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ORIGINAL PAPER

The antibiosis of nodule-endophytic agrobacteria and its potential effect on nodule functioning of *Phaseolus vulgaris*

Seif-Allah Chihaoui · Haythem Mhadhbi · Ridha Mhamdi

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Abstract The effect of the nodule-endophytic *Agrobacterium* strain 10C2 on nodulation, plant growth and nodule functioning of *Phaseolus vulgaris* was investigated using two rhizobial strains differing in their sensitivity to the in vitro antibiosis of the *Agrobacterium* strain. In the case of the sensitive strain, *Agrobacterium* sp. 10C2 induced a significant decrease in the proportion of pink nodules, probably by an antibiosis effect leading to the reduction in the number of bacteroids and thereby a decrease in total soluble proteins, leghaemoglobin content, photosynthesis and nitrogen fixation. In this case, the *Agrobacterium* strain behaved like a plant pathogen and the nodule reacted by increasing guaiacol peroxidase (POX) activity, which assures some physiological processes linked to pathogen control. By contrast, in the case of the resistant strain, the proportion of pink nodules increased, and thereby total soluble proteins, leghaemoglobin content, biomass production and nitrogen fixation were enhanced. The *Agrobacterium* strain is regarded in this case as a plant growth–promoting rhizobacterium and the POX-pathogen reaction was not observed. There was even a decrease in superoxide dismutase activity. The results suggested also that the *Agrobacterium* strain may be also involved in retarding nodule senescence in the case of the resistant strain.

Keywords *Agrobacterium* · Antioxidant enzymes · Endophyte · Nitrogen fixation · Nodule senescence · *Rhizobium*

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S.-A. Chihaoui · H. Mhadhbi · R. Mhamdi (✉)
Laboratory of Legumes, Centre of Biotechnology of Borj-Cédria, BP 901, Hammam-Lif 2050, Tunisia
e-mail: ridha.mhamdi@cbbc.rnrt.tn

Introduction

Various soil microorganisms were found in legume nodules together with symbiotic rhizobia (Tokala et al. 2002; Valverde et al. 2003; Scheublin et al. 2004; Mhamdi et al. 2005; Muresu et al. 2008; Deng et al. 2011; Saidi et al. 2011). Some of the nodule endophytes featured taxa known as human pathogens such as *Enterobacter cloacae*, *Enterobacter kobei*, *Escherichia vulneris*, *Pantoea agglomerans* and *Leclercia adecarboxylata* (Muresu et al. 2010). The high diversity of these nodule endophytes and the worldwide distribution of as yet uninvestigated legumes raise the concern that these represent a general niche that could enhance the hazards posed by microorganisms, mainly those of clinical nature.

The former *Agrobacterium* (syn. *Rhizobium*) species represent one of the endophytes most frequently isolated from root nodules of a wide range of wild and cultivated legumes (de Lajudie et al. 1999; Mhamdi et al. 2002; Wang et al. 2006; Kan et al. 2007; Lei et al. 2008; Murugesan et al. 2010). For commodity reasons and to avoid confusion with symbiotic rhizobia, we will use the epithet "*Agrobacterium*" in this study to designate the endophytic nodule isolates affiliated to the former *Agrobacterium* genus. These agrobacteria were non-pathogenic and failed to nodulate their original host when re-examined for nodulation (de Lajudie et al. 1999). However, when co-inoculated with an infective rhizobial strain, they were able to colonize nodules (Mhamdi et al. 2005). The number of invaded nodules increased with time and may exceed 50 % at 45 days after inoculation (Mhamdi et al. 2005). These endophytic agrobacteria were very diverse; most of them were affiliated to the species *Agrobacterium radiobacter* (syn. *Rhizobium radiobacter*), but others constituted new genomovars or species (Tiwary et al. 2007; Saidi et al.

