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Mycorrhiza-induced protection against pathogens is both genotype-specific and graft-transmissible

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Abstract In addition to the nutrient exchange that is promoted by the arbuscular mycorrhiza symbiosis (AMS) between plants and fungi, AMS triggers mycorrhiza-induced protection against plant pathogens. Although the induction of this protection against diverse plant pathogens has been described for several plant species, it is not clear if its onset differs among genotypes within a species. To address this, we have examined if and how this defense response is triggered by AMS in common bean and tomato. Leaflets from three different genotypes of mycorrhizal common beans and two genotypes of tomato were challenged with the pathogens *Sclerotinia sclerotiorum* and *Xanthomonas campestris* pv. *vesicatoria*, respectively, to determine if disease protection induced by mycorrhiza is genotype-specific. We have found that one tomato and two common bean genotypes display this type of protection, although this was not observed in Az Hig common bean and Micro-Tom tomato. These findings indicate that mycorrhiza-induced disease protection is genotype-specific for the species and genotypes included in this study. Previous work has shown that defense induced by mycorrhiza colonization is effective against foliar pathogens, suggesting the existence of a signal that must move from colonized roots to shoots. We examined the possibility that this defense response can be triggered in scions from non-mycorrhizal plants when they were grafted onto mycorrhizal rootstock. Pathogen infection assays were then performed on leaflets of both scions and rootstock, and infection damage was compared to non-grafted plants. Our results indicate that in genotypes displaying mycorrhiza-induced disease protection, scions originating from non-mycorrhizal plants acquired the ability to decrease disease symptoms when grafted onto mycorrhizal rootstocks, indicating that they are responsive to the putative signal that moves from mycorrhizal roots to the upper part of the plant to trigger disease protection. This grafting experimental system may be useful in elucidating the molecular mechanisms involved in the systemic signaling of mycorrhiza-induced defense response.

Keywords Mycorrhiza · *Rhizophagus irregularis* · *Sclerotinia sclerotiorum* · *Phaseolus vulgaris* · *Solanum lycopersicum* · *Xanthomonas campestris* pv. *vesicatoria*

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1 Introduction

Arbuscular mycorrhiza symbiosis (AMS) is a phenomenon that establishes between fungi of the phyla Glomeromycota and the roots of about 80 % of terrestrial plant species (Schübler et al. 2001). The fungus absorbs mineral nutrients, mainly phosphorous and nitrogen from the soil, and transports them to the plant. In return, plants provide carbon compounds

that the fungus cannot synthesize (Smith and Read 1997). In addition to the pivotal role of arbuscular mycorrhizal fungi (AMF) in plant-improved nutrition, AMS may alleviate disease damage (Jung et al. 2012; Pozo and Azcon-Aguilar 2007; Whipps 2004). Extensive experimental evidence indicates that AMF colonization reduces damage caused by soil-borne pathogens or nematodes (Elsen et al. 2003; Elsen et al. 2008; Hol and Cook 2005; Li et al. 2006; Vos et al. 2012a, b, c, d, 2013; Whipps 2004). Furthermore, enhanced defense is manifested locally in colonized root sections of mycorrhizal plants (Cordier et al. 1998; Hao et al. 2012; Khaosaad et al. 2007; Li et al. 2006; Pozo et al. 2002), as well as systemically, in stems and leaves infected mainly by necrotrophic shoot pathogens. AMF-colonized tomato plants show a reduced severity of symptoms induced by phytoplasmas as compared to non-mycorrhizal plants (Lingua et al. 2002), and similar results have been observed for disease caused by *Alternaria solani* (Fritz et al. 2006; Noval et al. 2007). Non-mycorrhizal *Capsicum annuum* plants showed 25 % more necrotic lesions caused by *Phytophthora capsici* as compared to mycorrhizal plants (Alejo-Iturvide et al. 2008). *Medicago truncatula* plants colonized with *Glomus intraradices* and infected with *Xanthomonas campestris* pv. *alfalfae* showed significantly less bacterial proliferation in comparison to infected non-mycorrhizal plants (Liu et al. 2007). The induction of resistance against *Magnaporthe oryzae* in mycorrhizal rice plants has also been reported (Campos-Soriano et al. 2012). Systemic induction of shoot defense response, exemplified by the above cases, should be mediated by the translocation of a signal from the colonized roots toward the upper parts of the plant. This could possibly entail the movement of the signal via the plant vascular tissue, which would then trigger the disease protection response in distal tissues. However, no studies have been reported as of yet to offer insight into this phenomenon.

