RESEARCH ARTICLE

The bean rhizosphere *Pseudomonas aeruginosa* strain RZ9 strongly reduces *Fusarium culmorum* growth and infectiveness of plant roots

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Abstract

A faba bean rhizospheric *Pseudomonas aeruginosa* isolate RZ9 was used for studying its antifungal activity and protecting effects of faba bean and common bean against the root pathogen *Fusarium culmorum* strain MZB47. The dual culture tests showed that RZ9 inhibits MZB47 *in vitro* growth by 56%. When mixing RZ9 cell suspension with MZB47 macroconidia at equal proportion, the macroconidia viability was reduced with 70%. Pathogenicity tests conducted in sterile conditions showed that MZB47 caused an intense root rotting in faba bean 'Aquadulce' plantlets and a slight level in common bean 'Coco blanc'. This was associated to significant decreases in plant growth only in 'Aquadulce', reducing shoot dry weight (DW) by 82% and root DW by 70%. In soil samples, MZB47 caused severe root rotting and induced significant decreases in shoot DW (up to 51%) and root DW (up to 60%) for both beans. It was associated to a decrease in nodule number by 73% and 52% for faba bean and common bean, respectively. Biocontrol assays revealed that the inoculation of RZ9 to MZB47-treated plantlets enhanced shoot DWs (25% and 110%) and root DWs (29% and 67%), in faba bean and common bean, respectively. Moreover, root rotting levels decreased and nodule number increased in treated compared to untreated plantlets. Collected data highlighted the disease severity of *F. culmorum* and demonstrated the potential of using RZ9 in controlling Fusaria root diseases in beans. Thereby, the current study represents the first report on the biocontrol effectiveness of *P. aeruginosa* against *F. culmorum* in beans.

Additional keywords: biocontrol; root rot; *Vicia faba*; *Phaseolus vulgaris*.

Abbreviations used: DW (dry weight); LB (Luria-Bertani); PDA (potato-dextrose agar); PDB (potato-dextrose broth).

Introduction

Fusarium spp. are soil borne plant pathogens representing a major threat to grain legumes crops (Patkowska *et al.,* 2007; Pierre *et al.,* 2016). The *Fusarium* diseases consist on a loss of seed germination capacity, reduced emergence, vascular wilt, root rot, seed decay rot in host plants that could be responsible of up to 100% yield loss in susceptible cultivars (Patkowska *et al.,* 2007; de Toledo-Souza *et al.,* 2012; Belete *et al.,* 2013). Among *Fusarium* spp*., F. culmorum* is mentioned among the major fungal pathogens of beans associated to severe damages as described above (Tseng *et al.,* 1995; Pięta *et al.,* 2005). In Tunisia,

Fusarium root rot is being a serious threat to bean crops and associated with severe losses in their production, particularly in intensive farming systems and are caused mainly by various *Fusarium* spp. Few options are available for the control of root rot diseases caused by *Fusarium* spp. and other fungi in beans (O'Brien *et al.,* 1991). Seed treatments with chemical fungicides provide only a small zone of protection around the seed, which limit their efficacy (Conner *et al.,* 2014). Beside this fact, healthy and ecological considerations in management of fungal diseases provide a special interest to the biological control strategy based on microbial agents such as bacteria (Winding *et al.,* 2004; Karthikeyan & Gnanamanickam, 2008).

Among bacteria, *Pseudomonas aeruginosa* (common soil and rhizosphere inhabitant) is known for its plant growth promoting effects since it has showed effective biocontrol capacities of some fungal diseases (Perneel *et al.*, 2008; D'aes *et al.*, 2010; Verma *et al.*, 2013). It has been used in the biocontrol of *Phytophtora* spp*., Phythium* spp*., Rhizoctonia* spp*.,* and *Fusarium* spp. in tomato, pepper, cocoyam and beans and has showed effective biocontrol capacities associated with plant growth promotion effects (D'aes *et al.*, 2010; Verma *et al.*, 2013). Their biocontrol effectiveness has been reported to be due to its ability to produce diverse antifungal compounds (Bajpai *et al.*, 2008; Höfte & Altier, 2010). For example, it was found that *P. aeruginosa* produces a specific protease resulting in inhibition of *Fusarium oxysporum* and *Fusarium solani* hyphal extension and spore germination helping in controlling the fungus on host plants (Yen *et al.*, 2006). Hence, these attributes make *P. aeruginosa* suitable target for searching effective biocontrol strains. However, the species is characterized by an immense ecological diversity and their biocontrol characteristics are strain-dependent (Kulasekara & Lory, 2004; Höfte & Altier, 2010). So, the assessment of biocontrol capacity is crucial to select convenient strains among this bacterial species.