2011). The interaction of endophytic agrobacteria with host plants appears to be unspecific; nevertheless, it was reported that a specific interaction could exist with *Phaseolus vulgaris* (Wang et al. 2006; Saïdi et al. 2011). Mrabet et al. (2006) showed that some endophytic agrobacteria exercised an antagonistic effect against *Rhizobium gallicum* in antibiosis assays and significantly reduced the nodulation by this species under soil conditions, where nodulation dropped from more than 400 nodules per plant to only five. By contrast, the nodulation by the resistant rhizobia was enhanced. A further study revealed that inoculation with endophytic agrobacteria may affect *Ensifer meliloti* host specificity by inducing a non-specific nodulation on some woody legumes (Liu et al. 2010). However, a recent study showed that inoculation of *Phaseolus vulgaris*, *Medicago laciniata* and *Medicago polymorpha* with an endophytic *Agrobacterium* strain may enhance nodulation and shoot dry weight, but does not affect host range specificity of *S. meliloti* and *Sinorhizobium medicae* (Salem et al. 2012). Other studies showed that endophytic agrobacteria could strongly solubilize phosphates and produce growth hormones (Hameed et al. 2004) or have no significant effect on nodulation and plant growth (Wang et al. 2006). To date, the implications of these endophytic agrobacteria on nodule functioning remain unclear and need to be investigated further.

The main focus of this study was to investigate the effect of the endophytic *Agrobacterium* sp. 10C2 on nodulation, biomass production and nodule functioning of common bean, via the analysis of certain parameters related to nitrogen fixation, protein and leghaemoglobin contents, and mainly the nodule antioxidant enzymes SOD and POX. The experiment was carried out using two rhizobial strains showing contrasting abilities (resistant or sensitive) towards the in vitro antibiosis exercised by the *Agrobacterium* strain.

Materials and methods

Bacterial strains

Three bacterial strains (10C2, 8a3 and Ma1A32) previously isolated from root nodules of common bean grown in Tunisian soils were used in this study. The non-pathogenic endophytic *Agrobacterium* strain 10C2 (syn. AGR2) was shown to exercise in vitro antibiosis against some rhizobial strains (Mhamdi et al. 2005; Mrabet et al. 2006). *Rhizobium gallicum* 8a3 is a very effective and competitive inoculant strain (Mrabet et al. 2005; Mnasri et al. 2007a) that was shown to be sensitive to the antagonistic activity exercised by *Agrobacterium* sp. 10C2 (Mrabet et al. 2006). *Rhizobium etli* Ma1A32 is an effective strain that was found to be resistant to the antibiosis of strain 10C2 (Mrabet et al. 2006).

Co-inoculation experiments

The white-seeded common bean (cv. coco) was used as the host plant for the co-inoculation experiments. Seeds were surface-sterilized with 95 % alcohol then 0.2 % $HgCl_2$ and germinated on perlite as previously described (Mhamdi et al. 1999). Seedlings were cultivated separately in 0.5-L plastic pots filled with sterile sand as previously described (Mnasri et al. 2007b). The bacterial strains were grown to late exponential phase in YEM medium (Vincent 1970), adjusted to an optical density equal to 1 at 620 nm and then diluted to 1/100. An aliquot of one ml from each bacterial strain was used for inoculation of the emerging seedlings. Four treatments were considered: plants mono-inoculated with *R. gallicum* 8a3 or *R. etli* Ma1A32 and plants co-inoculated with *Agrobacterium* sp. 10C2 and one of the rhizobial strains. Non-inoculated plants were also included as negative controls. The experiment was conducted in the glasshouse, and plants were irrigated with the nitrogen-free nutrient solution of Vadez et al. (1996).

Stomatal conductance and chlorophyll content

Net photosynthesis (μmol CO_2 m^{-2} s^{-1}) and stomatal conductance (mol H_2O m^{-2} s^{-1}) were measured with a portable photosynthesis system (Lc-Pot$^+$ 6200, Nebraska, USA) at ambient CO_2 of about 350 μmol mol^{-1}, 75 % relative humidity, 1,000 μmol m^{-2} s^{-1} of photon flux density and 25 °C. All measurements were carried out at 66 days after inoculation between 10:00 and 12:00 h. Leaf chlorophyll content was spectrophotometrically determined according to Torrecillas et al. (1984) at 66 days after inoculation from 100 mg fresh leaf tissues extracted in dark for 72 h in 80 % acetone. Extract absorbance was measured at 649 and 665 nm.

