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SPECIAL FEATURE

PLANT-MEDIATED INTERACTIONS BETWEEN ABOVE- AND BELOW-GROUND COMMUNITIES

Elicitation of foliar resistance mechanisms transiently impairs root association with arbuscular mycorrhizal fungi

Miriam de Román[1], Iván Fernández[2], Timon Wyatt[2], Mariam Sahrawy[3], Martin Heil[1,4] and María J. Pozo[2]*

[1]*Department of Botany–Plant Ecology, University of Duisburg-Essen, Universitätsstrasse 5, 45117 Essen, Germany;* [2]*Department of Soil Microbiology and Symbiotic Systems, Estación Experimental del Zaidín, CSIC, Profesor Albareda 1, 18008 Granada, Spain;* [3]*Department of Biochemistry and Molecular and Cellular Biology of Plants, Estación Experimental del Zaidín, CSIC, Profesor Albareda 1, 18008 Granada, Spain; and* [4]*Departamento de Ingeniería Genética, CINVESTAV-Irapuato, km 9.6 Libramiento Norte, Carretera Irapuato-León, 36821 Irapuato, Guanajuato, México*

Summary

1. Plants possess numerous mechanisms to control infections by deleterious organisms. Unspecific resistance mechanisms may, however, also exert ecological costs when they have a negative effect on beneficial plant–microbe interactions. Such negative effects may even cross the border between a plant's aerial parts and its roots and then affect very central functions such as nutrient uptake and root resistance to micro-organisms. Whereas an impaired nodulation indeed appears common after resistance expression in the leaves, contradictory results have been published for the case of arbuscular mycorrhizal (AM) fungi.

2. We analysed the effect of induction of resistance mechanisms in foliar tissues on AM colonization in soybean plants, using acibenzolar-S-methyl (ASM) as chemical elicitor. By determining different physiological and biochemical parameters, we assessed whether the effects are related to the activation of the plant defence mechanisms or rather to the re-allocation of primary metabolites.

3. Colonization with AM fungi transiently decreased after pathogen resistance mechanisms were elicited in the aerial parts of the plant. The induction with ASM led to a significant, yet moderate, defence response in the roots, which was modulated in mycorrhizal plants. No allocation or fitness costs associated with the induction of resistance were detected in this study.

4. *Synthesis*. Our study confirms a transient negative impact of the elicitation of foliar defences on root–AM interactions. The results show that induced resistance to foliar pathogens can (i) move from the above-ground to the below-ground compartment and (ii) affect mutualistic micro-organisms as well as plant pathogens. We also conclude that (iii) the negative effect is likely linked to changes in the defence status of the plant rather than to changes in resource allocation patterns and (iv) the AM association can modulate the activation of the plant defence mechanisms and overcome such effects.

Key-words: allocation costs, arbuscular mycorrhizal fungi, fitness costs, pathogenesis-related proteins, photosynthesis, plant–soil (below-ground) interactions, PR-1 gene expression, β-1,3-glucanase; chitinase, systemic acquired resistance

Introduction

Higher plants largely rely on mutualisms with a diverse set of organisms such as pollinators, natural enemies of herbivores, and micro-organisms such as leaf endopyhtic fungi and soil-borne rhizobia and fungi. Fungi forming arbuscular mycorrhizas (AM) are believed to associate with the roots of over 80% of all higher plants in nature and have significant influences on the nutrition of the plant (Parniske 2008; Smith & Read 2008) as well as on its defensive status (Pozo & Azcón-Aguilar 2007;

*Correspondence author. E-mail: mjpozo@eez.csic.es

Kempel *et al*. 2010). As a consequence, AM have a significant impact on plant interactions with other organisms and, ultimately, on ecosystem functioning (van der Heijden, Bardgett & van Straalen 2008).

Plant must, however, also cope with pathogenic micro-organisms, and they do so by activating various defence mechanisms (Pieterse *et al*. 2009). Because plants interact simultaneously with multiple other organisms, their responses to specific events may have a significant impact on other interactions, and these effects can even cross the border between the aerial compartments and the roots (van Dam 2009). In fact, the relevance of induced plant defence responses for above-ground and below-ground communities and their interactions is now being recognized (Bezemer & van Dam 2005; Erb *et al*. 2009; van Dam and Heil 2011). Because of the intimate association of plants with mutualistic micro-organisms, several cross-reactions may occur between plant resistance to pathogens and beneficial associations with, for example, nodulating rhizobia, free-living bacteria in the rhizosphere and mycorrhizal fungi (Pozo & Azcón-Aguilar 2007; Soto *et al*. 2009; Yang *et al*. 2011). In this study, we investigate whether the induction of resistance mechanisms to pathogens in foliar tissues can cross the above-ground–below-ground border and have an impact on the symbiosis of roots with AM fungi.

Systemic acquired resistance (SAR) is an induced resistance in plants that is expressed upon local pathogen infection and confers resistance to a broad spectrum of attackers (Ryals *et al*. 1996; Sticher, Mauch-Mani & Métraux 1997). In most plant species, this response requires the synthesis of the endogenous signalling molecule salicylic acid (SA) in the infected tissue and its accumulation in the systemic tissue (Heil & Ton 2008; Xu *et al*. 2009) and it is characterized by the synthesis of pathogenesis-related (PR) proteins (Durrant & Dong 2004). Fourteen families of PR proteins have been described so far, and chitinases and β-1,3-glucanases are among them (van Loon & van Strien 1999). Chitinases and β-1,3-glucanases catalyse the hydrolysis of chitin and β-D-glucans, respectively, so both possess direct antimicrobial activity by degrading common microbial cell wall components (Dumas-Gaudot *et al*. 1996; van Loon 1997). Thus, PR proteins, together with other elements of SAR, contribute to a broad-spectrum resistance against diverse pathogenic bacteria, fungi and viruses. Because of this lack of specificity, they may also have a negative impact on microbial mutualists of the plant, then leading to significant 'ecological costs' of SAR (Heil 2002).

In fact, several studies have reported negative effects of SAR on beneficial plant–microbe interactions: Martínez-Abarca *et al*. (1998), Ramanujam, Jaleel & Kumaravelu (1998), Lian *et al*. (2000), Heil (2001b) and Faessel *et al*. (2010) reported an inhibition of root nodule formation after chemical induction of pathogen resistance in several Fabaceae species. We can conclude that an above-ground elicitation of SA-dependent resistance responses generally impairs the capacity of plant roots to consecutively establish a symbiosis with nodulating bacteria (van Dam & Heil 2011). By contrast, variable results have been reported concerning AM associations, depending on the application method of the chemical elicitor and experimental conditions (Salazar Costa, Ríos-Ruiz & Rodrigues Lambais 2000; Tosi & Zazzerini 2000; Sonnemann, Finkhaeuser & Wolters 2002; Faessel *et al*. 2010).

How does a resistance against leaf pathogens affect below-ground symbioses with mutualistic micro-organisms? Unfortunately, the physiological mechanisms of the above-ground–below-ground interactions are unclear in most cases, although a re-allocation of primary metabolites or a systemic resistance expression appears to be the most common reason (van Dam & Heil 2011). Besides the induction of active resistance, elicitation of SAR can cause a reduction in plant productivity and fitness, which results from a shift in the allocation pathways from growth to defence (Heil 2001a; Walters & Heil 2007). A common phenomenon associated to this 'shift from housekeeping to resistance expression' is a reduction in photosynthesis (Logemann *et al*. 1995; Scheideler *et al*. 2002; Swarbrick, Schulze-Lefert & Scholes 2006; Schwachtje & Baldwin 2008; Heil & Walters 2009). Thus, negative effects of foliar resistance expression on below-ground mutualisms could result from re-allocation of assimilates, as described from those cases in which plant tolerance responses increased root susceptibility to root herbivores (Kaplan *et al*. 2008). The goal of our present study was, therefore, to elucidate the physiological mechanisms that underlie any putative phenotypic effects of elicitation of resistance in above-ground tissues on the interaction of roots with AM fungi.