Here, we have examined the hypothesis that a plant genotype-specific disease protection, triggered by colonization with the AMF *R. irregularis* (previously named *Glomus intraradices*), occurs in common bean and tomato. To do so, we have assayed two different plant-pathogen systems: 1) three genotypes of common beans challenged with *S. sclerotiorum*, and 2) two genotypes of tomato plants challenged with *X. campestris* pv. *vesicatoria.* The foliar pathogen *S. sclerotiorum* affects many crops and causes white mold disease in common beans (Williams et al. 2011). *X. campestris* pv. *vesicatoria* is the causal agent of bacterial spot in tomatoes (Leyns et al. 1984). Finally, the possibility that a shoot branch of a non-mycorrhizal plant could acquire disease protection was investigated by grafting scions of non-mycorrhizal plants onto mycorrhizal plants. Scions were then challenged with the pathogen. We found that the mycorrhiza-induced protection response is observed in grafted tissue. These substantial results will improve our understanding of systemic signaling in mycorrhiza-induced defense response, and eventually how its manipulation can be used to benefit agricultural systems.

2 Material and methods

2.1 Plant material

Three common bean genotypes grown in northern Mexico were used in this study. A-55 is a common bean breeding line developed at the Centro Internacional de Agricultura Tropical (CIAT) in Cali, Colombia, which bears small black seeds. It is a tall upright genotype with a type II indeterminate growth habit (Singh et al. 2003). Azufrado Regional 87 (Az Reg87) and Azufrado Higuera (Az Hig) are varieties that produce yellow seeds and have a type I determinate growth habit. Both lines were developed at the Valle del Fuerte Experimental Field Station (CEVAF) in Sinaloa, Mexico. A-55, Az Reg87 and Az Hig were obtained from the Instituto Nacional de Investigaciones Forestales, Agrícolas y Pecuarias (INIFAP) in Mexico (Rosales-Serna et al. 2004).

Two tomato genotypes were included in the present study. Micro-Tom is a small commercial cultivar that is easily transformable and fast-growing, which makes it a convenient model system for molecular research (Marti et al. 2006). Micro-Tom was produced for ornamental purposes by crossing Florida Basket and Ohio 4013-3 cultivars; it displays a dwarf phenotype with small red, ripened fruits (Scott and Harbaugh 1989). Missouri is a determinate commercial variety (Seminis Vegetable Seeds Co.).

2.2 AMF inoculum preparation

Rizophagus irregularis (DAOM 197198) (Schenck & Smith) is a ubiquitous arbuscular mycorrhizal species that is extensively used as an AMF model species in many experimental studies worldwide. *R. irregularis* was maintained in cultured carrot roots (*Daucus carota* L.) on M medium ($MgSO_4 \cdot 7H_2O$, 3 mM; KNO_3, 0.80 mM; KCl, 0.9 mM; KH_2PO_4, 35.3 μM; $Ca(NO_3)_2 \cdot 4H_2O$, 1.2 mM; Na-FeEDTA, 21.8 μM; KI, 4.5 μM; $MnCl_2 \cdot 4H_2O$, 30.3 μM; $ZnSO_4 \cdot 7H_2O$, 9.2 μM; H_3BO_3, 24.3 μM; $CuSO_4 \cdot 5H_2O$, 0.5 μM; $Na_2MoO_4 \cdot 4H_20$, 0.009 μM; glycine, 40 μM; thiamine HCl, 0.3 μM; pyridoxine HCl, 0.5 μM; nicotinic acid, 4.1 μM; myo-inositol, 0.3 mM; and sucrose, 29.2 mM to pH 5.5), as described by Chabot et al. (1992). Spore suspensions of *R. irregularis* were prepared as described by St-Arnaud et al. (1996). Briefly, spores were obtained by blending fungal mycelia with a milk shaker (Braun), using a 1:3 (*v*/*v*) proportion of M medium:10 mM citric acid sterile solution. After blending, the spores were passed through a 50 μm exclusion size mesh and abundantly washed with sterile water. Spores retained in

the mesh were suspended in sterile distilled water and counted under a stereo microscope.

2.3 AMF colonization and plant growing conditions

Common bean seeds (*P. vulgaris*) were surface-sterilized by immersion in ethanol for two minutes followed by five minutes in 0.5 % sodium hypochlorite. Seeds were then rinsed four times with distilled water and germinated in a mix of sterile fine sand and vermiculite (1:1). Eight days after seeding, five plantlets each of A-55, Az Reg 87, and Az Hig were transferred to 0.5 L pots with the same substrate and inoculated with 500 spores of *R. irregularis*. An equal number of plantlets were mock-inoculated by adding sterile distilled water as the non-mycorrhizal control. After inoculation, all plants were maintained in a growth chamber (Binder, model KBW 400) on an 8 h light (25 °C)/16 h dark (19 °C) photoperiod during six weeks, to allow plant colonization by AMF. Plants were fertilized on a weekly basis with Hoagland's solution (Hoagland and Arnon 1950) ($Ca(NO_3)_2 \cdot 4H_2O$, 2.5 mM; KNO_3, 2.5 mM; $MgSO_4 \cdot 7H_2O$, 1 mM; NaFe EDTA, 0.05 mM; H_3BO_3, 10 μM; $Na_2MoO_4 \cdot 2H_20$, 0.2 μM; $ZnSO_4 \cdot 7H_2O$, 1 μM; $MnCl_2 \cdot 4H_2O$, 2.0 μM; $CuSO_4 \cdot 5H_2O$, 0.5 μM; $CoCl_2 \cdot 6H_2O$, 0.2 μM; HCL, 25 μM; MES buffer 0.5 mM), containing either low phosphate (20 μM KH_2PO_4 for both sets of mycorrhizal and non-mycorrhizal plants) or regular phosphate (200 μM KH_2PO_4 for an additional non-mycorrhizal set of plants). Since mycorrhizal colonization is favored by low phosphate conditions, 20 μM KH_2PO_4 was used to induce colonization. A mock-inoculated set of plants was also fertilized with 200 μM KH_2PO_4, as controls. The set of non-mycorrhizal plants fertilized with regular phosphate (200 μM KH_2PO_4) was used as a control for responses derived by high-phosphate nutritional status instead of by mycorrhizal colonization.

