays a major influence on plant growth responses to AM fungi (Johnson, 2010). We therefore determined the P, N and C content in shoots of AM plants and compared it with that of the NAM counterparts. *R. irregularis* strain RI increased the concentration of P in WT shoots significantly more than strain FM, but the co-inoculation with RI and FM had no significant effect (Fig. V.3A; Table V.3). Thus, only RI enhanced the total P content in shoots of WT plants (Fig. V.3B; Table V.3). Although the concentration of P in shoots was increased by co-inoculation in both *W6:CKX1* and *35S:CKX2* and by RI in *35S:CKX2* (Fig. V.3A), these increases were associated with an unchanged total P content in shoots (Fig. V.3B).

The concentration and total content of P in shoots of *35S:CKX1* remained unaltered across fungal inoculations (Fig. V.3A and V.3B).

The concentration of N in shoots was also altered by AM fungal inoculations depending on the plant genotype (Fig. V.3C; Table V.3). Strain RI increased the concentration of N in shoots of WT and *35S:CKX2*, whereas strain FM increased only the concentration of N in shoots of *W6:CKX1* (Fig. V.3C). Co-inoculation with RI and FM suppressed the positive effect of RI on the concentration of N in shoots of WT, enhanced synergistically the concentration of N in shoots of *W6:CKX1*, and maintained the positive effects of RI on concentration of N in shoots of *35S:CKX2* (Fig. V.3C). These increases, however, were associated with an unchanged total N content in shoots (Fig. V.3D; Table V.3). As for P, the concentration and total content of N in shoots of *35S:CKX1* remained unaltered across fungal inoculations (Fig. V.3C and V.3D). Overall, the AM-mediated enhancement of these two important soil-derived nutrients was generally not associated with increased uptake, except for the P benefit provided by strain RI to WT plants (Fig. V.3A, B, C, D). Moreover, these increases were associated with AM-mediated reduction of plant growth (Fig. V.2A and V.2B; Fig. V.3A and V.3C), which suggests that neither P nor N were limiting growth factors in the present experiment.

According to Johnson (2010), when neither N nor P is limited for growth of AM plant, the fungal C demand can increase to the point where it may depress plant growth. We then questioned whether the depression of plant growth following *R. irregularis* colonization could be caused by a reduced concentration of C in shoots. Although the NAM *W6:CKX1* plants had a concentration of C in shoots comparable to that of WT, the co-inoculated strains reduced synergistically by 8 % the concentration of C in shoots of *W6:CKX1*, when compared with the NAM counterpart (Fig. V.4). The concentration of C in shoots of WT plants was increased by 17 % as compared to systemic CK-deficient plants (*35S:CKX1* and *35S:CKX2*) and was not affected by AM fungal inoculations in none of these genotypes (Fig. V.4). Taken together, our results suggest that C may have been a limiting growth factor for AM plants with lowered CK content in roots. Thus, normal CK levels in roots appear to be important for plants in order to compete with the fungal C sink when the CK levels in shoots are also normal.