Several chemical elicitors of plant resistance are available, which mimic the mechanism by which pathogen infection induces resistance and thereby elicits SAR (Oostendorp *et al*. 2001). The benzothiadiazole derivative benzo-[1,2,3]-thiadiazole-7-carbothioic acid-S-methyl ester (Görlach *et al*. 1996), also known as acibenzolar-S-methyl (ASM), is a functional analogue of SA and effectively induces systemic resistance against pathogens and nematodes in multiple plant species including soybean (Dann *et al*. 1998; Chinnasri, Sipes & Schmitt 2003; Meyer *et al*. 2006; Faessel *et al*. 2008), *Arabidopsis thaliana* (Dietrich, Ploss & Heil 2004, 2005), barley (Sonnemann, Streicher & Wolters 2005), tobacco (Friedrich *et al*. 1996; Ginzberg *et al*. 1998; Shaul *et al*. 1999), tomato (Baysal, Soylu & Soylu 2003; Rossi Cavalcanti *et al*. 2006) and papaya (Zhu *et al*. 2003).

Considering the contradictory studies on the effect of SAR on mycorrhizas cited above, and the lack of knowledge on the underlying mechanisms, detailed research is required to understand the impact of plant resistance regulation on AM. Using a well-characterized chemical elicitor and a mycotrophic model plant such as soybean allows a detailed investigation of such potential effects and the underlying biochemical and molecular mechanisms. We tested the hypothesis that SAR-expressing soybean plants are colonized by the AM fungus *Glomus mosseae* to a lower extent than untreated plants. In order to assess if such effects are related directly to the defence mechanisms or to the alloca-

tion costs that are associated with the expression of the defence, we analysed defence responses and growth-associated parameters. Protein content, the activity of chitinases and β-1,3-glucanases and the expression of the *PR-1a* gene were chosen as disease resistance markers, whereas plant weight, sugar content, photosynthetic activity and seed production were chosen as markers for allocation and fitness costs.

Materials and methods

PLANT AND FUNGAL MATERIAL, GROWTH CONDITIONS AND CHEMICAL INDUCTION OF RESISTANCE

Soybean seeds (*Glycine max* (L.) Merr. cv. Williams 82) were surface sterilized with a commercial bleach solution (10%, v/v) and germinated under sterile conditions on wet filter paper at 28 °C for 3 days. Plants were grown in 500 mL pots containing a sterile mixture of quartz sand and soil (1:1, v/v).

Arbuscular mycorrhizal fungal inoculum consisted of propagules from *G. mosseae* (Nicol. and Gerd.) Gerd. and Trappe (BEG 12) including spores and chopped *Allium porrum* L. roots colonized by the fungus in a sand:sepiolite (1:1, v/v) substrate. Mycorrhizal inoculation was carried out by mixing with the growing substrate 7% of the inoculum. In control plants 7% sand:sepiolite (1:1, v/v) was mixed with the growing substrate. At potting, all plants received a filtrate (< 20 μm) of the AM fungal inoculum in order to provide the microbial populations accompanying the mycorrhizal inocula but free from AM propagules.

Soybean plants were cultivated in a controlled environment room (25/18 °C day/night temperature, 60% relative humidity, 16 h photoperiod, with a photosynthetic photon flux of 400 μmol photons m^{-2} s^{-1}). Plants were watered three times a week, the first 3 weeks only with water, and then twice a week with water and once a week with 50% Long Ashton nutrient solution (Hewitt 1966) at one-quarter phosphorus strength.

Six weeks after planting half of the plants were sprayed with an aqueous 400 mg L^{-1} Bion® solution at 10 mL per plant in order to induce resistance. Bion® is the name under which ASM is marketed by Syngenta (Basel, Switzerland). The rest of the plants were sprayed with the same amount of distilled water. Plant roots had no direct contact with ASM as the application was done by spraying only the shoots. The experimental design consisted, thus, of four different treatments: non-induced non-mycorrhizal plants (Nm−), induced non-mycorrhizal plants (Nm+), non-induced plants inoculated with *G. mosseae* (Gm−), and induced plants inoculated with *G. mosseae* (Gm+).

PLANT HARVEST AND ASSESSMENT OF MYCORRHIZAL COLONIZATION

Plants were harvested 1 and 2 weeks after the application of ASM (7 and 8 weeks after planting). The root system was carefully washed in running tap water and then the fresh weight of shoots and roots was annotated. About one-third of the root system from each plant was kept for determination of mycorrhizal colonization, and the rest was immediately frozen in liquid nitrogen and stored at −80 °C until protein and RNA extraction. Leaf samples were also frozen and stored at −80 °C.

For the estimation of mycorrhizal colonization, samples from the root system were cleared and stained (Phillips & Hayman 1970), and the percentage of total root length colonized by the AM fungi was determined by using the gridline intersect method (Giovannetti & Mosse 1980).

The remaining plants were harvested at the end of their growth period (17 weeks after planting) to record the seed dry weight per plant and the number of seeds per plant.

SUGAR EXTRACTION AND QUANTIFICATION

Sugars were extracted following a modified procedure of the method described by Bligh & Dyer (1959). One gram of fresh leaf material from the plants harvested 1 and 2 weeks after the application of ASM was homogenized in a mortar filled with 7.5 mL methanol for 2 min. Then 7.5 mL chloroform was added and the mixture was homogenized for 1 min. Finally, 3.75 mL of water with 0.88% NaCl were added, the solution was centrifuged at 4500 ***g*** for 10 min at 0 °C, and the upper layer containing the sugars was kept for further analysis.

Quantification of sugars was carried out following the anthrone method (Morse 1947). One hundred microlitre sugar solution, 900 μL water and 3 mL anthrone solution (200 mg anthrone + 100 mL 72% H_2SO_4) were mixed and boiled for 10 min at 100 °C. Samples were then put on ice to stop the staining reaction. The absorbance was measured at 620 nm in a spectrophotometer. The amount of sugars released was determined by comparison with a glucose standard curve with concentrations ranging from 0 to 400 μg mL^{-1}. One millilitre of each glucose concentration was mixed with 3 mL anthrone solution, boiled and the absorbance measured as described above.

PHOTOSYNTHESIS DETERMINATION

Photosynthetic assimilation was measured with a portable photosynthesis system (Li-Cor 6400, Lincoln, NE, USA) 1 week after ASM treatment. The CO_2 concentration was set at 400 p.p.m. and the photosynthetically active radiation at 1000 μmol m^{-2} s^{-1}. All measurements were performed in the morning, between 9:00 and 12:00 AM.

PROTEIN EXTRACTION AND QUANTIFICATION

Frozen plant material (root and leaf samples) from the plants harvested 1 week after the application of ASM was ground at 4 °C in an ice-chilled mortar with liquid nitrogen and the resulting powder was suspended in 100 mM MacIlvaine extracting buffer (citric acid/Na_2HPO_4), pH 6.8 (1:1, w/v). Crude homogenates were centrifuged at 15 000 ***g*** for 30 min at 4 °C and the supernatant fractions were kept frozen at −20 °C. Protein contents were determined by the method of Bradford (1976) using BSA as standard.

CHITINASE AND B-1,3-GLUCANASE ACTIVITY ASSAYS

Chitinase activity was determined with a fluorimetric assay (Ren, Wee & Chang 2000). Five microlitre methylumbelliferyl β-D-*N,N',N'*-triacetylchitotrioside hydrate (0.5 mg mL^{-1}; Sigma, Alcobendas, Madrid, Spain) were added to 95 μL plant extract (root and leaf samples) in a black 96-well microplate. Samples were incubated at 40 °C for 30 min in a shaker. The fluorescence was measured using a Perkin Elmer luminescence spectrometer LS50 (excitation 365 nm, emission 450 nm). All values were reported as fluorescence units.