Tomato seeds (*S. lycopersicum*) were surface-sterilized with a commercial bleach solution (10 % *v*/*v*), rinsed with distilled water and germinated in germinating trays containing a 3:1 sterile mixture (*v*/*v*) of vermiculite and sterile fine sand previously washed with running water. Pots were maintained in a growth chamber (Binder, model KBW 400) or a growth room on a 16 h light (25 °C)/8 h dark (22 °C) photoperiod. Plantlets were transplanted upon expansion of the first true leaf and grown in 0.5 L pots containing the same substrate. Plants were then either inoculated with 400 axenically-produced spores of the AMF *R. irregularis* or mock inoculated, and fertilized twice per week with Hoagland's solution containing low phosphate (20 μM KH_2PO_4). Another set of mock-inoculated plants was fertilized with regular phosphate (200 μM KH_2PO_4) as in the common bean experiments. Tomato var Missouri plants were maintained for four or six weeks, while Micro-Tom plants were grown for two weeks post-inoculation with *R. irregularis*, due to the shorter life cycle of the latter tomato genotype.

2.4 *Sclerotinia sclerotiorum* inoculum preparation

S. sclerotiorum mycelia were obtained from sclerotia collected in common bean commercial fields in northern Sinaloa, Mexico. This fungus, also known as white mold, is one of the most common fungal diseases in this region. Sclerotia were surface-sterilized for one minute in 0.5 % sodium hypochlorite, rinsed twice for one minute in distilled water, blotted dry on a paper towel for several seconds, and then plated on PDA (potato dextrose agar) medium for germination. Mycelia were transferred to PDA plates and incubated for three days at 19 °C. Actively growing mycelia were taken from the plates for use in infection experiments. Experiments were established using mycelia originating from one individual sclerotium per experiment. The identity of *S. sclerotiorum* was determined by sequencing the ITS1, 5.8S, and ITS2 regions of rDNA fragment amplicons, using the primers ITS1 and ITS4 (White et al. 1990) (data not shown).

2.5 *Xanthomonas campestris* pv. *vesicatoria* inoculum

X. campestris pv. *vesicatoria* was maintained in nutritive agar at room temperature, and its identity was determined by sequencing the rDNA 16S region amplicon using the universal primers F2C and C (Shi et al. 1997) (data not shown).

2.6 Pathogen infection and measurement

2.6.1 Common bean whole-plant S. sclerotiorum assay

We implemented a whole-plant leaflet assay to evaluate *S. sclerotiorum* infection in the different genotypes. Agar plugs with actively growing *S. sclerotiorum* mycelia were placed on the third node abaxial leaflet surface of six-week-old common bean plants. Inoculated leaflets were introduced into a clear polyethylene bag previously sprayed with sterile distilled water to ensure 100 % relative humidity (RH). The polyethylene bag was sealed at the petiole of the leaflet with a rubber band without damaging the plant tissues. The progression of *S. sclerotiorum* infection was followed by measuring the diameter of the necrotic lesion on the leaflet 48 h after inoculation. One leaflet per plant was infected for each of the five plants used per treatment (both mycorrhizal and non-mycorrhizal plants). Independent experiments were performed twice with similar results. Data from only one of the experiments are presented here.

2.6.2 Tomato whole-plant X. campestris pv. vesicatoria assay

X. campestris pv. *vesicatoria* was grown in nutritive agar for 48 h at room temperature and suspended in 10 L of sterilized water to a final concentration of 1.6×10^5 CFU/mL for use in immersion infection assays. Complete tomato shoots were immersed in the inoculum suspension and the density of bacteria remaining on the foliar surface was determined to be 29 ± 4.5 CFU/cm^2. The plants were then placed in tightly closed clear plastic bags for 24–48 h to ensure 100 % RH, which is required during the early stages of bacterial infection. Following bacterial inoculation of Missouri and Micro-Tom tomato plants (for 10 and 7 days, respectively) the disease severity was determined by counting the number of bacterial spots per square centimeter of leaf tissue. Independent experiments were performed twice, with similar results for each tomato variety. Data from only one of the experiments are presented here.