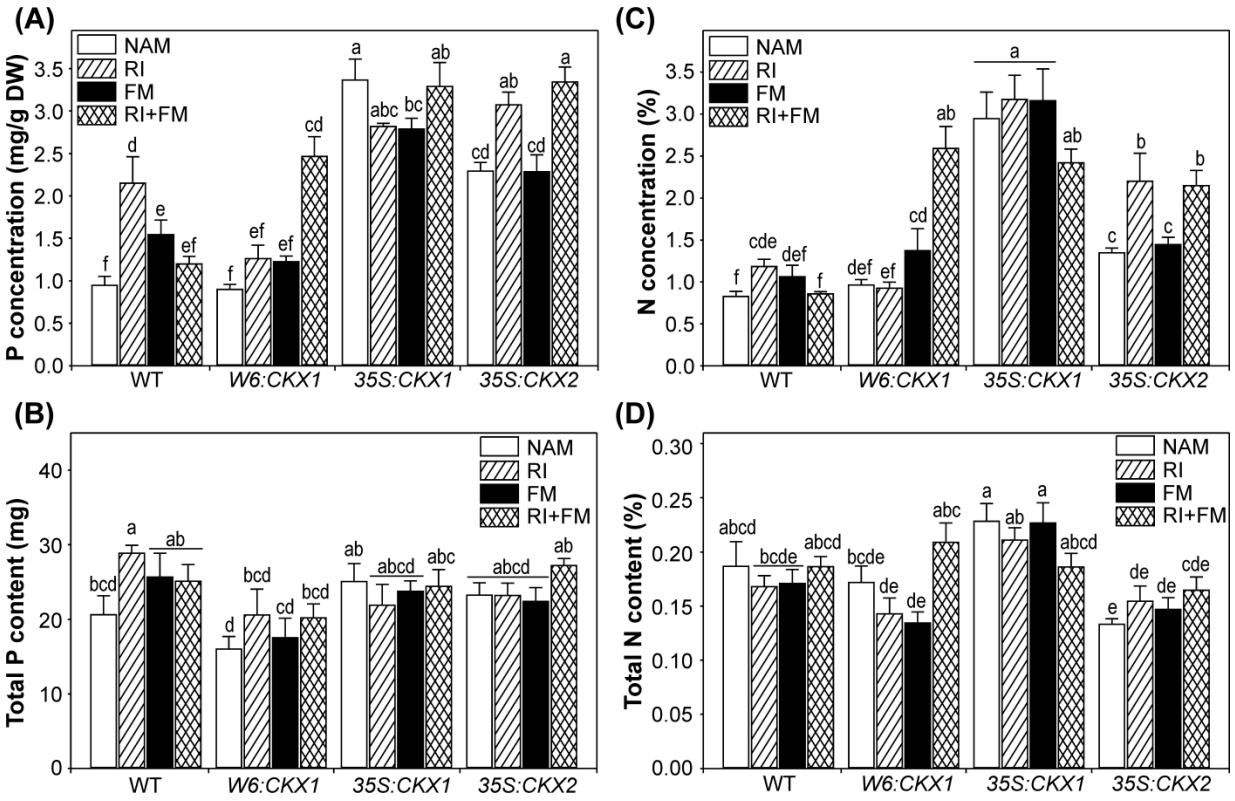

Figure V.3. *Influence of AM inoculation on phosphorus and nitrogen content of tobacco plants*. The concentration (A) and total content (B) of phosphorus (P) and nitrogen (N)(C, D) was measured in shoots of eight-week-old tobacco wild type (WT) plants and transgenic lines with root-specific (*W6:CKX1*) or systemic (*35S:CKX1* and *35S:CKX2*) cytokinin deficiency following inoculation with *R. irregularis* strain RI, FM or their co-inoculation (RI+FM) and compared to the respective non-arbuscular mycorrhizal (NAM) control plants. Values are means + SE. $n = 10$ for N and $n = 5$ for P. For each parameter, bars with similar letters are not significantly different ($P < 0.05$) according to Duncan's multiple range test.

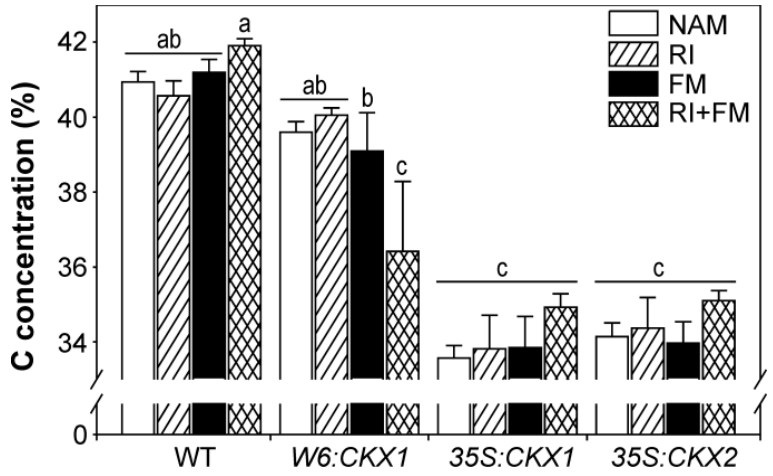

Figure V.4. *Influence of AM inoculation on carbon concentration in shoots of tobacco plants*. The concentration of carbon (C) was measured in shoots of eight-week-old tobacco wild type (WT) plants and transgenic lines with root-specific (*W6:CKX1*) or systemic (*35S:CKX1* and *35S:CKX2*) cytokinin deficiency following inoculation with *R. irregularis* strain RI, FM or their co-inoculation (RI+FM) and compared to the respective non-arbuscular mycorrhizal (NAM) control plants. Values are means + SE. $n = 10$. For each parameter, bars with similar letters are not significantly different ($P < 0.05$) according to Duncan's multiple range test.

Table V.3. *Content of phosphorus, nitrogen and carbon in shoots*. Tobacco line *W6:CKX1* with root-specific CK-deficiency, the *35S:CKX1* and *35S:CKX2* transgenic lines with systemic CK deficiency and their corresponding wild type where inoculated or not with the AM fungus *Rhizophagus irregularis* strain RI or strain FM. Plants were grown in a glasshouse for eight weeks and sampled to determine the concentration and total content of phosphorus (P) and nitrogen (N) as well as the concentration of carbon (C) in dry shoots. Three-way ANOVAs with the categorical factors tobacco lines, *R. irregularis* RI, and *R. irregularis* FM were used to determine the significant levels of *F* statistics. For the mean values see Fig. V.3 for N and P and Fig. V.4 for C. df, degrees of freedom.

Factors	P concentration			P total content			N concentration			N total content			C concentration		
	df	*F*	*P*	df	*F*	*P*	*F*	*P*		*F*	*P*		*F*	*P*	
Tobacco line (T)	3	**104.5**	***	3	**7.0**	***	**111.5**	***		**12.2**	***		**69.7**	***	
R. irregularis RI	1	**42.2**	***	1	*4.0*	(*)	**19.0**	***		0.0			0.0		
R. irregularis FM	1	**5.5**	*	1	0.6		**9.0**	**		0.2			0.1		
T x RI	3	**6.0**	**	3	1.2		**6.2**	***		*2.6*	(*)		1.4		
T x FM	3	**7.4**	***	3	0.1		**14.9**	***		0.7			**3.3**	*	
RI x FM	1	0.0		1	0.1		0.1			*3.8*	(*)		0.0		
T x RI x FM	3	**13.8**	***	3	2.0		**10.0**	***		**3.8**	*		1.9		
Residuals	64			144											

For $P < 0.05$, 0.01 and 0.001, significance levels of *F* values are presented as *, ** and ***, respectively, and are in bold. *F* values accompanied by (*) are marginally non-significant and are in italic. $n = 5$ for P and $n = 10$ for N and C.

Root transcript levels of phosphate transporter genes

AM fungi are known to induce in roots a higher transcript level of specific phosphate (Pi)-transporter (*PT*) genes that regulate the mycorrhizal pathway for Pi supply inside the arbusculated cortex cells (Chen *et al.*, 2007). Among these, *PT4* is considered to be one of the best indicators of a functional AM association (Helber *et al.*, 2011). At the same time, the *PT* genes that regulate the direct pathway for Pi uptake via root hairs and epidermis may be suppressed by AM fungi (Chen *et al.*, 2007). To test whether plant CK homeostasis affects the impact of *R. irregularis* on the transcript levels of *PT* genes in the roots, we determined the relative transcription levels of *NtPT4* and *NtPT1* that regulate the mycorrhizal and direct pathway for Pi uptake in tobacco, respectively (Chen *et al.*, 2007). The results confirm that *R. irregularis* induces a higher transcript level of *NtPT4*, which was increased up to 6000-fold upon fungal inoculation (Fig. V.5A; Table V.4). This induction did not differ significantly among *R. irregularis* strains or their co-inoculation across the different plant lines (Fig. V.5A). However, *35S:CKX1* expression reduced by approximately 80 % the induction of *NtPT4* transcripts in roots following *R. irregularis* colonization compared with the induction noted in WT, *W6:CKX1*, and *35S:CKX2* plants (Fig. V.5A). The relative transcript levels of *NtPT1* did not differ among the plant lines neither were they strongly affected by *R. irregularis* colonization, as the transcript levels varied only between 0.5 and 1.5-fold (Fig. V.5B). Overall, the AM fungus-induced transcription of *NtPT4* was not altered by the root CK status but was dramatically reduced by a strongly CK-deficient shoot phenotype. Furthermore, we found no strong evidence for a suppressed direct Pi uptake pathway following AM fungal colonization.