The activity of β-1,3-glucanase was performed in 96-well microplates using an adaptation of the method described by Somogyi (1952). *Laminaria digitata* laminarin (Sigma) was used as substrate. The total volume of 180 μL reaction preparation contained 50 μL

Table 1. Primers used for the quantitative real-time PCR

Organism	Gene	Accession number	Primer sequence (5′–3′)
Glomus intraradices	*GintEF* (Benabdellah *et al.* 2009)	DQ282611	F-(5′-GCTATTTTGATCATTGCCGCC-3′) R-(5′-TCATTAAAACGTTCTTCCGACC-3′)
Glycine max	*Gm18S* (Porcel *et al.* 2006)	X02623	F-(5′-CCATAAACGATGCCGACCAG-3′) R-(5′-CAGCCTTGCGACCATTACTCC-3′)
Glycine max	*GmPR-1a*	AF136636	F-(5′-ATGTGTGTGTTGGGGTTGGT-3′) R-(5′-ACTTTGGCACATCCAAGACG -3′)

plant extract (root and leaf samples), 10 μL laminarin (20 mg mL^{-1} in 50 mM Na-acetate buffer, pH 5.0), 60 μL copper reactive and 60 μL arsenic reactive. The absorbance was measured at 650 nm in a spectrophotometer. The amount of sugars released was determined by comparison with a glucose standard curve with concentrations ranging from 0 to 200 μg mL^{-1}.

RNA EXTRACTION AND GENE EXPRESSION ANALYSIS BY QUANTITATIVE REAL-TIME PCR

Total RNA was isolated from root and leaf samples using Tri Reagent® (Ambion, Foster City, CA, USA) and treated with DNase RQ1 (Promega, Madison, WI, USA). cDNA synthesis was performed using iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA, USA) starting with 1 μg of total DNAse-treated RNA.

Gene expression levels were determined by quantitative real-time PCR, using the primers detailed in Table 1. The sets of primers for *Gm18S* and *GintEF* have been previously used as constitutive expression controls for soybean and *Glomus intraradices*, respectively (Porcel *et al.* 2006; Benabdellah *et al.* 2009). The soybean *PR-1a* gene was selected as marker for SA-regulated defence responses, and the corresponding set of primers was designed using the PRIMER 3 software (Rozen & Skaletsky 2000). Prior to real-time PCR analysis, the specificity of the selected primers was checked by conventional RT-PCR. The efficiency of the primer sets was evaluated by performing real-time PCR on several dilutions of genomic DNA. All quantitative real-time PCR reactions were performed using iQ SYBR Green Supermix (Bio-Rad Laboratories) on an iQ5 Multicolor Real-Time PCR Detection System (Bio-Rad Laboratories). The amplification protocol included an initial denaturation at 95 °C for 3 min followed by 35 cycles of 95 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s. The specificity of the PCR amplification procedure was checked with a heat dissociation protocol (from 70 to 100 °C) after the final cycle of the PCR. The results obtained for the different treatments were standardized to the soybean 18S rRNA levels, which were amplified with the primers *Gm18S* shown in Table 1.

Real-time PCR experiments were carried out at least three times, with the threshold cycle (C_T) determined in triplicate. The relative levels of transcription were calculated by using the 2-DDCT method (Livak & Schmittgen 2001). Negative controls without cDNA were included in all PCR reactions.

REPLICATION AND STATISTICAL ANALYSIS

Eighteen replicated plants were used per treatment, four of them were harvested at each time point (7 and 14 days after treatment) and used for the physiological and biochemical analyses and the rest (10 replicated plants) were used for the quantification of seed production. STATISTICA 6.1 software (StatSoft Inc., Tulsa, OK, USA) was used for the statistical analysis. A Student's *t*-test was carried out to detect significant effects of induction of resistance on AM colonization. Mann–Whitney *U* tests were conducted to detect significant effects of induction of resistance on fresh weight, sugar content, photosynthetic activity, protein content, chitinase and β-1,3-glucanase activity, and seed production parameters. Mean ± SE were calculated. Significance levels were set at 5%.

Results

Soybean plants inoculated with *G. mosseae* harvested 1 week after the application of ASM (Gm+) showed significantly lower AM colonization than Gm plants sprayed only with water (Gm−) (Fig. 1a; $t = 2.63$; d.f. = 6; $P = 0.038$; *t*-test). A second harvest of Gm+ plants 2 weeks after ASM treatment revealed that the differences in AM colonization between Gm+ and Gm− plants had levelled off ($t = 0.35$; d.f. = 6; $P = 0.735$; *t*-test). Thus, the negative effect of the induction of systemic resistance on the AM colonization of soybean plants appeared transitory. The absence of AM colonization was confirmed in non-inoculated plants (Nm) (data not shown).

Quantifying the expression level of a fungal 'housekeeping gene' is known to be an accurate and sensitive method to estimate the amount of active, living AM fungus within root tissues (Isayenkov, Fester & Hause 2004). The quantitative real-time PCR of transcript levels of *GintEF*, constitutively expressed in AM fungi (Benabdellah *et al.* 2009), confirmed a significant reduction of the AM fungus in Gm+ roots 1 week after treatment (0.4-fold compared to untreated plants; Fig. 1b). The fungal presence increased with time in both treated and untreated plants, and 2 weeks after the chemical treatment the inhibition was completely overridden. The results confirm that the negative effect of ASM application on AM colonization was transitory.

Resistance induction has been associated to a shift in resources allocation from growth to defence, and this shift may potentially limit the resources available for the fungal symbiont, thus limiting AM colonization. To assess if this is the case, we compared plant biomass, sugar content and photosynthetic activity in induced and non-induced plants. Neither the fresh weight, nor the sugar content, nor the photosynthetic activity changed significantly in either Nm or Gm plants after the induction of resistance ($P > 0.05$; Mann–Whitney *U* test; Table 2).

Nm+ plants produced fewer seeds than Nm− ones (Fig. 2a,b), although the difference was insignificant both for

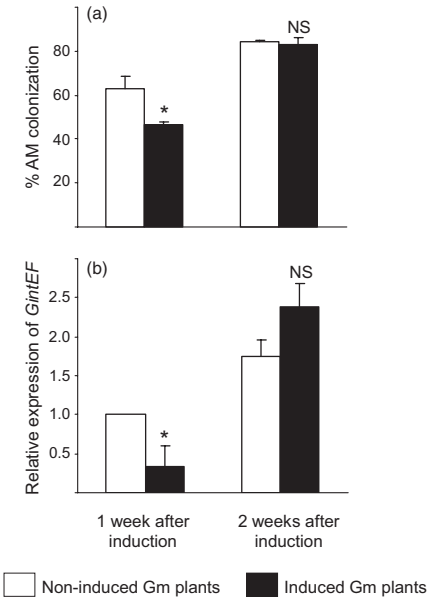

Fig. 1. Determination of arbuscular mycorrhizal (AM) fungal colonization of soybean roots by histochemical and real-time PCR analysis. Effect of the chemical induction of plant resistance to pathogens on AM colonization in soybean plants inoculated with *Glomus mosseae* (Gm) harvested 1 and 2 weeks after the application of acibenzolar-S-methyl (ASM). (a) Percentage of root length colonized by Gm estimated under the dissecting microscope upon staining of fungal structures with trypan blue. Measurements summarize mean percentage of AM colonization + SE of induced plants (black bars, ■) compared with non-induced plants (white bars, □). The sample size was four plants per treatment. $*P < 0.05$, ns $P > 0.05$ according to *t*-test. (b) Relative transcript levels of the *Glomus* elongation factor gene *GintEF*. Total RNA was extracted from soybean mycorrhizal roots, RNAs were reverse transcribed and gene expression was determined by quantitative real-time RT-PCR using gene-specific primers for *Glomus* elongation factor and normalized to soybean 18S rRNA. The figure represents mean fold change compared to the non-induced plants 1 week after treatment, set at 1. Black bars (■) represent induced plants and white bars (□) non-induced plants. $n = 3$. Changes in gene expression were calculated by using the 2-DDC method. Bars represent SE.