Therefore, the objectives of the current work were: (i) to evaluate the diseases incidence of *F. culmorum* strain MZB47 on *Vicia faba* 'Aquadulce' and on *Phaseolus vulgaris* 'Coco blanc', largely cultivated in Tunisia; and (ii) to assess the antagonistic activity of a novel rhizospheric isolate RZ9 of *P. aeruginosa* against *F. culmorum* and whether this antagonistic activity translates into plant growth protecting effects on both beans under soil conditions.

Material and methods

Bacterial, fungal strains growth and preparation of the inoculums

The fungal (*F. culmorum*) strain MZB47 was isolated from faba bean (*Vicia faba* L.) roots from an infested field usually cultivated with cereals/beans located in the region of Medjez Elbab (North Tunisia) according to the protocol of Narayanasamy (2011), and maintained on potato-dextrose agar (PDA) medium at 4°C. The bacterial strain RZ9 was selected among the laboratory collection of faba bean endophytic and rhizospheric bacterial strains on the basis of its antagonistic activity against MZB47. The bacterial strain RZ9 was isolated from the rhizosphere of healthy and well field-grown faba bean plants in the region of Hajeb Elayoun

(Central Tunisia) using the plate dilution technique according to Kumar *et al.* (2012), maintained on Luria-Bertani medium (LB) (Miller, 1972) and stored in 20% glycerol at -80°C. The bacterial inoculant was prepared in 2 litres-Erlenmeyer flasks containing 500 mL of LB broth by inoculating the medium with a pure colony of strain RZ9 and incubating in shaker at 150 rpm, and 28 °C during 24h (exponential phase). Cell density was determined by dilution plating method on solid LB medium and bacterial inoculant was adjusted to 10^8 cfu/mL for use in the biocontrol assays. The MZB47 inoculum was prepared by growing the fungus in Petri plates containing Joffe medium (KH_2PO_4, 1 g/L; $MgSO_4.7H_2O$, 0.5 g/L; starch, 0.2 g/L; sucrose, 0.2 g/L; KNO_3, 1 g/L; KCl, 0.5 g/L; glucose, 0.2 g/L; agar, 20 g/L) at 25°C for 3 weeks in darkness. The macroconidia were harvested in sterile distilled water and filtered through a double layer of sterile gauze. The density of the macroconidia suspension was determined using a cell of Malassez and an hematocytometer (Precicolor, HBG, Germany) and adjusted to 10^6 macroconidia/mL for plant tests.

Molecular identification

The 16S rRNA from the bacterial isolate was amplified using primers fD1 and rD1 (Weisburg *et al.*, 1991) and PCR conditions were as described by Laguerre *et al.* (1994). The primers ITS1 and ITS4 (White *et al.*, 1990) were used to amplify the intergenic sequences flanking the the 5.8S rRNA gene of the fungal isolate MZB47 according to Ko & Jung (2002). PCR products were purified from agarose gels using the Wizard SV Gel and PCR-clean up system from Promega (F-Lambda company, Tunisia). The amplified fragments were sequenced (Macrogen Europe) using the same primers and assembled using the CAP program available on the NCBI website (http://www.ncbi.nlm.njh.gov/blast).

In vitro dual culture tests *P. aeruginosa* strain RZ9/*F. culmorum* strain MZB47

The dual culture tests of the bacterial strain RZ9 and the fungal strain MZB47 were conducted both in PDA and on potato-dextrose broth (PDB) growth media focussing on mycelia growth and on macroconidia germination. The dual culture conducted on solid PDA medium was performed according to Mrabet *et al.*, (2011). Briefly, a fungal plug from a PDA five days-culture (nine mm in diameter) was placed in the centre of 90-mm-diameter Petri dishes containing PDA medium. The antagonistic strain was applied in two opposite streaks at approximately 20 mm from

the fungal plug. The ability of the bacterial strain to inhibit the fungal growth was assessed by measuring the diameter of mycelial growth after three days of incubation at 24°C. Six replicates were considered for each treatment according to the following formula:

$$\text{Growth inhibition (\%)} = [(A-B)/A] \times 100$$

where A = diameter of mycelia growth in control growth; and B = diameter of mycelia growth in dual culture tests.