2.7 Experimental design and data analysis

Three treatments per genotype were included in the common bean and tomato experiments as follows: 1) plants inoculated with *R. irregularis* that were fertilized with low phosphate Hoagland solution (M 20 μM-P); 2) control plants (non-mycorrhizal) that were fertilized with low phosphate Hoagland's solution (NM 20 μM-P), and 3) control plants (non-mycorrhizal) that were fertilized with regular phosphate Hoagland's solution (NM 200 μM-P). The treatments were arranged in a completely randomized design with four replicates. Each experiment was repeated twice.

The effect of the treatments in reducing the severity of white mold (caused by *S. sclerotiorum*) and bacterial spot (caused by *X. campestris* pv. *vesicatoria*) was determined by measuring the lesion diameter in the former and counting the spots in the latter. Data were assayed for homogeneity of variance and subjected to square root transformation before performing analysis of variance (ANOVA). Tukey's HSD test was used for separation of means (Little and Hills 1973). All statistical analyses were performed using the SAS/STAT statistical software.

2.8 Percentage of AMF colonization

Roots were harvested at the end of the *S. sclerotiorum* and *X. campestris* pv. *vesicatoria* challenge experiments. Roots were then washed with distilled water to remove substrate particles and were fixed in 50 % (*v*/*v*) ethanol for one hour, washed twice with distilled water and clarified in 20 % (*w*/*v*) KOH for two day at room temperature. Subsequently, roots were washed twice with distilled water and incubated for two hours in 1 % (*v*/*v*) HCl, washed twice in distilled water, and stained with a solution containing trypan blue 0.05 % (Phillips and Hayman 1970) for two days. Roots were maintained in lactoglycerol 1:1:1 (water:lactic acid:glycerol) at room temperature. Percentage of colonization was determined for each plant by the gridline intersection method, as previously described (Giovannetti and Mosse 1980). Non-mycorrhizal control plants were also analyzed for colonization; no mycorrhiza-related structures or hyphae were detected in the roots at any point.

2.9 Grafting experiments

Eight-day-old common bean Az Reg87 plants were inoculated with 500 spores of *R. irregularis* and grown under growth chamber conditions. Scions (e.g. a complete trifoliate leaf) of non-mycorrhizal plants were grafted onto mycorrhizal rootstocks once plants were two weeks old. As a control, scions of non-mycorrhizal plants were grafted onto non-mycorrhizal controls (Fig. 1). Four weeks later, the *S. sclerotiorum* infection assay was performed on a leaflet from the grafted leaf. Infection progress was monitored by measuring the diameter of necrotic lesions formed 48 h after inoculation with the pathogen.

Tomato var. Missouri plants were germinated and maintained in a climate chamber as indicated above. Three-week-old tomato plants were inoculated with 400 spores of *R. irregularis*; two weeks later, scions (e.g. a complete leaf) of non-mycorrhizal plants were grafted onto mycorrhizal rootstocks. As a control, scions of non-mycorrhizal plants were grafted onto non-mycorrhizal controls. After four weeks, the plants were inoculated with *X. campestris* as follows: a pathogenic isolate of the bacterium was grown in nutritive agar and re-suspended in sterile deionized water at 1.1 OD units (580 nm). Using a hypodermic syringe (without the needle), 100 μL of the bacterial suspension was carefully infiltrated in three leaflets of one pentafoliate leaf per tomato plant for all treatments. Nine days after bacterial infiltration the diameter of the bacterial infection was measured in each leaflet. At this time, roots were sampled to estimate mycorrhizal colonization. No colonization was ever detected in non-mycorrhizal plants.

3 Results

3.1 Mycorrhizal colonized A-55 and Az Reg87 common bean display mycorrhiza-induced disease protection against *S. sclerotiorum* but not Az Hig

Three common bean genotypes (A-55, Az Reg87, and Az Hig) that colonize with the AMF *R. irregularis* were challenged with *S. sclerotiorum*, and necrotic lesion size was monitored and compared to non-mycorrhizal plants. Percentage of root colonization ranged from 20 to 48 % depending on the