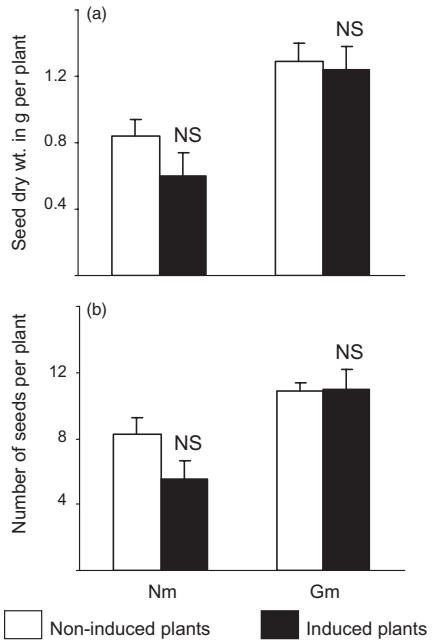

Fig. 2. Seed production. (a) Seed dry weight per plant and (b) number of seeds per plant of soybean. Nm, non-mycorrhizal plants; Gm, plants inoculated with *Glomus mosseae*. Measurements summarize mean parameters + SE of induced plants (black bars, ■) compared with non-induced plants (white bars, □). The sample size was 8–10 plants per treatment. $*P < 0.05$, ns $P > 0.05$ according to Mann–Whitney *U* test.

seed dry weight per plant ($P = 0.155$; Mann–Whitney *U* test) and numbers of seeds per plant ($P = 0.073$; Mann–Whitney *U* test). No hints towards changes in either the seed dry weight per plant ($P = 0.934$; Mann–Whitney *U* test) or the number of seeds per plant ($P = 0.866$; Mann–Whitney *U* test) were observed between Gm+ and Gm− plants.

We also analysed the impact of ASM treatment on the protein content and defence-related enzyme activities. The chemical induction of resistance led to a significant increase in protein content both in leaves and roots ($P < 0.05$; Mann–Whitney *U* tests) of Gm+ plants harvested 1 week after the application of ASM as compared to Gm− plants (Fig. 3a,b). No significant differences in protein content were observed between Nm+ and Nm− plants ($P > 0.05$; Mann–Whitney *U* test).

Chitinase activity (Fig. 4a,b) in leaves of Gm+ plants ($P < 0.01$; Mann–Whitney *U* test) 1 week after the induction of resistance was significantly higher than in Gm− plants. No

Table 2. Photosynthetic activity, sugar content and fresh weight of soybean plants 1 and 2 weeks after the application of acibenzolar-S-methyl (ASM)

	One week after induction				Two weeks after induction		
Treatment	Photosynthesis (μmol CO_2 m^{-2} s^{-1})	Sugar content (mg g^{-1} fresh wt. leaves)	Fresh wt. shoots (g)	Fresh wt. roots (g)	Sugar content (mg g^{-1} fresh wt.leaves)	Fresh wt. shoots (g)	Fresh wt. roots (g)
Nm−	7.08	3.69	5.19	4.77	10.13	6.61	5.08
Nm+	5.84 ns	4.59 ns	5.47 ns	4.81 ns	9.24 ns	6.82 ns	5.56 ns
Gm−	5.62	3.74	5.70	4.45	8.74	6.99	5.08
Gm+	6.99 ns	3.74 ns	5.40 ns	4.21 ns	8.60 ns	7.59 ns	5.03 ns

Fresh wt., fresh weight; Nm−, non-induced non-mycorrhizal plants; Nm+, induced non-mycorrhizal plants; Gm−, non-induced plants inoculated with *Glomus mosseae*; Gm+, induced plants inoculated with *G. mosseae*. Mean values of Nm− and Nm+ and Gm− and Gm+ in each column were compared using paired Mann–Whitney *U* tests. $n = 4$; ns $P > 0.05$.

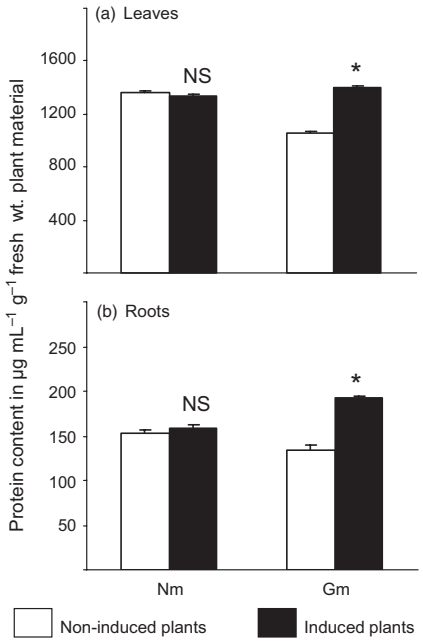

Fig. 3. Protein content. Protein content in µg mL^{-1} g^{-1} fresh weight (fresh wt.) of plant material in (a) leaves and (b) roots of soybean plants 1 week after the application of acibenzolar-S-methyl (ASM). Note that *Y*-axes differ between panels. Nm, non-mycorrhizal plants; Gm, plants inoculated with *Glomus mosseae*. Measurements summarize mean protein content + SE of induced plants (black bars, ■) compared with non-induced plants (white bars, □). **P* < 0.05, ns *P* > 0.05 according to Mann–Whitney *U* test.

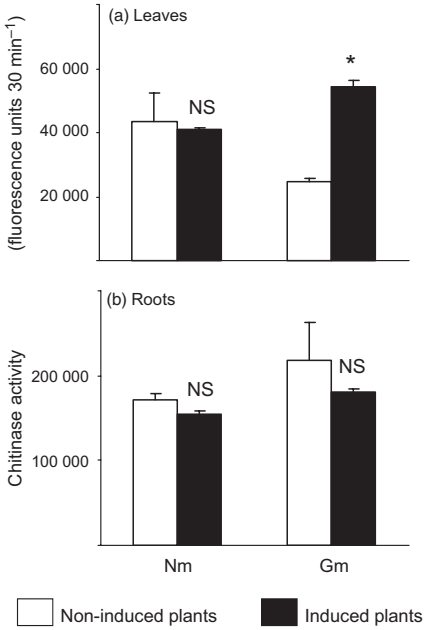

Fig. 4. Chitinase activity. Chitinase activity in fluorescence units min^{-1} in (a) leaves and (b) roots of soybean plants 1 week after the application of acibenzolar-S-methyl (ASM). Note that *Y*-axes differ between panels. Nm, non-mycorrhizal plants; Gm, plants inoculated with *Glomus mosseae*. Measurements summarize mean chitinase activity + SE of induced plants (black bars, ■) compared with non-induced plants (white bars, □). **P* < 0.05, ns *P* > 0.05 according to Mann–Whitney *U* test.

significant differences were observed in root chitinase activity between Gm+ and Gm− plants or in either roots or leaves of Nm+ and Nm− plants ($P > 0.05$; Mann–Whitney *U* test).

β-1,3-glucanase activity (Fig. 5a,b) was significantly higher in leaves of induced Nm+ and Gm+ plants compared to non-induced plants, as well as in roots of Nm+ plants when compared with Nm− plants ($P < 0.05$; Mann–Whitney *U* tests). No significant changes in β-1,3-glucanase activity were observed in roots of Gm plants after the induction of resistance ($P > 0.05$; Mann–Whitney *U* test).

Acibenzolar-S-methyl is a functional analogue of SA, and its application induces resistance in plants through the activation of the SA signalling pathway. In order to monitor this pathway we analysed the expression levels of the soybean *PR-1a* gene, coding for an acidic isoform of *PR-1*, a common marker for SA-regulated responses. ASM application resulted in an increase in *PR-1a* transcript levels in shoots in Nm and Gm plants, confirming the activation of the SA signalling pathway (Fig. 6a). In agreement with the reported systemic nature of the elicitor, the expression levels of the gene were also significantly higher in the roots of Nm+ plants. Although the basal level of *PR1a* expression was higher in mycorrhizal plants than in non-mycorrhizal controls, no increase in *PR-1a* expression was observed in roots of Gm plants upon induction by ASM treatment (Fig. 6b). Elevated levels of PR1a expression in roots were still found 2 weeks after induction in Nm+ plants, but again no difference was detected between Gm+ and Gm− plants (Fig. 6c).