Acetylene reduction assay

The in situ nitrogen-fixing activity was monitored at regular intervals (38, 42, 52, 62, 66 and 74 days after inoculation) by the acetylene reduction assay (ARA) using gaseous phase chromatography with Porapak T column (Hardy et al. 1968). At every sampling date, the root system was incubated in 10 % C_2H_2 atmosphere. After 1 h of incubation, the formed ethylene was measured by injecting 0.5 ml gas sample withdrawn from the root atmosphere of each plant (Mhadhbi et al. 2005).

Nodulation and growth parameters

At 77 days after inoculation, plants were uprooted carefully, washed with cold distilled water and divided into

shoots and roots. Nodules were detached, counted at 4 °C and stored at −80 °C until analysis. Shoot and root dry weights were measured after drying at 70 °C for 3 days.

Leghaemoglobin content

Leghaemoglobin was determined spectrophotometrically according to Shiffmann and Lobel (1970) by using bovine haemoglobin as standard. A sample of 100 mg of fresh nodules was homogenized in 3 ml Drabkin's solution. The homogenate was centrifuged at 5,000 *g* for 15 min. The supernatant was added to 10 ml of Drabkin's solution, homogenized and centrifuged for 30 min at 15,000 *g*. The supernatant was then collected, and absorbance was determined at 540 nm.

Total soluble proteins and antioxidant activities

Total soluble protein was measured according to the method of Bradford (1976). A sample of 0.5 g of fresh nodules was ground in a mortar with 10 % (w/w) polyvinyl-pyrrolidone in 1 mL of 50 mM phosphate buffer (pH 7.8) containing 0.1 mM EDTA, 0.1 % (v/v) Triton X-100, 1 mM phenylmethanesulfonyl fluoride (PMSF). The extract was centrifuged at 13,000 *g* for 20 min, and the supernatant was used to determine enzyme activities. Superoxide dismutase (SOD, EC 1.15.1.1) activity was determined spectrophotometrically by measuring its ability to inhibit the photoreduction of nitroblue tetrazolium (NBT) at 560 nm (Beauchamp and Fridovich 1971). The reaction solution contained 50 mM K-phosphate (pH 7.8), 0.1 mM EDTA, 10 mM L-methionine, 2.7 μM riboflavin and 75 μM NBT. One unit of SOD activity was defined as the amount of enzyme that inhibited 50 % of NBT photoreduction at 25 °C. Guaicol peroxidase (POX, EC 1.11.1.7) was assayed according to Anderson et al. (1995) following the evolution of the kinetics of the enzyme at 470 nm during 1 min ($\varepsilon = 36\ M^{-1}\ cm^{-1}$). POX activity was measured as oxidation of guaiacol (9 mM) in the presence of H_2O_2 (19 mM).

Lipid peroxidation assay

Lipid peroxidation in nodules was assayed using the thiobarbituric acid (TBARS) method modified according to Singh et al. (2007). A sample of 500 mg fresh nodules was homogenized in 3 ml of 0.1 % TCA solution. The homogenate was centrifuged at 10,000 *g* for 20 min, and 0.5 ml of the supernatant was added to 1 ml of 0.5 % TBA in 20 % TCA. The absorbance of the supernatant was determined at 532 nm. The value for non-specific absorption at 600 nm was subtracted. The amount of malondialdehyde (MDA) was calculated using the extinction coefficient $\varepsilon = 155\ mM^{-1}\ cm^{-1}$.

Statistical analysis

Data were submitted to analysis of variance (ANOVA) using the STATISTICA software (http://www.statsoft.com). Means were compared by the Fisher's LSD test ($P < 0.05$). Eight replicates per treatment were considered for nodule number and dry weights. Four replicates were considered for photosynthesis and stomatal conductance. Three replicates were considered for acetylene reduction assay, enzyme activities, total soluble proteins, lipid peroxidation and leghaemoglobin content.