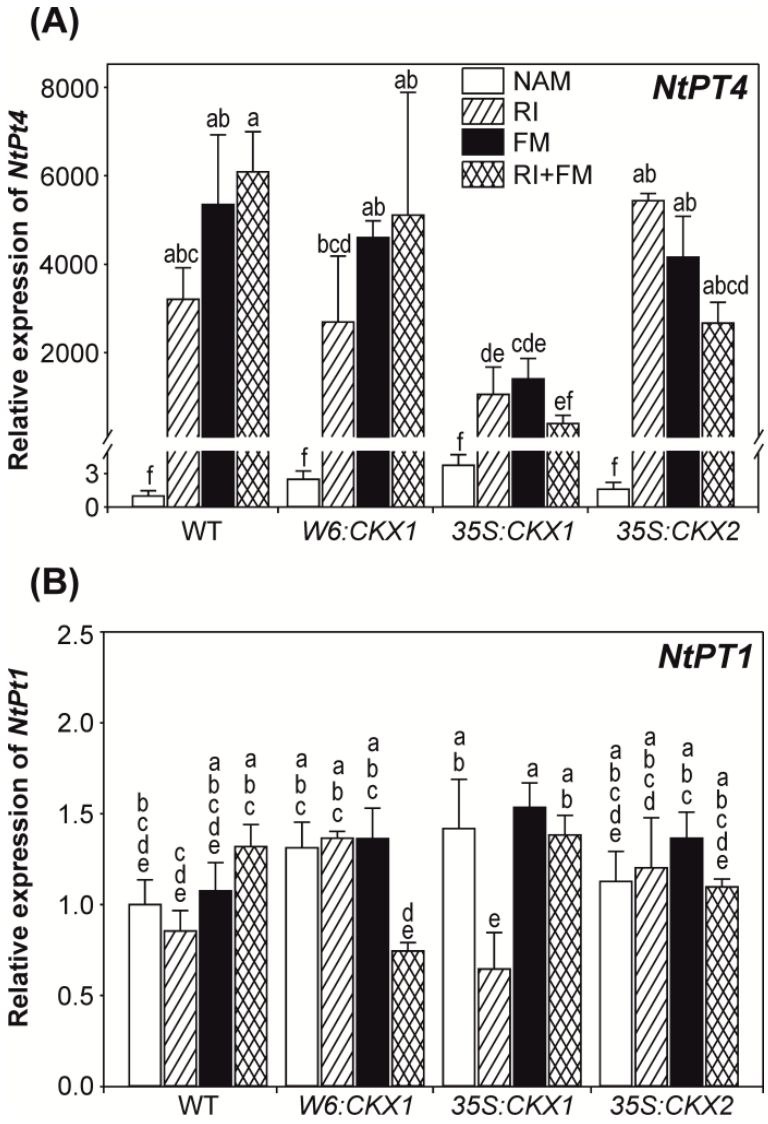

Figure V.5. *Influence of AM inoculation on the expression of phosphate transporter genes in tobacco.* Steady state mRNA levels of the phosphate transporter genes *NtPT4* (A) and *NtPT1* (B) was measured in roots of eight-week-old tobacco wild type (WT) plants and transgenic lines with root-specific (*W6:CKX1*) or systemic (*35S:CKX1* and *35S:CKX2*) cytokinin deficiency following inoculation with *R. irregularis* strain RI, FM or their co-inoculation (RI+FM) and compared to the respective non-arbuscular mycorrhizal (NAM) control plants. Values are means + SE. $n = 3$. Each biological replicate contained roots from at least three individual plants. In both cases the expression level of WT in non-AM controls was set to 1. For each parameter, bars with similar letters are not significantly different ($P < 0.05$) according to Duncan's multiple range test.

Table V.4. *Root transcript levels of phosphate transporter genes*. Tobacco line *W6:CKX1* with root-specific CK-deficiency, the *35S:CKX1* and *35S:CKX2* transgenic lines with systemic CK deficiency and their corresponding wild type where inoculated or not with the AM fungus *Rhizophagus irregularis* strain RI or strain FM. Plants were grown in a glasshouse for eight weeks and sampled to determine the relative expression levels of *NtPT4* and *NtPT1* using quantitative real-time PCR (qRT-PCR) analyses. Three-way ANOVAs with the categorical factors tobacco lines, *R. irregularis* RI, and *R. irregularis* FM were used to determine the significant levels of *F* statistics. For the mean values see Fig. 5. df, degrees of freedom.

Factors		*NtPT4*		*NtPT1*	
	df	*F*	*P*	*F*	*P*
Tobacco line (T)	3	**11.9**	***	1.2	
R. irregularis RI	1	**22.4**	***	**7.9**	**
R. irregularis FM	1	**55.3**	***	2.6	
T x RI	3	1.9		2.2	
T x FM	3	**4.0**	*	**4.4**	*
RI x FM	1	**43.6**	***	0.0	
T x RI x FM	3	1.2		**4.2**	*
Residuals	30				

For $P < 0.05$, 0.01 and 0.001, significance levels of *F* values are presented as *, ** and ***, respectively, and are in bold. *F* values accompanied by (*) are marginally non-significant and are in italic. $n = 3$.