Discussion

Plants live in a complex environment and interact with multiple micro-organisms that can behave as pathogens or as mutualists. The level of infection by mutualists such as rhizobia and AM fungi is in part controlled by the same physiological mechanisms that suppress infections by pathogens (Pozo & Azcón-Aguilar 2007; Soto *et al.* 2009). Therefore, plant resistance to pathogens can interfere with mutualistic interactions and *vice versa*, and these effects can systemically move through the plant, from the above-ground to the below-ground compartment and *vice versa* (see references in the Introduction, and van Dam & Heil 2011). Indeed, several studies found inhibitory effects of SAR expression on beneficial plant–microbe interactions, but all clear results have been obtained from experiments with nitrogen-fixing bacteria that form nodules in the roots (Martínez-Abarca *et al.* 1998; Ramanujam, Jaleel & Kumaravelu 1998; Lian *et al.* 2000; Heil 2001b; Faessel *et al.* 2010). In the case of AM fungi, by contrast, three seemingly different responses have been observed: the induction of resistance (i) inhibits AM colonization (Salazar Costa, Ríos-Ruiz & Rodrigues Lambais 2000; Faessel *et al.* 2010); (ii) leads to a transitory decrease in AM colonization (Tosi & Zazzerini 2000); and (iii) does not have any effect on AM colonization (Sonnemann, Finkhaeuser & Wolters 2002).

Our study agrees most with that of Tosi & Zazzerini (2000), because we also found a transitory decrease in AM coloniza-

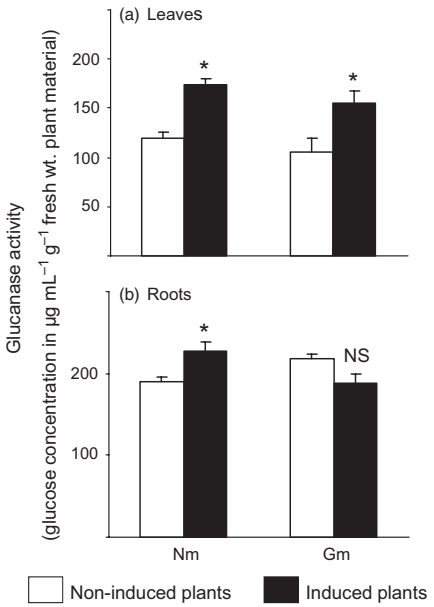

Fig. 5. β-1,3-Glucanase activity. β-1,3-glucanase activity as expressed by amount of glucose released in µg mL^{-1} g^{-1} fresh weight (fresh wt.) of plant material in (a) leaves and (b) roots of soybean plants 1 week after the application of acibenzolar-S-methyl (ASM). Note that *Y*-axes differ between panels. Nm, non-mycorrhizal plants; Gm, plants inoculated with *Glomus mosseae*. Measurements summarize mean chitinase activity + SE of induced plants (black bars, ■) compared with non-induced plants (white bars, □). $*P < 0.05$, ns $P > 0.05$ according to Mann–Whitney *U* test.

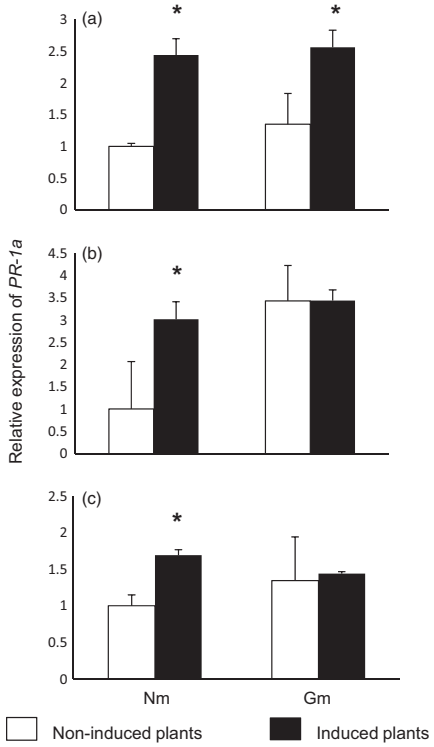

Fig. 6. Fold change in *PR-1a* gene expression upon acibenzolar-S-methyl (ASM) treatment. Total RNA was extracted from soybean (a) shoots and (b, c) roots of non-mycorrhizal (Nm) or mycorrhizal (Gm) plants harvested 1 (a, b) or 2 weeks (c) after resistance induction by treatment with ASM. RNAs were reverse transcribed and gene expression was determined by quantitative real-time RT-PCR using gene-specific primers for soybean *PR-1a* gene and *18S* rRNA. The figure represents mean fold change compared to the non-induced, non-mycorrhizal plants, set at 1. Black bars (■) represent induced plants and white bars (□) non-induced plants. $n = 3$. Changes in gene expression were calculated by using the 2-DDC method. Bars represent SE.

tion. Treating leaves with ASM sufficed to alter root colonization by *G. mosseae*. The long-term inhibitory effect reported by Salazar Costa, Ríos-Ruiz & Rodrigues Lambais (2000) followed the repeated application of SA as soil drench to bean plants, a very intensive treatment compared to standard studies in which chemical elicitors are applied just once (Baysal, Soylu & Soylu 2003; Dietrich, Ploss & Heil 2004, 2005; Sonnemann, Streicher & Wolters 2005; Rossi Cavalcanti *et al.* 2006). It is likely that the constantly low levels of AM colonization were due to this intensive induction of resistance. Furthermore, direct effects of the elicitor on the AM fungus cannot be ruled out at high concentrations of ASM (Faessel *et al.* 2010). In the study by Sonnemann, Finkhaeuser & Wolters (2002), the time lag between the application of ASM as foliar spray and the harvest of barley samples was very long (3 months), so any possible transitory effect of the induction of resistance on AM colonization could have levelled off at the time of data acquisition. In summary, the results of all available studies on negative effects of SAR on AM colonization (Salazar Costa, Ríos-Ruiz & Rodrigues Lambais 2000; Tosi & Zazzerini 2000; Sonnemann, Finkhaeuser & Wolters2002; Faessel *et al.* 2010; and the present study) are consistent with a transient inhibitory effect of SAR elicitation on mycorrhization. Because, the other way round, AM colonization affects the capacity of a plant to express above-ground resistance to pathogens and herbivores (van Dam & Heil 2011; Vannette & Hunter 2011), our results highlight the relevance of the correct timing of events in studies that aim at understanding the outcome of plant interactions with multiple organisms (Erb *et al.* 2011).

What is the biochemical or genetic mechanism that underlies this phenomenon? Negative effects of SAR induction on the interaction of plants with beneficial microbes can principally result from two different phenomena: (i) the 'metabolic shift' from primary to secondary metabolism that usually occurs during resistance elicitation might lower photosynthesis and, thus, the allocation of assimilates to the root system (as suggested by Russin *et al.* 1990), or (ii) defensive traits such as PR proteins might exert direct negative effects on the beneficial microbes (as suggested by Heil 2001b). In fact, the AM association, although a mutualistic one, is still a form of fungal invasion, and the plant activates its defence mechanisms (Riedel, Groten & Baldwin 2008). Remarkably, local defence responses such as increases of chitinase and β-1,3-glucanase activities are activated during early steps of compatible AM interactions (Ruiz-Lozano *et al.* 1999; Pozo & Azcón-Aguilar 2007), but these enzymatic activities are repressed at a later stage of mycorrhiza formation (Dumas-Gaudot *et al.* 1996; Gianinazzi-Pearson *et al.* 1996; Kapulnik *et al.* 1996). However,

induction of specific hydrolytic enzymes (chitinase, chitosanase and β-1,3-glucanase isoforms) as a response to the AM symbiosis that differ from those induced as the result of general defence mechanisms have been reported in different plants (Dumas-Gaudot *et al*. 1996; Pozo *et al*. 1996, 1998, 1999). The induction of these hydrolytic enzymes and other defence-related compounds in AM symbiosis seems to be involved in the plant control over the fungal symbiont, but it may affect other organisms (Pozo *et al*. 2002; López-Ráez *et al*. 2010). Indeed, the AM symbiosis is usually associated with an enhanced disease resistance or mycorrhiza-induced resistance (Pozo & Azcón-Aguilar 2007). Thus, besides a shift in assimilate allocation patterns, a shift in the delicate balance between host resistance and AM infection (Bennett, Bever & Bowers 2009) provides an alternative explanation of the pattern reported here.