Results

Nodulation and plant growth

Nodule number and plant biomass production were determined 77 days after the inoculation (dai) (Fig. 1). Both rhizobial strains induced similar numbers of nodules; However, *R. gallicum* 8a3 appeared more effective than *R. etli* Ma1A32. In comparison with plants inoculated with *R. gallicum* 8a3, analysis revealed no significant effect of inoculation with *Agrobacterium* sp. 10C2 on the total number of nodules and shoot biomass, but induced an increase in root dry weight. However, *Agrobacterium* sp. 10C2 showed a beneficial effect on the symbiotic parameters in the case of the resistant strain *R. etli* Ma1A32. Shoot and root dry weights were approximately increased twice, and the number of nodules was increased about 30 %. When pink (full active) and green (inactive or senescent) nodules were analysed separately, results showed that inoculation with strain 10C2 induced a reduction in the proportion of pink nodules in the case of *R. gallicum* 8a3 and an increase in the case of *R. etli* Ma1A32 (Fig. 1b). Conversely, the proportion of green nodules increased with *R. gallicum* 8a3 and decreased with *R. etli* Ma1A32.

Nitrogen fixation activity

Both rhizobial strains showed similar curves of acetylene-reducing activity (ARA) with a maximum being reached at flowering stage (around 62 dai), albeit that *R. gallicum* 8a3 appeared more effective in nitrogen fixation than *R. etli* Ma1A32 (Fig. 2). The inoculation with *Agrobacterium* sp. 10C2 induced a significant decrease in the acetylene-reducing

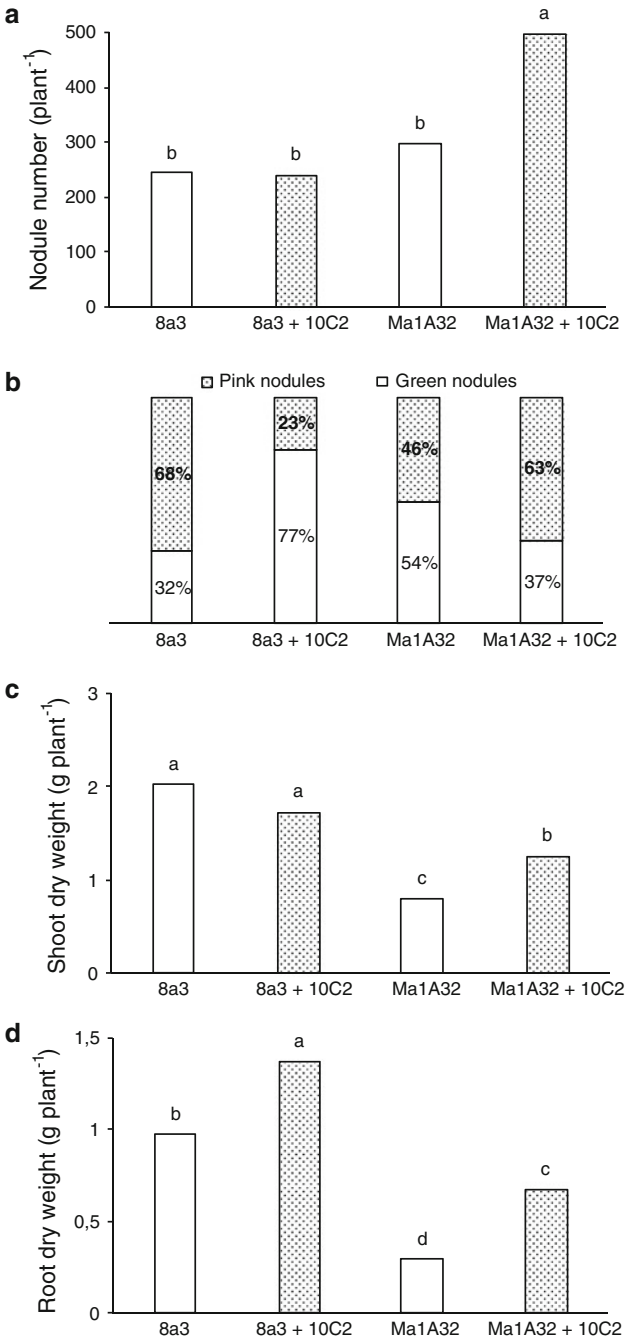

Fig. 1 Effect of co-inoculation of common bean with *Agrobacterium* sp. 10C2 and *R. gallicum* 8a3 or *R. etli* Ma1A32 on **a** total nodule number per plant, **b** proportion of *pink* and *green* nodules, **c** shoot dry weight and **d** root dry weight at 77 days after inoculation. Values with *identical letters* are not significantly different (LSD test, $P < 0.05$, $n = 8$)

activity along nodule life cycle in the case of *R. gallicum* 8a3. However, in the case of *R. etli* Ma1A32, the effect was insignificant till 66 dai, but seemed positive later. In fact, a higher nitrogen-fixing capacity was maintained at 77 dai (4.81 μmol h^{-1} $plant^{-1}$), while it dropped when *R. etli* was alone (2.41 μmol h^{-1} $plant^{-1}$).