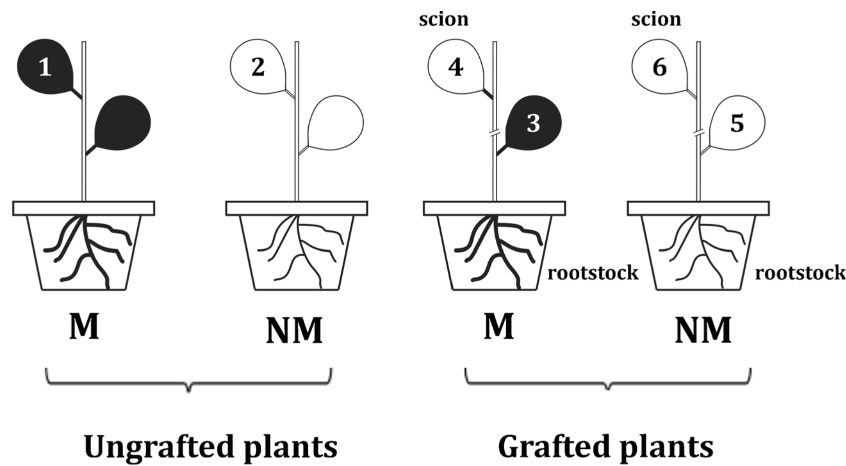

Fig. 1 Schematic representation of the grafting assays. 1) Leaf of control mycorrhizal non-grafted plant (M; black); 2) leaf of control non-colonized non-grafted plant (NM; white); (3) leaf of mycorrhizal rootstock (/M R); 4) scion obtained from a non-mycorrhizal plant grafted onto a mycorrhizal rootstock (NM Sc/M R); 5) leaf of non-mycorrhizal rootstock (/NM R); 6) scion obtained from a non-mycorrhizal plant grafted onto a non-mycorrhizal rootstock (NM Sc/NM R)

genotype (Table 1). Significantly smaller lesions caused by the pathogen were observed in leaflets from mycorrhizal A-55 and Az Reg87 (M 20 μM-P) plants fertilized with low phosphate, as compared to non-mycorrhizal plants fertilized with low (NM 20 μM-P) or regular (NM 200 μM-P) phosphate content. However, no differences in damage were observed in Az Hig common bean plants, regardless of mycorrhizal colonization or phosphate concentration (low or regular phosphate; Table 1), indicating that mycorrhiza-induced protection was not triggered in this genotype. With the exception of the A-55 NM 20 μM-P shoot, no significant differences in shoot or root biomass were observed among treatments in the common bean genotypes tested (Table 1).

3.2 Mycorrhizal colonized tomato var Missouri displays mycorrhiza-induced disease protection against *X. campestris* pv. *vesicatoria* but not Micro-Tom cv.

Mycorrhiza-induced protection against the foliar pathogen *X. campestris* pv. *vesicatoria* was analyzed in two different tomato varieties (Missouri and Micro-Tom). Fewer lesions were observed per leaflet in M 20 μM-P as compared to NM 20 μM-P and NM 200 μM-P Missouri variety plants. However, no differences in the number of lesions were found among treatments in the Micro-Tom plants (Table 2). Regarding biomass production, the two genotypes responded differently to phosphate fertilization.

Table 1 Percentage of root colonization by the AMF *R. irregularis*, growth (g), and lesion diameter (cm) caused by the foliar pathogen *S. sclerotiorum* in three common bean genotypes

Treatment	Root colonization (%)	Growth		Lesion diameter (cm)
		Root (g)	Shoot (g)	
A-55 M 20 μM-P [x]	48 [y] ± 23	3.5 ± 0.7 a[z]	2.98 ± 0.46 a	1.10 a [z]
A-55 NM 20 μM-P	–	3.36 ± 1.0 a	2.33 ± 0.51 b	2.12 b
A-55 NM 200 μM-P	–	2.67 ± 0.39 a	2.67 ± 0.75 ab	1.87 b
Az Reg 87 M 20 μM-P	20 ± 10	4.93 ± 0.46 a	3.23 ± 0.45 a	1.59 a
Az Reg 87 NM 20 μM-P	–	4.90 ± 1.5 a	2.90 ± 0.67 a	2.36 b
Az Reg 87 NM 200 μM-P	–	5.4 ± 0.44 a	3.10 ± 0.90 a	2.11 b
Az Hig M 20 μM-P	23 ± 10	3.51 ± 0.76 a	2.98 ± 0.46 a	2.67 a
Az Hig NM 20 μM-P	–	3.36 ± 1.36 a	2.33 ± 0.51 a	2.77 a
Az Hig NM 200 μM-P	–	2.64 ± 0.75 a	2.67 ± 0.40 a	2.33 a

[x] (M 20 μM-P) mycorrhizal and (NM 20 μM-P) non-mycorrhizal plants fertilized with low phosphate; (NM 200 μM-P) non-mycorrhizal plants were fertilized with regular phosphate. Lesion diameter measurements were taken 48 h after inoculation with *S. sclerotiorum*

[y] Eight-day-old common bean plants were inoculated with 500 spores of *R. irregularis*, maintained for six weeks, and challenged with *S. sclerotiorum* for 48 h. Percentage of colonization was determined after *S. sclerotiorum* assays

[z] Data labeled 'a' and 'b' are significantly different ($P = 0.05$) according to Tukey's means comparison test ($n = 5$)

± indicates standard deviation

Table 2 Percentage of root colonization by the AMF *R. irregularis*, growth (g), and number of lesions per leaflet caused by the foliar pathogen *X. campestris* pv. *vesicatoria* in two tomato genotypes