Discussion

AM symbiosis is functionally important for plant nutrition, productivity and fitness, and plants can regulate their symbiotic interaction with AM fungi. However, the regulatory role of CK is poorly understood although it has been often reported that AM plants generally have enhanced CK levels in both roots and shoots (Allen *et al.*, 1980; Baas & Kuiper, 1989; Drüge & Schonbeck, 1992; Danneberg *et al.*, 1993; van Rhijn *et al.*, 1997; Barker & Tagu, 2000; Torelli *et al.*, 2000; Shaul-Keinan *et al.*, 2002; Yao *et al.*, 2005). In tobacco, P supply alone had similar positive effects on the CK metabolite levels in leaves as *R. irregularis*, while in roots the fungus induced specifically zeatin riboside and increased by 16-fold the concentration of isopentenyl adenosine as compared with P supply (Shaul-Keinan *et al.*, 2002). This suggests a high degree of AM fungal specificity in increasing the CK levels in colonized roots. As a lowered CK status in tobacco may enhance *R. irregularis* hyphal colonization (Cosme & Wurst, 2013), it is plausible that increased levels of CK in roots may negatively feedback on further AM hyphal colonization. In the present study, a low systemic CK status in *35S:CKX1* plants reduced *R. irregularis* colonization. However, this reduction was not observed in the root-specific CK-deficient *W6:CKX1* plants, which suggests that the reduced colonization in *35S:CKX1* may have been caused by the lowered CK levels in shoots and not in roots. Although we observed strong plant-fungus interactions, possibly caused by specificities of plant and fungal genotypes, the use of different transgenic tobacco lines, *R. irregularis* strains and their co-inoculation unveiled several consistent functions of root CK: CK prevented root growth depression following *R. irregularis* colonization, restricted synergistic depression of plant growth and fitness as well as on C demand following strain co-inoculation, and secured a P benefit when the symbiotic cost on shoot growth and plant fitness was strong. Thus, contrary to the speculation that CK does not influence AM symbiosis (Foo *et al.*, 2013), our study provides genetic evidence that root CK is involved in regulating the AM symbiotic function and that this regulatory role is influenced by the shoot CK status.

The comparison of *R. irregularis* colonization in *W6:CKX1*, *35S:CKX1* and WT plants revealed a positive impact of shoot CK on AM symbiosis. This positive impact became apparent through the reduced AM colonization success in *35S:CKX1* plants which differed from the two other genotypes by a strongly reduced CK status of the shoot. The difference in AM colonization may

be caused by a lower sugar availability derived from source leaves, as *35S:CKX1* leaves have a 30 % reduction in sugar content compared with the WT (Werner *et al.*, 2008). In addition, *35S:CKX1* plants accumulate 80 % more starch in sink leaves than the WT (Werner *et al.*, 2008), which represent an aboveground sink that may contribute to restrict further the fungal access to C in roots, and consequently reduce the AM symbiotic development, i.e. decrease hyphal and arbuscules colonization and *NtPT4* transcription. Consistently, this C restriction protected *35S:CKX1* roots against fungal parasitism. The root transcription of *PT4* is specifically induced in cortex cells during arbuscule formation to equip the membranes for Pi transport (Chen *et al.*, 2007; Franken *et al.*, 2007) and was reported to require the transcription of an AM fungal monosaccharide transporter which in turn is induced by sugar availability (Helber et al., 2011). Thus, an indirect negative effect of a low shoot CK status on root *NtPT4* transcription seems very likely, i.e. a reduced source of sugars in *35S:CKX1* plants could limit the *NtPT4* transcription in response to AM colonization, reducing the AM pathway for P uptake (Fig. 6). Taken together, our study suggests that the general positive effects of AM fungi on shoot CK (e.g. Allen *et al.*, 1980) may positively feedback on the functioning of AM symbiosis, possibly through an enhanced source of C.

The CK-deficient roots in *W6:CKX1* and *35S:CKX2* supported higher AM fungal colonization than *35S:CKX1* and were susceptible to AM-mediated root growth depression, while the WT was not. Yet, the induction of *NtPT4* transcripts was not significantly affected by root CK, as similar induction levels were observed in WT, *W6:CKX1* and *35S:CKX2*. Furthermore, the plant uptake of P and N was not limited by the AM-mediated root growth depression. A remarkable and surprising result was the genotype-specific effect of fungal co-inoculation on plant growth. Co-inoculation with RI and FM neutralized their negative effects on shoot biomass formation of WT. However, it led to a synergistic reduction of the shoot and root biomass and C concentration in shoots of *W6:CKX1*, which suggests that a low CK status in roots may facilitate fungal acquisition of C independently of fungal P supply (Fig. 6). This might explain our previous results showing enhanced AM hyphal colonization without altered arbuscule formation in *35S:CKX2* plants (Cosme & Wurst, 2013). In line with this argument, several lines of evidence suggest that the intraradical hyphae may actively acquire C in roots independently of arbuscule formation: i) the extraradical mycelium begins to grow as soon as the intercellular hyphae colonize the root cortex and before the arbuscules are formed (Mosse & Hepper, 1975); ii) the

hyphae have superior longevity and are continuously connected to the external mycelium even when the arbuscules decline (Smith & Read, 2008); iii) the membranes of the intercellular hyphae have a high ATPase activity and therefore are energized for active transport of sugars (Harrison, 1999); iv) plant mutants with constitutive GA signaling and GA-treated roots were extensively colonized by intercellular hyphae without the presence of arbuscules (Floss *et al.*, 2013); and v) the expression of a fungal monosaccharide transporter gene in intercellular AM hyphae indicates that these are important sites of fungal assimilation of sugars (Helber *et al.*, 2011). Moreover, although CK-deficient roots have increased root growth rates (Werner *et al.*, 2001; Werner *et al.*, 2010), their sugar content was reduced, presumably because of rapid metabolic utilization due to the increased growth rate (Werner *et al.*, 2008). This further implies that transfer of sugars from the plant to the AM fungus in CK-deficient roots is most likely mediated by active hyphal transport, with an AM fungal sink competing effectively with the sink systems of the host plant. Thus, an AM-specific increase of CK levels in tobacco roots (Shaul-Keinan *et al.*, 2002) might be involved in enhancing the C sink capacity of roots in relation to that of the fungus, which in turn may limit hyphal proliferation in roots (Cosme & Wurst, 2013) or avert fungal parasitism.