Photosynthesis, stomatal conductance and chlorophyll content

Stomatal conductance and net photosynthesis of plants inoculated with *R. gallicum* 8a3 were twice higher than those of plants inoculated with *R etli* Ma1A32 (Fig. 3). The co-inoculation with *Agrobacterium* sp. 10C2 drastically affected both net photosynthesis and stomatal conductance in the case of *R. gallicum* 8a3; about 50 and 40 % decreases were respectively observed. However, insignificant effects were found with the resistant strain *R. etli* Ma1A32.

The plants inoculated with *R. gallicum* 8a3 exhibited also higher total chlorophyll content than plants inoculated with *R. etli* Ma1A32 (Fig. 3d). Likewise, *Agrobacterium* sp. 10C2 induced also a significant decrease in the leaf total chlorophyll content in the case of *R. gallicum* 8a3. However, it did not affect the total chlorophyll content with *R. etli* Ma1A32.

Nodule metabolism

The results showed that total soluble proteins and leghaemoglobin contents are significantly higher in nodules induced by *R. gallicum* 8a3 than in nodules induced by *R. etli* Ma1A32 (Fig. 4). However, co-inoculation with *Agrobacterium* sp.10C2 drastically affected both parameters with *R. gallicum* 8a3. Conversely, *Agrobacterium* sp.10C2 caused a significant increase in the same parameters in the case of *R. etli* Ma1A32. Nevertheless, no significant effects were observed on MDA content with both rhizobial strains (data not shown).

To investigate the activated oxidative stress control within nodules, the antioxidant enzymes SOD and POX were assessed (Fig. 5). The co-inoculation with *Agrobacterium* sp. 10C2 induced a significant increase in POX activity in the case of *R. gallicum* 8a3, but did not statistically affect the SOD activity. By contrast, in the case of *R. etli* Ma1A32, *Agrobacterium* sp. 10C2 induced a clear decrease in SOD activity, while POX activity was statistically unchanged.

In an independent experiment, SOD and POX activities were re-assessed separately in green and pink nodules at the flowering stage (48 dai). Results showed that SOD and POX antioxidant activities in green nodules are higher than those in pink nodules with both rhizobial strains and irrespective of *Agrobacterium* sp. 10C2 (Fig. 6). In the case of *R. gallicum* 8a3, *Agrobacterium* sp. 10C2 induced a decrease in SOD activity in pink nodules and an increase in POX activity in green nodules. Meanwhile, it induced an increase in both SOD and POX activities in pink nodules in the case of *R. etli* Ma1A32 and conversely their decrease in green nodules (Fig. 6).