Treatment	Root colonization (%)	Growth		No. lesions/leaflet
		Root (g)	Shoot (g)	
Missouri M 20 μM P [x]	66.34 ± 3.43 [y]	5.03 + 0.4 a[z]	19.10 + 1.9 a	34 a[z]
Missouri NM 20 μM P	–	5.15 + 0.9 a	19.13 + 2.5 a	75 b
Missouri NM 200 μM P	–	5.18 + 0.7 a	18.72 + 2.3 a	63 b
Micro-Tom M 20 μM P	72.99 ± 9.79	2.32 + 0.5 a	1.24 + 0.2 a	10.27 a
Micro-Tom NM 20 μM P	–	1.57 + 0.8 a	1.16 + 0.3 a	10.57 a
Micro-Tom NM 200 μM P	–	5.46 + 0.7 b	1.87 + 0.1 b	10.31 a

[x] (M 20 μM-P) mycorrhizal and (NM 20 μM-P) non-mycorrhizal plants fertilized with low phosphate; (NM 200 μM-P) non-mycorrhizal plants fertilized with regular phosphate

[y] Tomato plants were inoculated with 400 spores of *R. irregularis* and challenged with *X. campestris* pv. *vesicatoria*. Percentage of mycorrhizal colonization was determined after *X. campestris* pv. *vesicatoria* assays

[z] Data labeled 'a' and 'b' are significantly different ($P = 0.05$) according to Tukey's means comparison test ($n = 4$)

± indicates standard deviation

Whereas the Missouri variety did not show any difference in growth under different phosphate nutrition and mycorrhizal conditions, a positive growth response was observed in the Micro-Tom cultivar when plants were fertilized with 200 μM as compared to 20 μM phosphate (M and NM). These differences in biomass production in the Micro-Tom cultivar were not associated with a defense response, since no differences in the number of lesions were observed in M and NM plants, irrespective of their phosphate nutrition (Table 2).

3.3 Mycorrhiza-induced disease protection is acquired by scions from non-mycorrhizal plants grafted onto mycorrhizal rootstocks

Common bean Az Reg87 and tomato var Missouri were used for the grafting experiment, since they displayed mycorrhiza-induced protection against *S. sclerotiorum* and *X. campestris* pv. *vesicatoria*, respectively. As expected, leaves from mycorrhizal plants exhibited smaller lesion diameters than leaves from non-mycorrhizal plants in common bean (Fig. 2a), as well as in tomato (Fig. 2b). Scions obtained from non-mycorrhizal plants and grafted onto mycorrhizal rootstocks (3, in Fig. 2) showed similar lesion sizes as compared to common bean and tomato leaves from mycorrhizal rootstocks (4, in Fig. 2). Conversely, scions from non-mycorrhizal plants (5, in Fig. 2) grafted onto non-mycorrhizal rootstocks exhibited larger lesions than scions from non-mycorrhizal plants grafted onto mycorrhizal rootstocks (3, in Fig. 2), and were similar to leaves from non-mycorrhizal rootstocks (6, in Fig. 2). In these experiments, the percentage of root colonization by *R. irregularis* in common bean (Az Reg87) and in tomato (var. Missouri) was 18 and 31 %, respectively.

4 Discussion

We found that *R. irregularis* colonization of the root system in Az Reg87 and A-55 common bean genotypes, as well as tomato var Missouri, induced protection against *S. sclerotiorum* and *X. campestris* pv. *vesicatoria*, respectively. These results represent novel experimental evidence in support of the existence of mycorrhiza-induced disease protection against these two plant-pathogen systems. Since the establishment of mycorrhizal symbiosis is favored by low phosphate fertilization, AMF-inoculated and non-inoculated plants were fertilized with Hoagland's solution containing one-tenth of the regular content of phosphate. A regular phosphate (200 μM) non-mycorrhizal control was included to control for any response that could be attributed to phosphate nutrition rather than mycorrhizal colonization. Low and regular phosphate-fertilized non-mycorrhizal plants showed similar levels of pathogen infection, which was higher than in mycorrhizal plants fertilized with low phosphate. This indicates that the enhanced disease protection is triggered by AMF colonization and not by the nutritional status of the plant, as previously reported in other plant-pathogen systems (Fritz et al. 2006; Liu et al. 2007; Trotta et al. 1996). Notably, this reduction in disease symptoms was neither observed in common bean Az Hig nor in tomato Micro-Tom. These two genotypes were colonized at the same level as the genotypes that displayed an increased protection (Tables 1 and 2) then; the absence of mycorrhiza-induced protection was not related to deficient mycorrhiza colonization, indicating that this disease protection is a genotype-specific trait. This is consistent with a previous report in which mycorrhizal symbiosis enhanced accumulation of root chemical defenses in a plant genotype-dependent way (De Deyn et al. 2009). Although regular phosphate fertilization (200 μM) in Micro-Tom tomato induced an increase in growth compared to low phosphate fertilization (20 μM); this was not associated with an increase in protection