The effect of strain RZ9 on MZB47 macroconidia germination in PDB medium and mycelia development was performed by incubation of macroconidia (10^6 cfu/mL) with bacterial strain RZ9 cell suspension in exponential phase (10^8 cfu/mL) in 500 µL eppendorf tubes containing a fixed final volume of RZ9 cells/MZB47 macroconidia preparation. The RZ9 cell suspension was applied at percentages of 0, 5, 25, and 50 of the final volume to the MZB47 macroconidia suspension. The differences of volumes between various mixes were completed to the final volume (200 µL) by addition of the convenient volume of LB sterile medium to standardize the manipulation. The germination of macroconidia was examined by light microscope observation at 24 h after incubation at 25°C. A control macroconidia growth which consists on their incubation in pure PDB medium was included. Macroconidia and mycelia were stained with methylene blue and revealed in light microscope. The ability of macroconidia to germinate was assessed in the basis on the emission of mycelia. For each mix of RZ9/MZB47 macroconidia suspensions, the macroconidia were counted using a cell of Malassez with three repetitions for each counting.

Pathogenicity of *F. culmorum* strain MZB47 under sterile growth conditions

The pathogenicity of the fungal strain MZB47 was assessed on germinated seeds at 1-2 cm radicle length, and on growing plants at two leaf stage of faba bean 'Aquadulce' and common bean 'Coco blanc'. For this reason, faba bean (*Vicia faba* L.) 'Aquadulce' and common bean (*Phaseolus vulgaris* L.) 'Coco blanc' seeds were surface-disinfected with 0.1% mercuric chloride for 2 min and abundantly rinsed and then imbibed with sterile distilled water. Disinfected seeds were germinated on autoclaved 0.9% agar medium at 25°C in darkness for 3-4 days. Then, germinated seeds were sown in a mix of peat and vermiculite (1:2) in 0.5 L bottles. Each emerging plantlet was inoculated with 5 mL of 10^6 macroconidia/mL suspension (3-4 days post plantation). The fungal inoculant was spread to the plant stem base using a sterile syringe. Then, treated plants were incubated in greenhouse under controlled conditions (25°C, 60% RH, 10 h/14 h photoperiod, and 500 µE ligth). Plants were watered with sterile distilled water when needed. The disease symptoms of strain MZB47 was assessed at 21 after fungal treatment on the basis of shoot and root dry weight (DW) measurements, and root rotting level using a scoring method of four levels score (0-3), where 0 means no visible infection symptoms and 3 coresponds to the most severe fungal attacks (severe discoloration of roots) according to the scale published by Al-Hamdany & Salih (1986).

Biocontrol of *F. culmorum* strain MZB47 with *P. aeruginosa* strain RZ9 in soil samples under greenhouse conditions

The capacity of the strain RZ9 of *P. aeruginosa* to control *F. culmorum* strain MZB47 on faba bean 'Aquadulce' and common bean 'Coco blanc' was carried out in soils sampled from the Cap Bon region (North Tunisia). Both types of bean seeds were surface-disinfected, germinated, planted and incubated for two months in a greenhouse as previously described. Germinated seeds were planted separately in 2-L PVC pots containing agricultural soil and grown in greenhouse conditions. Four treatments were considered: (i) not inoculated plants, (ii) plants inoculated with the fungal strain MZB47, (iii) plants not inoculated with the pathogen and treated with strain RZ9, and (iv) inoculated plants with the pathogen and treated with the antagonistic strain RZ9. In each biocontrol trial, 5 replications were performed for each treatment. Treatments with *P. aeruginosa*, 10 mL of RZ9 suspension (10^8 cfu/mL) were spread immediately on the pot surface at sowing. MZB47 was inoculated as previously described in the pathogenicity test section. Plants were watered as needed and harvested 60 days post plantation; shoot and root DWs, infected-root biomass fraction showing rotting disease (%), root-infection score as previously described, and the number of nodules were recorded and subjected to analysis of variance (ANOVA) and means were compared with Fisher's LSD test (at *p*=0.05) (www.statsoft.com). Dry weights were measured by incubating shoot and root plant parts of both beans in a drying incubator (WTC binder, 78532 TUTTLINGEN/ Germany) at 60°C till obtaining constant weights. The plant tests were conducted in three in time-separated biocontrol assays under greenhouse controlled conditions (60% RH, 16 h/8 h photoperiod, 26°C).