The relation between CK and growth of AM plants has been so far unclear. A strong correlation between increased CK levels and improved photosynthesis and growth of AM plants led to the hypothesis that CK is part of the positive AM effect on plant performance (Allen *et al.*, 1980; Drüge & Schonbeck, 1992). However, this hypothesis has been disputed as a correlation was not always observed (Baas & Kuiper, 1989; Danneberg *et al.*, 1993). Furthermore, AM fungi do not always increase simultaneously the CK levels in shoots and roots and may even reduce it in roots under specific conditions. The CK levels might decrease in AM roots under high P amendment (Torelli *et al.*, 2000) or temporarily during early colonization (Drüge & Schonbeck, 1992), which is often associated with plant growth depression (Smith & Read, 2008; Johnson, 2010). Neutral growth responses in AM plants have been associated with elevated root CK levels combined with small or no changes in shoot CK content (Baas & Kuiper, 1989; Danneberg *et al.*, 1993; Shaul-Keinan *et al.*, 2002). A stronger increase of the shoot CK content accompanied by elevated root CK levels could be causally involved in a positive plant growth response to AM symbiosis (Allen *et al.*, 1980; Drüge & Schonbeck, 1992; Yao *et al.*, 2005). A comparison between *in vitro* root organ cultures and *in planta* experiments suggested that auxin regulation of

AM colonization is shoot-dependent (Hanlon & Coenen, 2011). An organ-dependent phytohormone regulation of AM symbiosis is corroborated *in planta* by the CK effects observed in our study, in which increased shoot CK levels enhance AM symbiotic functioning in roots while increased levels of CK in roots enhance the C sink capacity of roots in relation to that of the fungus. Together this is likely to contribute to a balanced C for P exchange between symbionts (Fig. 6) and leads potentially to an enhanced growth of AM plants (Johnson, 2010). Although it remains to be determined how other phytohormones may interact with CK to regulate AM symbiosis, overall our study adds to the evidence that the phytohormone regulation of plant-supplied C and fungal-supplied P may be uncoupled (Floss *et al.*, 2013) and clearly demonstrates that plant CK status can affect profoundly the growth and fitness of a higher plant in response to an ubiquitous AM fungus.

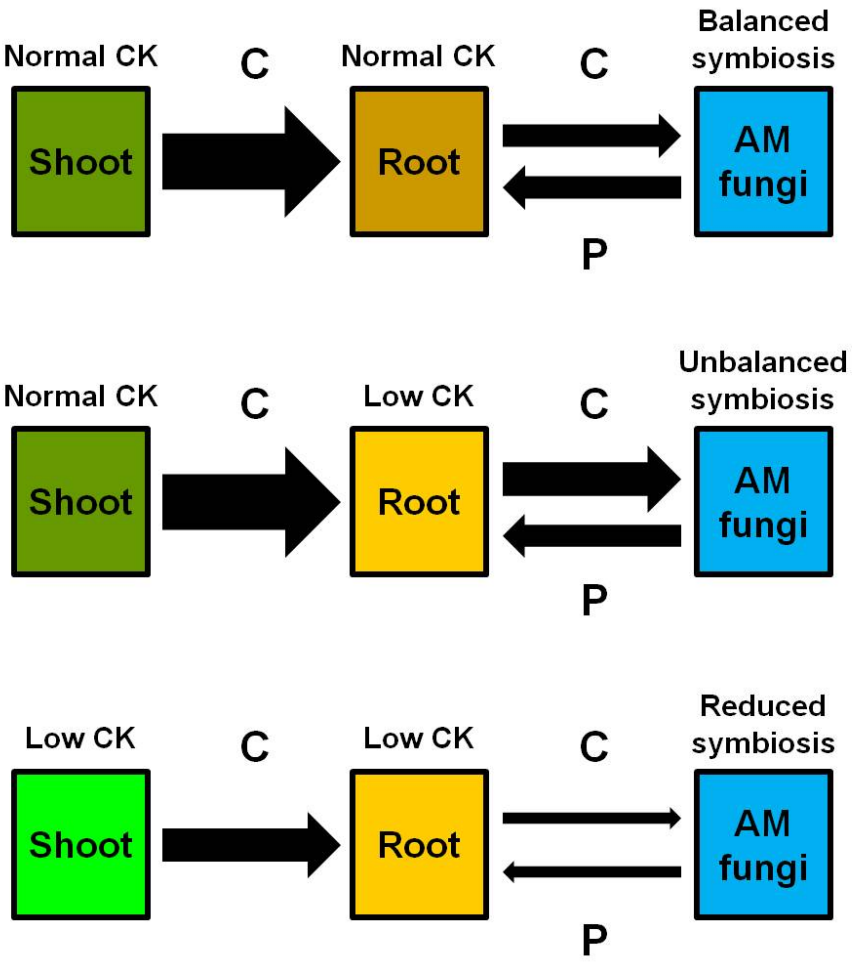

Figure V.6. *Proposed model for the regulation of bidirectional exchange of carbon and phosphorus in AM symbiosis by cytokinin.* A normal CK status in shoots and in roots contributes to balance the bidirectional flow of carbon (C) and phosphorus (P) between symbionts (balanced symbiosis), which is required for a greater potential growth of AM plants (Johnson, 2010). A normal CK status of the shoots combined with reduced CK status in the roots maintain a strong source of C from the shoots into the roots but may reduce the sink capacity of the roots in relation to that of the AM fungi, irrespective of P supply, causing an unbalanced C for P exchange between symbionts (unbalanced symbiosis), which can lead to fungal parasitism and reduced plant growth (Johnson, 2010). A strongly reduced CK status of the shoots negatively regulates the source of C from the shoots by reducing the availability of sugars (Werner *et al.*, 2008), which may be causally involved in reducing the AM pathway for P uptake (Nouri *et al.*, 2014), irrespective of the root CK status (reduced symbiosis). Arrow thickness illustrates the relative flow strength of C or P.