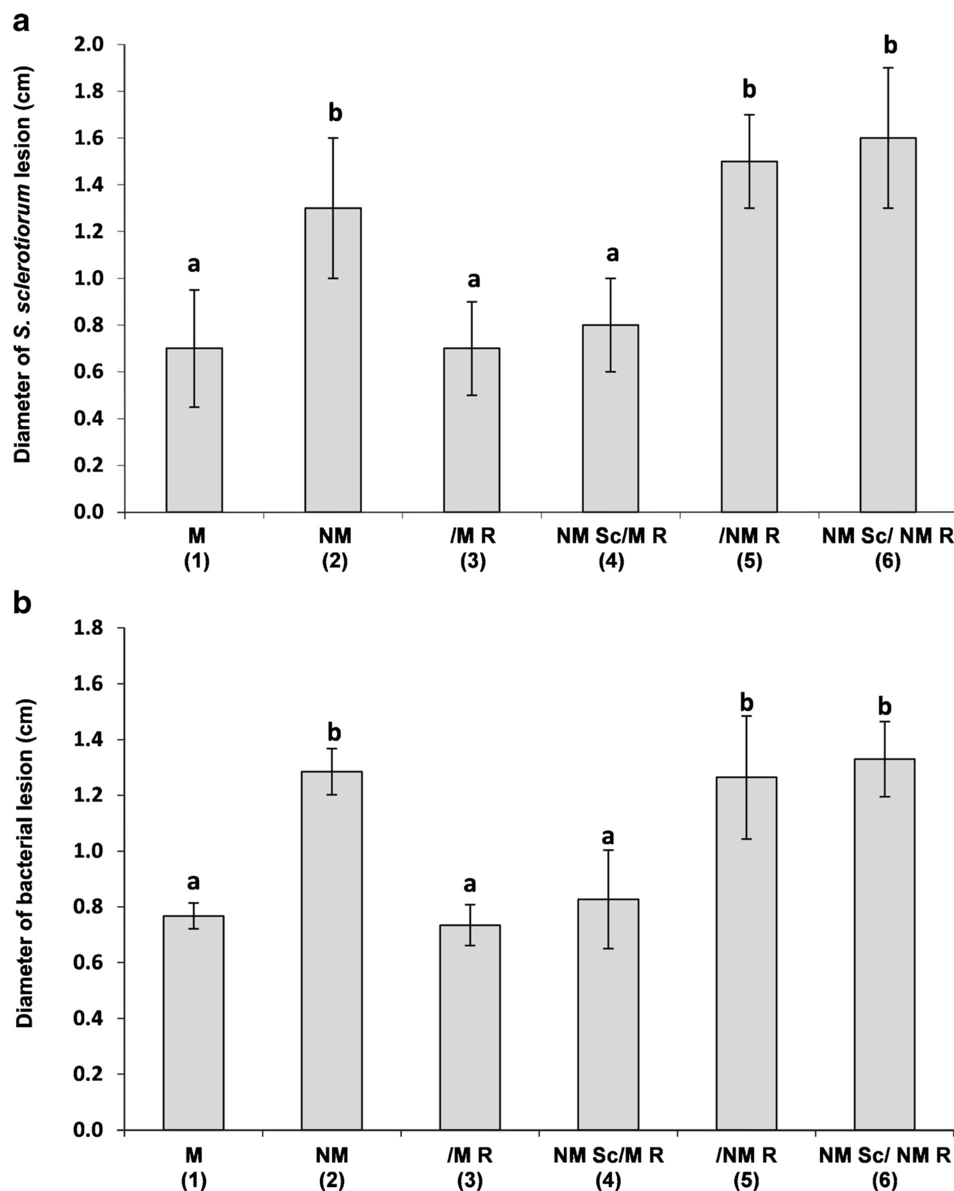

Fig. 2 Diameter (cm) of lesions caused by *S. sclerotiorum* infection in leaflets of Az Reg87 common beans (**a**), and diameter (cm) of lesions caused after 9 days of *X. campestris* pv. *vesicatoria* infiltration in leaflets of tomato var Missouri (**b**). M (1) Leaf of mycorrhizal non-grafted plant; NM (2) leaf of non-mycorrhizal non-grafted plant;/M R (3) leaf of mycorrhizal rootstock; NM Sc/M R (4) scion obtained from a non-mycorrhizal plant grafted onto a mycorrhizal rootstock;/NM R (5) leaf of non-mycorrhizal rootstock; NM Sc/NM R (6) scion obtained from non-mycorrhizal plant grafted onto a non-mycorrhizal rootstock. Data labeled '*a*' and '*b*' are significantly different ($P < 0.05$) according to Tukey's mean test, $n = 4$. Error bars indicate standard deviations

to *X. campestris*. These results support the hypothesis that the induced protection observed in these experiments is regulated by mycorrhiza colonization rather than by phosphate fertilization and/or growth.