Results

Molecular identification

The nearly full lenght 16S rRNA gene sequence of strain RZ9 (1409 bp) was deposited in Genbank under Acc. No. KF152932. Comparison of the 16S rRNA gene sequences showed that RZ9 exhibited 100% identity to the *P. aeruginosa* type strain DSM 50071 (Acc. No. NR_117678.1) in 1409 bp sequenced DNA region.

The rRNA gene region including partial sequence of 18S rRNA, ITS1, 5.8S rRNA, ITS2 and a partial sequence of 28S rRNA (517 bp) of the fungal strain MZB47 was deposited in Genbank under accession number KF152933. The MZB47 had 100% identity in the internal transcribed spacers flanking the 5.8S rRNA gene to *Fusarium culmorum* strains (*i.e.* KF896, FC55, CWB501 and ITCC148) in 517 bp sequenced DNA region.

Pathogenicity of *F. culmorum* strain MZB47 under sterile growth conditions

When inoculated to germinated seeds of faba bean 'Aquadulce', MZB47 induced 100% seeds damping-off. However, common bean 'Coco blanc' germinated seeds were not significantly affected by the pathogen. In growing plantlets, strain MZB47 induced a total root rotting (100% of plant roots) in faba bean 'Aquadulce' and at score 3 of disease scoring scale adopted in this study. MZB47 decreased significantly shoot and root growth ($p<0.05$) only for 'Aquadulce', reducing shoot DW by 82% and root DW by 70%, comparatively to control plants (Figs. 1a,b).

In vitro dual culture tests *P. aeruginosa* strain RZ9/*F. culmorum* strain MZB47

Regarding the aggressiveness of MZB47, colonies grew quickly on PDA and invaded all the 90 mm-Petri dish surface after four days of incubation at 24°C. The bacterial strain was found to inhibit the growth of MZB47 by 56% of its control growth (Fig. 2).

Light microscopy observations showed that RZ9 resulted in no germination of MZB47 macroconidia (Figs. 3b,d). In control treatment, MZB47 macroconidia germinated and showed densely mycelial development after 24h of incubation (Figs. 3a,c).

Macroconidia counting showed that when RZ9 cell suspension (10^8 cfu/mL) was mixed with MZB47 macroconidia suspension at 50% of the total volume, the macroconidia number was declined by 70% in comparison to the control treatment (Fig. 4).

a
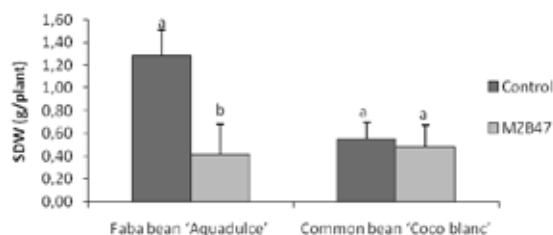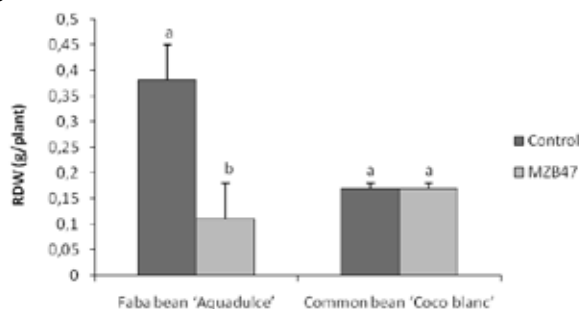
b

Figure 1. Effect of the inoculation with *Fusarium culmorum* strain MZB47 on faba bean 'Aquadulce' and common bean 'Coco blanc' shoot (a) and root (b) dry weights (SDW, RDW). Each value is an average of three replicates. Values with different letters are statistically different (Fishers LSD test, at $p=0.05$). Bars represent standard errors.