In order to distinguish among these two alternatives we quantified photosynthetic rates and resistance expression in our plants. No changes in photosynthetic activity, sugar content or plant growth were recorded in either mycorrhizal or non-mycorrhizal plants after the induction of resistance. We conclude, therefore, that the negative effects of resistance induction on AM colonization were linked to the biochemical changes associated with the defence response rather than a shift in the allocation pathways from growth to defence. The application of ASM elicited a significant, yet moderate, increase in protein content and PR protein activity. To further analyse the activation of defence responses by ASM, we monitored expression levels of the *PR-1a* gene, commonly used as marker for SA-dependent defence responses (Uknes *et al*. 1993). Gene expression levels confirmed the activation of this signalling pathway not only in shoots, but also in roots of ASM treated non-mycorrhizal plants. SA-dependent responses are effective against biotrophic pathogens (Glazebrook 2005), but they may also impact mycorrhizal fungi because of their biotroph condition (Pozo & Azcón-Aguilar 2007). It is, therefore, likely that the activation of the SA signalling pathway upon ASM treatment results in an inhibition of AM root colonization.

Interestingly, ASM treatment led to a significant change in leaf chitinase activity in Gm plants but not in Nm plants. This pattern was caused by both, a lowered chitinase activity in Gm controls and a higher chitinase activity in Gm-induced plants, compared to the respective groups of Nm plants (Fig. 4a). This pattern is consistent with a successful suppression of chitinase activity by *G. mosseae*, which is transiently lost after ASM treatment. Our results are, therefore, consistent with the hypothesis that a shift in the balance between host resistance and control of AM infection explains why resistance induction transiently inhibits mycorrhization.

It is assumed that the induction of disease resistance incurs allocation and/or fitness costs, but only few studies have demonstrated or quantified them (Heil *et al*. 2000; Cipollini 2002; Heil 2002). In other cases, the results depended on growing conditions (Dietrich, Ploss & Heil 2005). In our study, plant growth was not affected by the induction of resistance. Similar results were reported by Iriti & Faoro (2003), and Zhu *et al*. (2003) and Faessel *et al*. (2008) recorded only transitory negative effects on plant growth. As for the seed production, the induction of resistance was associated with a lower number of seeds per plant and a lower seed dry weight per plant in non-mycorrhizal plants, although the differences were not significant. This trend was not observed in plants inoculated with *G. mosseae*.

In conclusion, our results support the hypothesis that the elicitation of foliar plant defence responses may negatively affect beneficial plant–microbe interactions below-ground, as a decrease in AM colonization of soybean roots was detected after the application of the chemical elicitor ASM to the leaves. The negative effects of the induction of resistance on AM seemed to be directly linked to the biochemical changes associated with the defence response and not to a possible shift in the allocation pathways from growth to defence. Because AM colonization plays an important role in plant nutrition and resistance, these effects likely will affect the future capacity of the plant to survive in the presence of competitors and enemies. Thus, it appears adaptive from the perspective of the plant to minimize these effects as far as possible and indeed, the decrease in AM colonization was only transitory. Because the ability of AM fungi to modulate the plant defence responses likely explains the transitory nature of the effect, we speculate that traits of both the host and its symbiont may be involved in overcoming the negative effects of above-ground resistance expression on the below-ground mutualism. Future studies aiming at an identification of the mechanisms of this co-operative effort would involve studying the interaction under different nutrient conditions and densities of AM colonization (Vannette & Hunter 2011) and monitoring physiological and transcriptomic changes in both plant roots and AM fungi during the various phases of the interaction.

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References

Baysal, Ö., Soylu, E.M. & Soylu, S. (2003) Induction of defence-related enzymes and resistance by the plant activator acibenzolar-S-methyl in tomato seedlings against bacterial canker caused by *Clavibacter michiganensis* ssp. *michiganensis*. *Plant Pathology*, **52**, 747–753.

Benabdellah, K., Merlos, M.A., Azcón-Aguilar, C. & Ferrol, N. (2009) GintGRX1, the first characterized glomeromycotan glutaredoxin, is a multifunctional enzyme that responds to oxidative stress. *Fungal Genetics and Biology*, **46**, 94–103.

Bennett, A.E., Bever, J.D. & Bowers, M.D. (2009) Arbuscular mycorrhizal fungal species suppress inducible plant responses and alter defensive strategies following herbivory. *Oecologia*, **160**, 771–779.

Bezemer, T.M. & van Dam, N.M. (2005) Linking aboveground and belowground interactions via induced plant defenses. *Trends in Ecology and Evolution*, **20**, 617–624.

Bligh, E.G. & Dyer, W.J. (1959) A rapid method of total lipid extraction and purification. *Canadian Journal of Biochemistry and Physiology*, **37**, 911–917.

Bradford, M.M. (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, **72**, 248–254.

Chinnasri, B., Sipes, B.S. & Schmitt, D.P. (2003) Effects of acibenzolar-s-methyl application to *Rotylenchulus reniformis* and *Meloidogyne javanica*. *Journal of Nematology*, **35**, 110–114.

Cipollini, D. (2002) Does competition magnify the fitness costs of induced resistance in *Arabidopsis thaliana*? A manipulative approach. *Oecologia*, **131**, 514–520.

van Dam, N.M. (2009) How plants cope with biotic interactions. *Plant Biology*, **11**, 1–5.

van Dam, N.M. & Heil, M. (2011) Multitrophic interactions below and above ground: *en route* to the next level. *Journal of Ecology*, **99**, 77–88.

Dann, E., Diers, B., Byrum, J. & Hammerschmidt, R. (1998) Effect of treating soybean with 2,6-dichloroisonicotinic acid (INA) and benzothiadiazole (BTH) on seed yields and the level of disease caused by *Sclerotinia sclerotiorum* in field and greenhouse studies. *European Journal of Plant Pathology*, **104**, 271–278.

Dietrich, R., Ploss, K. & Heil, M. (2004) Constitutive and induced resistance to pathogens in *Arabidopsis thaliana* depends on nitrogen supply. *Plant, Cell and Environment*, **27**, 896–906.

Dietrich, R., Ploss, K. & Heil, M. (2005) Growth responses and fitness costs after induction of pathogen resistance depend on environmental conditions. *Plant, Cell and Environment*, **28**, 211–222.

Dumas-Gaudot, E., Slezack, S., Dassi, B., Pozo, M.J., Gianinazzi-Pearson, V. & Gianinazzi, S. (1996) Plant hydrolytic enzymes (chitinases and β-1,3-glucanases) in root reactions to pathogenic and symbiotic microorganisms. *Plant and Soil*, **185**, 211–221.

Durrant, W.E. & Dong, X. (2004) Systemic acquired resistance. *Annual Review of Phytopathology*, **42**, 185–209.

Erb, M., Lenk, C., Degenhardt, J. & Turlings, T.C.J. (2009) The underestimated role of roots in defence against leaf attackers. *Trends in Plant Science*, **14**, 653–659.

Erb, M., Robert, C.A.M, Hibbard, B.E. & Turlings, T.C.J. (2011) Sequence of arrival determines plant-mediated interactions between herbivores. *Journal of Ecology*, **99**, 36–45.

Faessel, L., Nassr, N., Lebeau, T. & Walter, B. (2008) Effects of the plant defence inducer, acibenzolar-S-methyl, on hypocotyl rot of soybean caused by *Rhizoctonia solani* AG-4. *Journal of Phytopathology*, **156**, 236–242.