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MC dedicates this article to Michael F. Allen and Martha Christensen who provided the first demonstration of different cytokinin content in arbuscular mycorrhizal (AM) versus non-AM plants more than thirty five years ago. We are thankful to Kerstin Fischer, Monika Fünning, Kerstin Schmidt, and Dominic Schmitz for technical help. MC was supported by the doctoral grant SFRH/BD/81785/2011 from Fundação para a Ciência e a Tecnologia, Portugal.

Supplementary information to Chapter V

Table V.S1. Primers used for quantitative real-time RT-PCR.

Gene name	Gene bank accession No.	Primer sequence
NtEF-1α	AF120093	5'- TGAGATGCACCACGAAGCTC -3' 5'- CCAACATTGTCACCAGGAAGTG -3'
NtL25	L18908	5'- CCCCTCACCACAGAGTCTGC -3' 5'- AAGGGTGTTGTTGTCCTCAATCTT -3'
NtPT1	AB020061	5'- AGCGTTCATTGCTGCTGTTT -3' 5'- AGAGCGTCGGCATGATATGT -3'
NtPT4	EF091672	5'- GTCAACTCGTGGGGCGTTTAT -3' 5'- CTCAGGCTCCGTGGACAAAAT -3'

Chapter VI: General discussion

The global human population has increased exponentially in recent years and brought major challenges to the human food supply, whereas modern intensification of crop production was followed by several environmental problems and still left many people hungry or malnourished. Although the goals of modern agriculture have been traditionally the enhancement of crop yields, a more sustainable production and a higher nutritional value of plant foods have emerged as the new agricultural paradigms. These paradigms are indirectly linked to the effectiveness of crop roots to overcome mineral nutrient and water limitations in soils, and a part of the solution to enhance yields and nutritional value of crop foods in a more sustainable manner might be found in the overlooked rhizosphere. Terrestrial plants have co-evolved with belowground mutualistic microbes that perform important ecological functions in the rhizosphere and whose complex interactions are far from being fully understood. These microbes can also interact actively with intrinsic developmental and physiological pathways of their host plants, which in turn can mediate not only the plant-microbe mutualism but also the plant interaction with other important ecological factors.

The present thesis had two main objectives as identified in the introduction: 1) to test novel effects of beneficial microbes on crop plants related with new agricultural paradigms; and 2) to investigate the role of intrinsic plant regulators involved in microbial effects on crop plants. In the **chapter II,** I have focused on the effects of AM fungal colonization on the aboveground oviposition on rice plants by RWW, an important global pest of rice. In the **chapter III**, I have tested the defensive effect of a Sebacinalean root endophyte against RWW attacks on rice, and demonstrated the intrinsic phytohormonal regulators involved in this defensive effect. To explore the potential of AM fungi as helper in the fight against micronutrient malnutrition, in the **chapter IV** I have focused on the AM fungal effects on the nutraceutical value of edible leaves of *M. oleifera*. Finally, in **chapter V**, I demonstrated how the root and shoot CK status regulate the AM symbiotic functioning in tobacco.

Chapter II

The study presented in chapter II shows that the AM fungus *R. irregularis* can increase the aboveground oviposition by rice water weevil (RWW) females, whose subsequent larval offspring feed on roots. The AM fungus-inoculated rice plants preferred for oviposition had higher concentrations of N and P in shoots and of N in roots than the non-AM control plants, suggesting that the female oviposition choice of RWW may be affected by the nutritional status of the plants. Positive effects of AM fungi on the survival of the root-feeding larvae of clover root weevil were associated with an AM fungus-mediated increase in N concentration of the whole plant (Currie *et al.*, 2011). This suggests that the oviposition preference of RWW females may be potentially related to a better performance of the root feeding larvae. However, further investigation is required to elucidate the effects of AM fungi and plant N status on the larval performance of RWW.

The aboveground feeding of RWW adults, measured as consumed leaf area per plant, was not affected by *G. intraradices*. Gange (2001) reported negative effects of different magnitude of *F. mosseae* and *R. fasciculatum* on the leaf area consumed on strawberry plants by black vine weevil adults, whose larvae are also root-feeders. The inconsistency between studies may be due to the host plant–AM fungal species specificity (Dhillion, 1992; Klironomos, 2003). Furthermore, as observed in previous studies (Stout & Riggio, 2002; Stout *et al.*, 2002; Tindall & Stout, 2003), the adult of RWW might be more tolerant to changes in plant quality for feeding than for oviposition.

The ability of insect females to adjust oviposition behavior depending on plant AM status is a novel aspect of below- and aboveground ecological interactions. The results also indicate that belowground AM colonization may decrease rice plant resistance against an important insect pest. In a field study conducted at the Rice Research Station Agricultural Center of the Louisiana State University (USA), in collaboration with Prof. Dr. Michael J Stout, the inoculation of rice plots led to an increase in numbers of RWW larvae compared with that of uninoculated rice plots (unpublished data), supporting the hypothesis that AM fungal inoculation decrease the resistance of rice plant against RWW. These results encouraged me to focus on other belowground plant-

fungus mutualism to investigate potential defensive effects, which became the aim of the next chapter.

Chapter III

The study presented in chapter III demonstrates that plant-herbivore interactions are affected by *P. indica*, a model endophyte with agronomic potential (Qiang *et al.*, 2012). *P. indica* enhanced rice defense to herbivory mediated by induced root tolerance, i.e. endophyte-colonized plants infested with RWW larvae gained more shoot biomass, tillers, root biomass and total root length compared with plants infested with larvae without *P. indica*, but the root resistance measured as larval survival and growth was not affected by the endophyte. Therefore, Sebacinalean root endophytes, in addition to protecting plants against root and shoot pathogens and salt stress (Waller *et al.*, 2005), can improve plant defense against root herbivores.