Biocontrol of *F. culmorum* strain MZB47 with *P. aeruginosa* strain RZ9 in soil samples under greenhouse conditions

In the biocontrol assays conducted in soil samples, we found absence of significant differences ($p>0.05$) between the three conducted soil assays when considering the recorded parameters (shoot and root
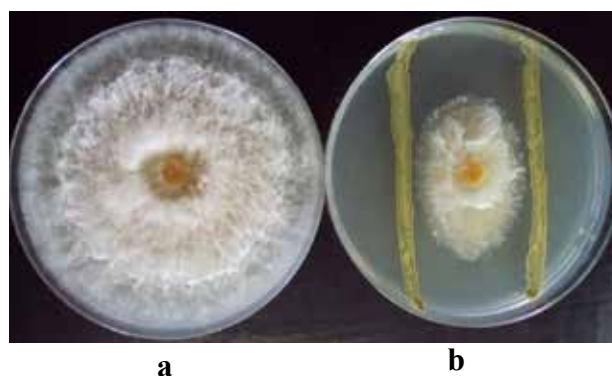

a   **b**

Figure 2. *In vitro* antibiosis of *Pseudomonas aeruginosa* strain RZ9 against *Fusarium culmorum* strain MZB47 in a dual culture test: MZB47 incubated for four days at 25°C (a); inhibition of MZB47 by RZ9 after four days of incubation at 25°C (b).
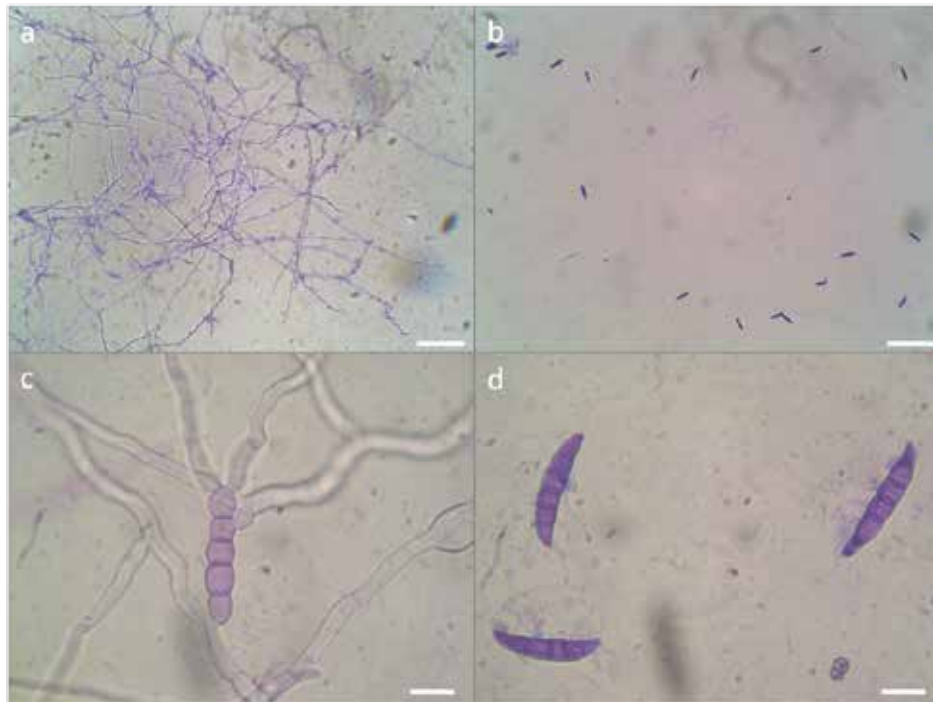
Figure 3. Light microscopy observations of *Fusarium culmorum* strain MZB47 macroconidia germination after 24h incubation in presence or not of *Pseudomonas aeruginosa* strain RZ9. (a) and (c) show the germination of *F. culmorum* macroconidia and mycelia development; (b) and (d) show the complete inhibition of *F. culmorum* macroconidia germination and mycelia development after incubation in presence of *P. aeruginosa* strain RZ9. Bars: 100 µm (a, b), 10 µm (c, d).