Faessel, L., Nassr, N., Lebeau, T. & Walter, B. (2010) Chemically-induced resistance on soybean inhibits nodulation and mycorrhization. *Plant and Soil*, **329**, 259–268.

Friedrich, L., Lawton, K., Ruess, W., Masner, P., Specker, N., Gut Rella, M. *et al.* (1996) A benzothiadiazole derivative induces systemic acquired resistance in tobacco. *Plant Journal*, **10**, 61–70.

Gianinazzi-Pearson, V., Dumas-Gaudot, E., Gollotte, A., Tahiri-Alaoui, A. & Gianinazzi, S. (1996) Cellular and molecular defence-related root responses to invasion by arbuscular mycorrhizal fungi. *New Phytologist*, **133**, 45–57.

Ginzberg, I., David, R., Shaul, O., Elad, Y., Wininger, S., Ben-Dor, B., Badani, H., Fang, Y.W., van Rhijn, P., Li, Y., Hirsch, A.M. & Kapulnik, Y. (1998) *Glomus intraradices* colonization regulates gene expression in tobacco roots. *Symbiosis*, **25**, 145–157.

Giovannetti, M. & Mosse, B. (1980) An evaluation of techniques for measuring vesicular arbuscular infections in roots. *New Phytologist*, **84**, 489–500.

Glazebrook, J. (2005) Contrasting mechanisms of defence against biotrophic and necrotrophic pathogens. *Annual Review of Phytopathology*, **43**, 205–227.

Görlach, J., Volrath, S., Knauf-Beiter, G., Hengy, G., Beckhove, U., Kogel, K.H., Oostendorp, M., Staub, T., Ward, E., Kessmann, H. & Ryals, J. (1996) Benzothiadiazole, a novel class of inducers of systemic acquired resistance, activates gene expression and disease resistance in wheat. *Plant Cell*, **8**, 629–643.

van der Heijden, M.G.A., Bardgett, R.D. & van Straalen, N.M. (2008) The unseen majority: soil microbes as drivers of plant diversity and productivity in terrestrial ecosystems. *Ecology Letters*, **11**, 296–310.

Heil, M. (2001a) The ecological concept of costs of induced systemic resistance (ISR). *European Journal of Plant Pathology*, **107**, 137–146.

Heil, M. (2001b) Induced systemic resistance (ISR) against pathogens – a promising field for ecological research. *Perspectives in Plant Ecology, Evolution and Systematics*, **4**, 65–79.

Heil, M. (2002) Ecological costs of induced resistance. *Current Opinion in Plant Biology*, **5**, 345–350.

Heil, M. & Ton, J. (2008) Long-distance signalling in plant defence. *Trends in Plant Science*, **13**, 264–272.

Heil, M. & Walters, D. (2009) Ecological consequences of plant defence signalling. *Plant Innate Immunity* (ed. L.C. van Loon), pp. 667–716. Elsevier, London.

Heil, M., Hilpert, A., Kaiser, W. & Linsenmair, K.E. (2000) Reduced growth and seed set following chemical induction of pathogen defence: does systemic acquired resistance (SAR) incur allocation costs? *Journal of Ecology*, **88**, 645–654.

Hewitt, E.J. (1966) Sand and water culture methods used in the studies of plant nutrition. *Technical Communication*, **Vol. 22**, pp. 430–434. Commonwealth Agricultural Bureau, London.

Iriti, M. & Faoro, F. (2003) Does benzothiadiazole-induced resistance increase fitness cost in bean? *Journal of Plant Pathology*, **85**, 41–47.

Isayenkov, S., Fester, T. & Hause, B. (2004) Rapid determination of fungal colonization and arbuscule formation in roots of *Medicago truncatula* using real-time (RT) PCR. *Journal of Plant Physiology*, **161**, 1379–1383.

Kaplan, I., Halitschke, R., Kessler, A., Rehill, B.J., Sardanelli, S. & Denno, R.F. (2008) Physiological integration of roots and shoots in plant defense strategies links above- and belowground herbivory. *Ecology Letters*, **11**, 841–851.

Kapulnik, Y., Volpin, H., Itzhaki, H., Ganon, D., Galili, S., David, R., Shaul, O., Elad, Y., Chet, I. & Okon, Y. (1996) Suppression of defence responses in mycorrhizal alfalfa and tobacco roots. *New Phytologist*, **133**, 59–64.

Kempel, A., Schmidt, A.K., Brandl, R. & Schadler, M. (2010) Support from the underground: Induced plant resistance depends on arbuscular mycorrhizal fungi. *Functional Ecology*, **24**, 293–300.

Lian, B., Zhou, X., Miransari, M. & Smith, D.L. (2000) Effects of salicylic acid on the development and root nodulation of soybean seedlings. *Journal of Agronomy and Crop Sciences*, **185**, 187–192.

Livak, K.J. & Schmittgen, T.D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(T)(-Delta Delta C) method. *Methods*, **25**, 402–408.

Logemann, E., Wu, S.C., Schröder, J., Schmelzer, E., Somssich, I.E. & Hahlbrock, K. (1995) Gene activation by UV light, fungal elicitor or fungal infection in *Petroselinum crispum* is correlated with repression of cell cycle-related genes. *Plant Journal*, **8**, 865–876.

van Loon, L.C. (1997) Induced resistance in plants and the role of pathogenesis-related proteins. *European Journal of Plant Pathology*, **103**, 753–765.

van Loon, L.C. & van Strien, E.A. (1999) The families of pathogenesis-related proteins, their activities, and comparative analysis of PR-1 type proteins. *Physiological and Molecular Plant Pathology*, **55**, 85–97.

López-Ráez, J.A., Verhage, A., Fernández, I., García, J.M., Azcón-Aguilar, C., Flors, V. & Pozo, M.J. (2010) Hormonal and transcriptional profiles highlight common and differential host responses to arbuscular mycorrhizal fungi and the regulation of the oxylipin pathway. *Journal of Experimental Botany*, **61**, 2589–2601.

Martínez-Abarca, F., Herrera-Cervera, J.A., Bueno, P., Sanjuan, J., Bisseling, T. & Olivares, J. (1998) Involvement of salicylic acid in the establishment of the *Rhizobium meliloti*-alfalfa symbiosis. *Molecular Plant-Microbe Interactions*, **11**, 153–155.

Meyer, M.C., Bueno, C.J., de Souza, N.L. & Yorinori, J.T. (2006) Effect of doses of fungicides and plant resistance activators on the control of *Rhizoctonia* foliar blight of soybean, and on *Rhizoctonia solani* AG1-IA *in vitro* development. *Crop Protection*, **25**, 848–854.

Morse, E.E. (1947) Anthrone in estimating low concentrations of sucrose. *Analytical Chemistry*, **19**, 1012–1013.

Oostendorp, M., Kunz, W., Dietrich, B. & Staub, T. (2001) Induced resistance in plants by chemicals. *European Journal of Plant Pathology*, **107**, 19–28.

Parniske, M. (2008) Arbuscular mycorrhiza: the mother of plant root endosymbioses. *Nature Reviews Microbiology*, **6**, 763–775.

Phillips, J.M. & Hayman, D.S. (1970) Improved procedures for clearing roots and staining parasitic and vesicular-arbuscular mycorrhizal fungi for rapid assessment of infection. *Transactions of the British Mycological Society*, **55**, 158–161.

Pieterse, C.M.J., León-Reyes, A., van der Ent, S. & van Wees, S.C.M. (2009) Networking by small-molecule hormones in plant immunity. *Nature Chemical Biology*, **5**, 308–316.

Porcel, R., Aroca, R., Azcón, R. & Ruiz-Lozano, J.M. (2006) PIP aquaporin gene expression in arbuscular mycorrhizal *Glycine max* and *Lactuca sativa* plants in relation to drought stress tolerance. *Plant Molecular Biology*, **60**, 389–404.

Pozo, M.J. & Azcón-Aguilar, C. (2007) Unraveling mycorrhiza-induced resistance. *Current Opinion in Plant Biology*, **10**, 393–398.