An apparent synergistic positive effect of RWW adults and larvae on induced JA in roots was associated with a significant additive negative effect on total root length accompanied by similar negative effects on root biomass, shoot biomass and number of tillers, while the negative effect of RWW on WT roots was not detected in *coi1-18* roots. Taken together, this suggests that RWW effects on plant growth were mediated by induced JA signaling in roots. Two key mechanisms leading to JA-mediated growth inhibition are the JA-modulation of GA biosynthesis and the JAZ interference with the interaction between DELLAs and growth-promoting PIF transcription factors (Yang *et al.*, 2012; Heinrich *et al.*, 2013; Matschi *et al.*, 2015). In the study presented in chapter III, *P. indica*-induced GA signaling was required to establish a mutualistic association with rice, while the stronger plant growth inhibition of *Eui1-OX* plants due to root herbivory suggests that negative effects of RWW larvae on plant growth are counteracted by GA signaling in WT. Taken together, as illustrated by the proposed model presented in Fig. III.5, an enhanced GA signaling and suppressed JA signaling in roots is one mechanism by which *P. indica* induces plant tolerance to RWW. This study appears to be the first showing the impact of a root endophyte on plant defense against belowground herbivory.

Chapter IV

The study present in chapter IV appears to be the first evidence for systemic effects of AM fungi on glucosinolates in aboveground plant tissues, which are often consumed as vegetables. To date, only two studies have shown that AM fungi can enhance the levels of glucosinolates in roots of *Tropaeolum* sp. in a non-species-specific manner (Vierheilig *et al.*, 2000; Ludwig-Müller *et al.*, 2002). Consistently, in the study presented in chapter IV, the systemic effects of AM fungal colonization of roots on the levels of glucosinolates in leaves of *M. oleifera* were not specifically dependent on *R. intraradices*, *F. mosseae*, or their combination.

Several carotenoids are bioactive against chronic diseases in humans when consumed as part of a diet (Baldermann *et al.*, 2013). All the measured carotenoids in *M. oleifera* leaves, including the important β-carotene (pro-vitamin A) targeted for biofortification (Mayer *et al.*, 2008), were significantly reduced by AM fungal colonization of roots (only lutein showed a marginal reduction by *R. intraradices*). *F. mosseae* had stronger negative impacts than *R. irregularis* on all carotenoids, which suggest these effects were species-specific. These results contrast with others showing positive effects of AM fungi on carotenoids in crop plants (Krishna *et al.*, 2005; Mena-Violante *et al.*, 2006; Farmer *et al.*, 2007; Baslam *et al.*, 2011a; Baslam *et al.*, 2013; Tong *et al.*, 2013). Also contrary to these studies, in chapter IV the plant growth was not enhanced by AM fungi, and the negative effects of AM fungal colonization on carotenoid levels could be related with a redundant fungal sink for sugars, which combined with reduced levels of chlorophylls (data not shown) could have restricted carotenoid biosynthesis in leaves.

Zn and Cu levels in *M. oleifera* leaves were enhanced by AM fungi, which are a well documented effects (Marschner, 1995). The lack of these mineral elements is an important cause of micronutrient malnutrition in humans (Mayer *et al.*, 2008; White & Broadley, 2009). Interestingly, Zn in *M. oleifera* leaves was only increased by the co-inoculation with *R. intraradices* and *F. mosseae*, but not when each fungal species was inoculated alone, which suggests a functional complementarity among species in term of enhanced Zn in leaves. Overall, the study presented in chapter IV encourages research on other AM fungi and their combinations to achieve general benefits on bioactive compounds in edible tissues of *M. oleifera*.

Chapter V

In study presented in chapter V, a low systemic CK status in *35S:CKX1* tobacco plants reduced *R. irregularis* colonization and induction of *NtPT4* transcription. However, this reduction was not observed in the root-specific CK-deficient *W6:CKX1* plants, which suggests that the reduced colonization in *35S:CKX1* was caused by the lowered CK levels in shoots and not in roots. Moreover, the use of different transgenic tobacco lines, *R. irregularis* strains and their co-inoculation unveiled several consistent functions of root CK: CK prevented root growth depression following *R. irregularis* colonization, restricted synergistic depression of plant growth and fitness as well as on C demand following strain co-inoculation, and secured a P benefit when the symbiotic cost on shoot growth and plant fitness was strong.

The negative effects of lower shoot CK levels on hyphal and arbuscules colonization and *NtPT4* transcription may be caused by a lower sugar availability derived from source leaves, as *35S:CKX1* leaves have a 30 % reduction in sugar content compared with the WT (Werner *et al.*, 2008), while the root transcription of *PT4* was reported to require the transcription of an AM fungal monosaccharide transporter which in turn is induced by sugar availability (Helber et al., 2011). Consistently, this sugar restriction protected *35S:CKX1* roots against fungal parasitism. Hence, these results suggests that the general positive effects of AM fungi on shoot CK (e.g. Allen *et al.*, 1980) may positively feedback on the functioning of AM symbiosis, possibly through an enhanced source of C.

The CK-deficient roots in *W6:CKX1* and *35S:CKX2* supported higher AM fungal colonization than *35S:CKX1* and were susceptible to AM-mediated root growth depression, while the WT was not. Yet, the induction of *NtPT4* transcripts was not significantly affected by root CK. Co-inoculation with RI and FM neutralized their negative effects on shoot biomass formation of WT. However, it led to a synergistic reduction of the shoot and root biomass and C concentration in shoots of *W6:CKX1*, which suggests that a low CK status in roots may facilitate fungal acquisition of C independently of fungal P supply. Several lines of evidence suggest that the intraradical hyphae may actively acquire C in roots independently of arbuscules formation (Mosse & Hepper, 1975; Harrison, 1999; Smith & Read, 2008; Helber *et al.*, 2011; Floss *et al.*, 2013). As CK-deficient roots have lower sugar contents (Werner *et al.*, 2008), this implies that

transfer of sugars from the plant to the AM fungus in CK-deficient roots is most likely mediated by active hyphal transport, with an AM fungal sink competing effectively with the sink systems of the host plant. Thus, an AM-specific increase of CK levels in tobacco roots (Shaul-Keinan *et al.*, 2002) might be involved in enhancing the C sink capacity of roots in relation to that of the fungus.