DW, root rot biomass, and root infection score). It raised that when inoculated separately, MZB47 resulted in significant decreases of shoot DWs by an average of 51% and 54%, and of root DWs by an average of 60% and 34% in faba bean and common bean, respectively
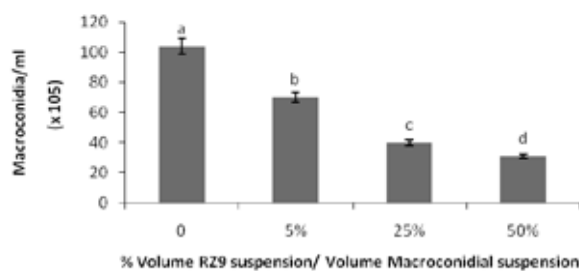

Figure 4. Effect of *Pseudomonas aeruginosa* RZ9 cell suspension (10^8 cfu/mL) on *Fusarium culmorum* MZB47 macroconidia suspension after 24 h of incubation at 25°C. RZ9 cell suspension was mixed at 0%, 5%, 25%, and 50% of the final volume to the MZB47 macroconidia suspension grown in PDB medium. The germination of macroconidia was examined by light microscope observation at 24 h after incubation at 25°C. MZB47 macroconidia were counted using a cell of Malassez with three repetitions for each counting. Values with different letters are statistically different (Fishers LSD test, at *p*=0.05). Bars represent standard errors.

(Fig. 5). Roots were 100% infected with high score levels (score 3) in faba bean 'Aquadulce' and moderate level (score 2) in case of common bean 'Coco blanc'. Moreover, the *F. culmorum* strain MZB47 caused significant decreases in the number of root nodules by an average of 73% and 52% in 'Aquadulce' and 'Coco blanc', respectively. Interestingly, the application of RZ9 to MZB47-treated plantlets resulted in significant increases of shoot DWs by an average of 62% and 81%, and of root DWs by an average of 51% and 143% for 'Aquadulce' and 'Coco blanc', respectively, as compared to MZB47-treated plants used as control. The scores of infection also decreased for both beans (Fig. 5). Moreover, RZ9 significantly reduced the percentage of infected roots and infection levels to low score (Fig. 5). RZ9 also significantly enhanced the root nodule number and partially re-established the decreases caused by *F. culmorum* strain MZB47 strain for both beans (Fig. 5).