Pozo, M.J., Dumas-Gaudot, E., Slezack, S., Cordier, C., Asselin, A., Gianinazzi, S., Gianinazzi-Pearson, V., Azcón-Aguilar, C. & Barea, J.M. (1996) Induction of new chitinase isoforms in tomato roots during interactions with

Glomus mosseae and/or *Phytophthora nicotianae* var. *parasitica*. *Agronomie*, **16**, 689–697.

Pozo, M.J., Azcón-Aguilar, C., Dumas-Gaudot, E. & Barea, J.M. (1998) Chitosanase and chitinase activities in tomato roots during interactions with arbuscular mycorrhizal fungi or *Phytophthora parasitica*. *Journal of Experimental Botany*, **49**, 1729–1739.

Pozo, M.J., Azcón-Aguilar, C., Dumas-Gaudot, E. & Barea, J.M. (1999) β-1,3-Glucanase activities in tomato roots inoculated with arbuscular mycorrhizal fungi and/or *Phytophthora parasitica* and their possible involvement in bioprotection. *Plant Science*, **141**, 149–157.

Pozo, M.J., Cordier, C., Dumas-Gaudot, E., Gianinazzi, S., Barea, J.M. & Azcón-Aguilar, C. (2002) Localized versus systemic effect of arbuscular mycorrhizal fungi on defence responses to *Phytophthora* infection in tomato plants. *Journal of Experimental Botany*, **53**, 525–534.

Ramanujam, M.P., Jaleel, V.A. & Kumaravelu, G. (1998) Effect of salicylic acid on nodulation, nitrogenous compounds and related enzymes of *Vigna mungo*. *Biologia Plantarum*, **41**, 307–311.

Ren, Y., Wee, K.E. & Chang, F.N. (2000) Deficiency of current methods in assaying endochitinase activity. *Biochemical and Biophysical Research Communications*, **268**, 302–305.

Riedel, T., Groten, K. & Baldwin, I.T. (2008) Symbiosis between *Nicotiana attenuata* and *Glomus intraradices*: ethylene plays a role, jasmonic acid does not. *Plant, Cell & Environment*, **31**, 1203–1213.

Rossi Cavalcanti, F., Vilela Resende, M.L., Matos Santos Lima, J.P., Gomes Silveira, J.A. & Abreu Oliveira, J.T. (2006) Activities of antioxidant enzymes and photosynthetic responses in tomato pre-treated by plant activators and inoculated by *Xanthomonas vesicatoria*. *Physiological and Molecular Plant Pathology*, **68**, 198–208.

Rozen, S. & Skaletsky, H.J. (2000) Primer 3 on the WWW for general users and for biologist programmers. *Bioinformatics Methods and Protocols: Methods in Molecular Biology* (eds S. Krawetz & S. Misener), pp. 365–386. Humana Press, Totowa, NJ.

Ruiz-Lozano, J.M., Roussel, H., Gianinazzi, S. & Gianinazzi-Pearson, V. (1999) Defense genes are differentially induced by a mycorrhizal fungus and *Rhizobium* sp. in wild-type and symbiosis-defective pea genotypes. *Molecular Plant-Microbe Interactions*, **12**, 976–984.

Russin, J.S., Layton, M.B., Boethel, D.J., McGawley, E.C., Snow, J.P. & Berggren, G.T. (1990) Growth, nodule development, and N_2-fixing ability in soybean damaged by an insect-fungus-nematode pest complex. *Journal of Economic Entomology*, **83**, 247–254.

Ryals, J.A., Neuenschwander, U.H., Willits, M.G., Molina, A., Steiner, H.Y. & Hunt, M. (1996) Systemic acquired resistance. *Plant Cell*, **8**, 1809–1819.

Salazar Costa, H., Ríos-Ruiz, W.F. & Rodrigues Lambais, M. (2000) Acido salicilico inibe a formacao de micorrizas arbusculares e modifica a expressao de quitinases e β-1,3-glucanases em raizes de feijoeiro. *Scientia agricola*, **57**, 19–25.

Scheideler, M., Schlaich, N.L., Fellenberg, K., Beissbarth, T., Hauser, N.C., Vingron, M., Slusarenko, A.J. & Hoheisel, J.D. (2002) Monitoring the switch from housekeeping to pathogen defense metabolism in *Arabidopsis thaliana* using cDNA arrays. *Journal of Biological Chemistry*, **277**, 10555–10561.

Schwachtje, J. & Baldwin, I.T. (2008) Why does herbivore attack reconfigure primary metabolism? *Plant Physiology*, **146**, 845–851.

Shaul, O., Galili, S., Volpin, H., Ginzberg, I., Elad, Y., Chet, I. & Kapulnik, Y. (1999) Mycorrhiza-induced changes in disease severity and PR protein expression in tobacco leaves. *Molecular Plant-Microbe Interactions*, **12**, 1000–1007.

Smith, S.E. & Read, D.J. (2008) *Mycorrhizal Symbiosis*, 3rd edn. Academic Press, London.

Somogyi, M. (1952) Notes on sugar determination. *Journal of Biological Chemistry*, **195**, 19–23.

Sonnemann, I., Finkhaeuser, K. & Wolters, V. (2002) Does induced resistance in plants affect the belowground community? *Applied Soil Ecology*, **21**, 179–185.

Sonnemann, I., Streicher, N.M. & Wolters, V. (2005) Root associated organisms modify the effectiveness of chemically induced resistance in barley. *Soil Biology & Biochemistry*, **37**, 1837–1842.

Soto, M.J., Domínguez-Ferreras, A., Pérez-Mendoza, D., Sanjuán, J. & Olivares, J. (2009) Mutualism versus pathogenesis: the give-and-take in plant-bacteria interactions. *Cellular Microbiology*, **11**, 381–388.

Sticher, L., Mauch-Mani, B. & Métraux, J.P. (1997) Systemic acquired resistance. *Annual Review of Phytopathology*, **35**, 235–270.

Swarbrick, P.J., Schulze-Lefert, P. & Scholes, J.D. (2006) Metabolic consequences of susceptibility and resistance (race-specific and broad-spectrum) in barley leaves challenged with powdery mildew. *Plant, Cell & Environment*, **29**, 1061–1076.

Tosi, L. & Zazzerini, A. (2000) Interactions between *Plasmopara helianthi*, *Glomus mosseae* and two plant activators in sunflower plants. *European Journal of Plant Pathology*, **106**, 735–744.

Uknes, S., Dincher, S., Friedrich, L., Negrotto, D., Williams, S., Thompson, T.H., Potter, S., Ward, E. & Ryals, J. (1993) Regulation of pathogenesis-related protein-1a gene expression in tobacco. *The Plant Cell*, **5**, 159–169.

Vannette, R.L. & Hunter, M.D. (2011) Plant defence theory re-examined: nonlinear expectations based on the costs and benefits of resource mutualisms. *Journal of Ecology*, **99**, 66–76.

Walters, D.R. & Heil, M. (2007) Costs and trade-offs associated with induced resistance. *Physiological and Molecular Plant Pathology*, **71**, 3–17.

Xu, M., Dong, J., Wang, H. & Huang, L. (2009) Complementary action of jasmonic acid on salicylic acid in mediating fungal elicitor-induced flavonol glycoside accumulation of *Ginkgo biloba* cells. *Plant, Cell & Environment*, **32**, 960–967.

Yang, J.W., Yi, H.-S., Kim, H., Lee, S., Ghim, S.-Y. & Ryu, C.-M. (2011) Whitefly infestation of pepper plants elicits defence responses against bacterial pathogens in leaves and roots and changes the below-ground microflora. *Journal of Ecology*, **99**, 46–56.

Zhu, J.Z., Qiu, X., Moore, P.H., Borth, W., Hu, J., Ferreira, S. & Albert, H.H. (2003) Systemic acquired resistance induced by BTH in papaya. *Physiological and Molecular Plant Pathology*, **63**, 237–248.

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