A strong correlation between increased CK levels and improved photosynthesis and growth of AM plants led to the hypothesis that CK is part of the positive AM effect on plant performance (Allen *et al.*, 1980; Drüge & Schonbeck, 1992). However, this hypothesis has been disputed as a correlation was not always observed (Baas & Kuiper, 1989; Danneberg *et al.*, 1993). Moreover, the CK levels might be decreased in AM roots under high P amendment (Torelli *et al.*, 2000) or temporarily during early colonization (Drüge & Schonbeck, 1992), which is often associated with plant growth depression (Smith & Read, 2008; Johnson, 2010). Neutral growth responses in AM plants have been associated with elevated root CK levels combined with small or no changes in shoot CK content (Baas & Kuiper, 1989; Danneberg *et al.*, 1993; Shaul-Keinan *et al.*, 2002). The study presented in chapter V proposes a model (Fig. V.6) where the CK effects on AM symbiosis are organ-specific, in which increased shoot CK levels enhance AM symbiotic functioning in roots while increased levels of CK in roots enhance the C sink capacity of roots in relation to that of the fungus. Together this is likely to contribute to a balanced C for P exchange between symbionts and potentially lead to an enhanced growth of AM plants (Johnson, 2010).

Synthesis

In order to address the two main objects of my thesis, I started by testing the effects of two model plant-microbe mutualisms on the interaction between rice plants and RWW (**chapter II and III**). Rice is one of the major staple foods providing 20 % of the energy intakes by the global human population. The RWW is a belowground pest of rice present in the biggest rice producing regions of the world (Stout *et al.*, 2002) and poses a threat to food security. Past management programs for RWW relied almost exclusively on insecticides, some of which were banned due to health- and environmental-related concerns (Stout *et al.*, 2001), creating a need for new management practices. In a greenhouse study, I found that AM fungi can enhanced the aboveground oviposition by RWW, which is a novel aspect of agro-ecological interactions. This

suggests that AM colonization may decrease rice resistance against RWW, which was supported by a field study conducted in collaboration with Prof. Dr. Michael J Stout. This support the notion that soil fungi that are generally considered beneficial in terms of nutrient uptake may not be beneficial in respect to anti-herbivore protection. In contrast, the Sebacinalean root endophyte *P. indica* could enhance rice defense to herbivory by RWW, mediated by induced root tolerance and without affecting root resistance. Using a set of laboratory experiments, I provided evidence that the endophyte-induced root tolerance was mediated by induction of GA signaling and suppression of JA signaling. Overall, belowground plant-microbe mutualisms can affect dramatically the interaction between a globally important crop plant and an important insect pest. These effects are at least partially mediated by plant intrinsic regulators and should be considered in future management practices. Next, I tested the impacts of AM fungi on the nutraceutical properties of edible leaves of *M. oleifera* (**chapter IV**). The enhancement of the nutritional value of plant foods is instrumental to combat diet-related diseases affecting the health of many people around the globe (Welch & Graham, 1999; Mayer *et al.*, 2008; Antunes *et al.*, 2012). The high nutritional value and the adaptability of *M. oleifera* to the climate of some of the most affected regions have generated research interest in this crop. In a pot experiment, I found that AM fungi can affect the levels of important bioactive compounds and mineral elements in leaves of *M. oleifera*. The non-specific positive effects of AM fungi on glucosinolates and their species-specific negative effects on carotenoids encourage research on other AM fungal species and their combinations to achieve general benefits on bioactive compounds in *M. oleifera*. Finally, I tested the role of root and shoot CK status on AM symbiosis (**chapter V**). CK is an important intrinsic regulator of plant growth and development (Werner & Schmülling, 2009), and AM plants generally have increased CK levels in roots and shoots (Allen *et al.*, 1980; Barker & Tagu, 2000; Shaul-Keinan *et al.*, 2002). I observed that organ-specific CK status can affect profoundly the plant performance in response to AM symbiosis, and I proposed that CK in roots and in shoots contribute to a balanced C for P exchange between symbionts.

The results presented here provide significant contributions to the field of plant-microbe interactions and have potential application in crop management practices targeted at sustainable production and nutritional enhancement of plant foods (Mayer *et al.*, 2008; Gewin, 2010). It is widely recognized that the function of plant-microbe mutualisms, generally measured in terms of plant yield, is depends on several ecological factors including fungal genotypes (Klironomos,

2003; Kogel *et al.*, 2006; Angelard *et al.*, 2010; Johnson, 2010). The present thesis also supports this notion and adds evidence that variation depend on the functional traits of interest under evaluation, sometimes independently of the fungal genotype or plant yield. For instance, AM fungi and *P. indica* had opposite effects on rice defense against RWW, while the effects of AM fungi on *M. oleifera* quality or tobacco growth dependent on the fungal genotypes or their co-inoculations, but the same fungal genotype could have positive and negatives effects on different parameters of interest. Furthermore, AM fungi are generally recognized as beneficial microbes that can contribute to improve agriculture production (Smith, F & Smith, S, 2011; Verbruggen *et al.*, 2013). However, this thesis suggests that the incorporation of AM fungi in wetland rice might have detrimental effects if the infestation by RWW is a major constraint. By contrast, in such case, the exploitation of *P. indica* could be a positive complement to rice management strategies. Thus, both considerations could potentially contribute to reduce chemicals inputs required to control RWW. Moreover, in this thesis the phytohormones were important regulators of the microbial mutualistic functions. For instance, root CK was an important mechanism to balance the nutrient exchange in AM symbiosis, while GA was an important mechanism of microbe-induced root tolerance against herbivory. These findings provide new mechanistic bases for plant biology research and should be integrated in molecular plant breeding. This might permit in turn a better agronomical exploitation of belowground microbes and contribute to improve our ability to meet the nutritional needs of an increasing global population.

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