There is also published evidence to support the idea that mycorriza-induced protection, as well as ISR (induced systemic resistance) elicited by rhizobacteria, is effective against necrotrophic pathogens (but not biotrophs), indicating that the effect of protection on pathogens induced by mycorrhiza depends on their lifestyle (Pozo and Azcon-Aguilar 2007). Our results are consistent with this view, since neither *S. sclerotiorum* nor *X. campestris* pv. *vesicatoria* have biotrophic lifestyles (Agrios 2005). Finally, as we observed, the genotype of the plant determines whether mycorrhizal colonization can induce disease protection (in addition to the lifestyle of the foliar attacker).

The role of AMF colonization in inducing protection to root and foliar pathogens has been largely overlooked by breeders, and it is possible that this trait has been inadvertently eliminated from bred lines (*cf.* Smith and Read 1997). So far, only partial physiological resistance to *S. sclerotiorum* has been identified in common bean, meaning that broad-spectrum defense mechanisms such as mycorrhiza-induced protection may be incorporated in an integrated program for management of white mold caused by *S. sclerotiorum* in common bean. On the other hand, grafting of vegetable crops has already been used to control some diseases, by using resistant varieties as rootstocks (King et al. 2008). Our results support the view that mycorrhiza-colonized rootstocks may be used as an additional strategy for increasing disease protection, in particular for foliar diseases. However, it remains to be demonstrated that mycorrhiza-induced protection can be triggered in

scions originating from a different variety other than the rootstock. Grafting incompatibility among the genotypes used in the present work prevented testing this hypothesis (Data not shown).

Changes in plant architecture, root exudates and activation of plant defense mechanisms have all been suggested to regulate the mycorrhiza-induced protection response (Pozo et al. 2010). However, the mechanism that regulates the systemic effect of mycorrhizal colonization upon induction of protection to fungal and bacterial foliar pathogens remains unknown. Mycorrhiza-induced protection has also been proposed to be the result of an additive effect of plant defense responses to mycorrhiza colonization and ISR responses to rhizobacteria in the mycorrhizosphere (Cameron et al. 2013). Currently we cannot rule out this possibility, since our experiments were not performed under sterile conditions. Furthermore, although we used sterilized substrates, it is certainly possible that some rhizobacteria may have been established in mycorrhiza-colonized roots in our experiments, as well as in the non-mycorrhizal controls. However, elucidating the existence of specific rhizobacteria interacting with mycorrhizal roots, or their potential cumulative effect, requires further experimentation.

It has been assumed that a mycorrhiza-generated root signal could be translocated to the shoot through vascular tissues in order to trigger a defense response in aboveground plant parts. However, it was not known whether a shoot branch could acquire the protection even though it comes from a non-mycorrhizal plant. We examined this hypothesis via grafting experiments using two different plant-pathogen systems: a legume challenged with a fungal pathogen (common bean/*S. sclerotiorum*), and a solanaceous plant challenged with a bacterial pathogen (tomato/*X. campestris* pv. *vesicatoria*). Our data indicate for both plant-pathogen systems that scions obtained from non-mycorrhizal plants acquire the ability to display mycorrhizal induced disease protection via their grafting onto mycorrhizal rootstocks (Fig. 2). This suggests that a signal moves from colonized roots to the upper parts of plants, enabling the induction of pathogen defense in non-mycorrhizal branches grafted to mycorrhizal rootstocks. This phenomenon could possibly involve the movement of specific proteins and RNA molecules (or even the plant hormone abscisic acid) as the potential signal, since the participation of these molecules has previously been demonstrated to be responsible for long-distance signaling, mainly through the vasculature (Harada 2010; Herrera-Medina et al. 2007; Lucas et al. 2001). Finally, recently published work suggests that 9-oxylipin pathway-derived molecules may be involved in the signaling process, since silencing of a specific common bean root-expressing lipoxygenase (*PvLOX2*) prevents mycorrhiza induced resistance onset in shoots (Mora-Romero et al. 2015).

Grafting experiments have already been performed on mycorrhizal plants as a mean to test the compatibility between host and non-host mycorrhizal plants (Gianinazzi-Pearson and Gianinazzi 1992). However, our study is the first to use grafting to investigate systemic mycorrhiza-induced protection against foliar pathogens. In addition to the abundant evidence in support of the view that a signal moves from mycorrhizal roots to shoots to confer disease protection, our results demonstrate that mycorrhiza-induced protection can be acquired by an already developed tissue which has not been exposed to the signal.

Future studies should aim to perform inter-variety grafting and rootstock-scion reciprocal grafting, which will provide insight on the possible nature of the moving signal, as well as the persistence of mycorrhiza induce protection in scions from a mycorrhizal plant grafted on to a non-mycorrhizal rootstock. Thus, the grafting experimental system may be a feasible technical approach for future studies on the molecular mechanisms involved in the systemic signaling of mycorrhiza-induced disease protection.

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