Discussion

The pathogenicity test conducted in sterile conditions showed that the inoculation of the *F. culmorum* strain MZB47 caused severe damages on faba bean 'Aquadulce' comparatively to common bean
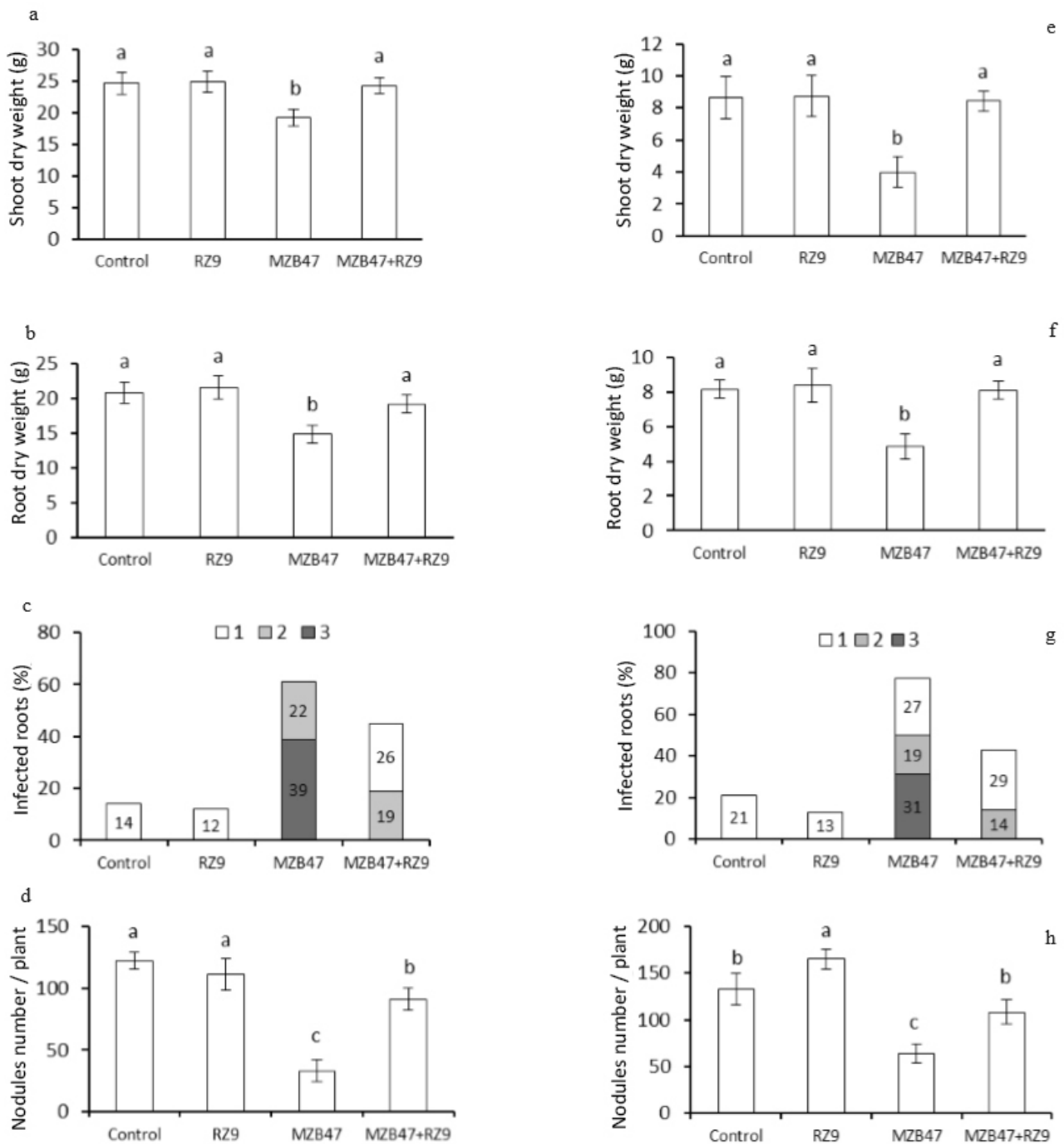
Figure 5. Effects of *Pseudomonas aeruginosa* strain RZ9 on the biocontrol of *Fusarium culmorum* strain MZB47 in faba bean 'Aquadulce' (a, b, c, d) and common bean 'Coco blanc' (e, f, g, h) grown in soil samples under greenhouse conditions. Plants were harvested 60 days post plantation. Each value is an average of three biocontrol assays of 5 replicates for each treatment. Values with different letters are statistically different (Fishers LSD test, at *p*=0.05). Root rot was assessed according to the infection scale of Al-Hamdany & Salih (1986) where (1) indicates slightly infected, (2) moderately infected and (3) highly infected roots.

'Coco blanc'. It resulted in significant decrease of shoot and root DWs in *Vicia faba* by 82% and 70%, respectively. The difference in beans susceptibility to the strain MZB47 could be related to the specificity of fungus-plant interaction since this fungal strain was

isolated from a *Vicia faba* infected plant. This finding underlines the aggressiveness of *F. culmorum* on beans, which corroborates other reports demonstrating its pathogenicity among other *Fusarium* species on various legumes crops (Pięta *et al.,* 2005; Tivoli *et al.,* 2006;

Biddle & Cattlin, 2007; Conner *et al.,* 2014) as well as on other crops such as cereals (Wagacha & Muthomi, 2007; Yli-mattila, 2010). Hence, the severity of the disease incidence of *F. culmorum* revealed in this study urged us to search for an eco-friendly alternative based on faba bean root rhizospheric bacteria with the aim to initiate a fighting-strategy against the pathogen.

Hence, the bacterial strain RZ9 of *P. aeruginosa* used in this work (which was isolated from the rhizosphere of healthy and well field-grown faba bean plants) was selected on the basis of its capacity to inhibit the *in vitro* growth of MZB47 by 56% (Fig. 2). It was the only strain able to inhibit the *in vitro* growth of MZB47 in a screening antibiosis test among near 100 bacterial strains including endophytic, symbiotic and rhizospheric isolates (data not shown). Light microscopy observations showed that inoculation with RZ9 resulted in complete loss of MZB47 macroconidia germination. The antagonistic activity of *P. aeruginosa* strains against other *Fusarium* species such *Fusarium oxysporum* and *Fusarium solani* had been previously reported (Yen *et al.,* 2006; Bajpai *et al.,* 2008; Verma *et al.,* 2013) and was attributed to their capacity to produce various antifungal metabolites such as hydrogen cyanide, 2,4-diacetylphloroglucinol (DAPG), chitinases and glucanases, pyoluteorin, pyrrolnitrin, phenazines, siderophores, and surfactants (Singh *et al.,* 2010; Liang *et al.,* 2011; Yin *et al.,* 2013).

Moreover, the RZ9 cell suspension mixed with the MZB47 macroconidia caused a significant reduction of viability of the macroconidia, as revealed by light microscopy counting-up to 70% decrease of macroconidia number when mixing equally the volumes of MZB47 macroconidia suspension (10^6 cfu/mL) and RZ9 cells suspension (10^8 cfu/mL). The macroconidia lysis could be the result of proteases activities (*i.e.* glucanases and chitinases) as attributed in previous works related to antifungal activities of *P. aeruginosa* strains (Yen *et al.,* 2006). Therefore, the selected strain RZ9 could reduce the host plant vulnerability to *Fusarium* attacks by reducing macroconidia abundance.

The plant experiments in soil samples confirmed the aggressiveness of the *F. culmorum* strain MZB47 on *Vicia faba* L. as revealed in sterile conditions. On 'Coco blanc' and in the soil experiment, the pathogen caused more damages than in sterile conditions. This pathogenicity, on soil experiment, could be linked to the effect of *F. culmorum* strain MZB47 on nodulation by native soil-rhizobia since nodules number was significantly reduced for both beans (Fig. 5). The negative incidence of root rot diseases caused by phytopathogenic fungi, including *Fusarium* spp*.,* on nodulation in legumes has been largely reported (Muthomi *et al.,* 2007; Chang *et al.,* 2013; Conner *et al.,* 2014) and was shown to be associated

with damages on plants growth and production. The present study shows that RZ9 significantly reduced the percentage of infected roots and infection levels to low scores. This finding confirm earlier works demonstrating the capacity of *Pseudomonas* spp. strains to control root rot caused by other *Fusarium* species in beans (Siddiqui & Shaukat, 2003; Verma *et al.,* 2013). In fact, Siddiqui & Shaukat (2003) showed that the inoculation of *P. aeruginosa* strains to tomato plants reduced significantly root-knot development and enhanced plant growth due to their capacity to control the phytopathogenic fungi and to act on soil-fungal composition. We reveal here that RZ9 also significantly enhanced nodules number for both beans. Such finding unveiled the promoting effect of the selected strains of *P. aeruginosa* on root nodulation by native soil rhizobial population. In corroboration with such results, it has been reported that seeds bacterization of chickpea with *P. aeruginosa* strains increased significantly nodules number associated to an increase of shoot and root growth (Sindhu & Dadarwal, 2001; Verma *et al.,* 2013). In fact, *Pseudomonas* species were found to secrete indol-acetic-acid (IAA) and to enhance phosphorus uptake having the properties to positively affect nodulation and plant growth (Verma *et al.,* 2013; Kumar *et al.,* 2016). Hence, the reduced disease incidence on roots of both beans associated to strain RZ9 should enhance nodulation process.

In summary, the present study highlighted the severity of *F. culmorum* attacks particularly on faba bean 'Aquadulce' and underlined the antagonistic effect of the selected *P. aeruginosa* strain RZ9 against the *F. culmorum* strain MZB47, and demonstrated its efficacy to control the pathogen in soil samples for both beans. Further investigations into the selected *P. aeruginosa* strain RZ9 for its antagonistic activity against various bean fungal diseases, and its biocontrol capacity *in planta*, as well as its plant growth promoting effects will be carried out in laboratory and pilot-scale in the future. Moreover, since the species *Pseudomonas aeruginosa* was mentioned to harbor opportunistic strains (Liang *et al.,* 2011), it will be necessary to investigate in cytotoxicity tests whether the strain RZ9 could be used safely or not in biocontrol strategies.

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