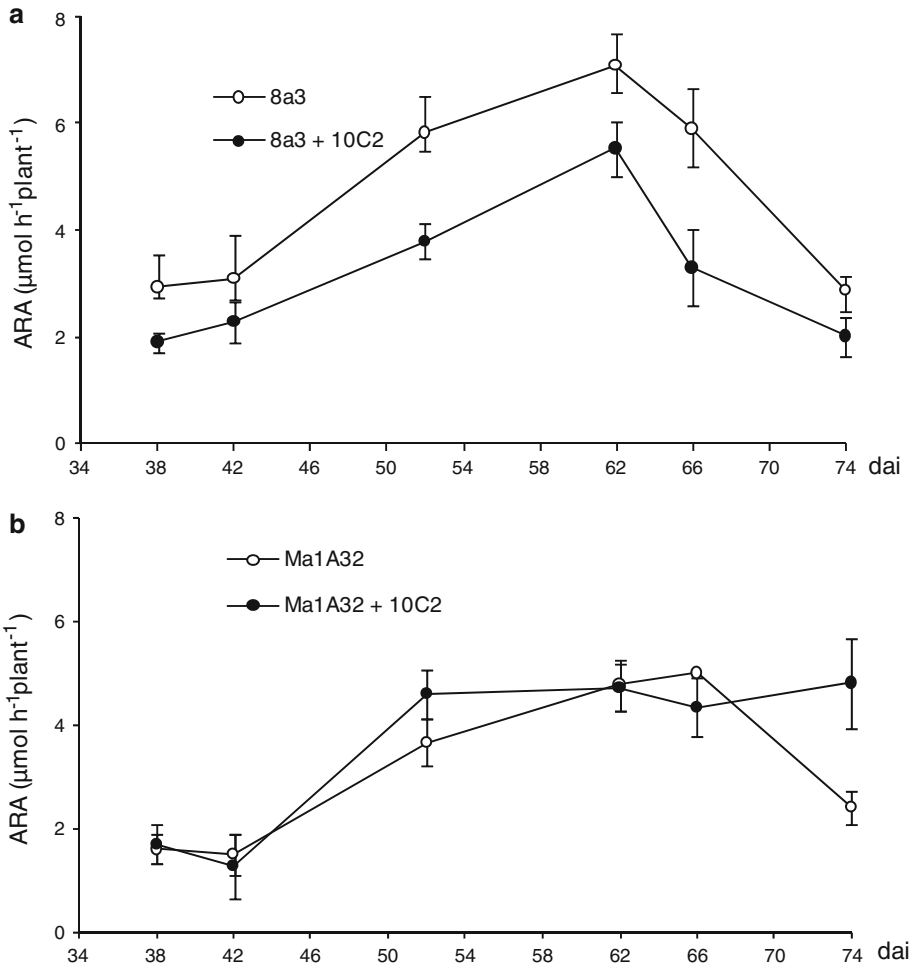

Fig. 2 Effect of *Agrobacterium* sp. 10C2 on nitrogen-fixing activity as estimated by the acetylene reduction assay (ARA) of **a** *R. gallicum* 8a3 and **b** *R. etli* Ma1A32. Each value is the average of three replicated plants. *dai* days after inoculation

Discussion

The non-pathogenic and non-symbiotic *Agrobacterium* strain 10C2 was previously isolated from root nodules of *P. vulgaris* and was shown to be able to colonize nodules when co-inoculated with an infective rhizobial strain (Mhamdi et al. 2005). In this work, we wanted to investigate further the effect of this strain on nodule functioning in relation to its in vitro antibiosis against two rhizobial strains, *R. gallicum* 8a3 (sensitive) and *R. etli* Ma1A32 (resistant).

The results showed that *Agrobacterium* sp. 10C2 did not affect the total nodule number of *R. gallicum* 8a3; however, it reduced the proportion of pink nodules, chlorophyll content, leghaemoglobin and protein contents, photosynthesis, and nitrogen fixation. Meanwhile, the same parameters were increased or unchanged with the resistant strain *R. etli* Ma1A32, suggesting that the observed in vitro antibiosis may play a critical role inside nodules. However, other strain-specific effects, such as the difference in effectiveness between both strains, could not be completely discarded.

The inoculation with *Agrobacterium* sp. 10C2 induced an inhibition of nitrogenase activity in the case of *R. gallicum* 8a3. This is likely due to a handicap in oxygen transport by leghaemoglobin to the respiratory chain of bacteroids. This idea is also supported by the increase in the proportion of green nodules, which also indicates an acceleration of nodule senescence. The most famous, nodule senescence is due to a rapid decline in nitrogen fixation and leghaemoglobin content (Pfeiffer et al. 1983; Mhadhbi et al. 2011). By contrast, in the case of *R. etli* Ma1A32, inoculation with *Agrobacterium* sp. 10C2 showed positive effects on total nodule number, proportion of pink nodules, protein and leghaemoglobin contents, whereas chlorophyll content, stomatal conductance and photosynthesis remained unchanged. There was also no effect on nitrogen fixation till 66 dai. However, beyond this date, nitrogen fixation dropped with *R. etli* alone, but it was maintained in the presence of *Agrobacterium*. These results suggest that *Agrobacterium* could retard the nodule senescence in the case of *R. etli*. Retarding nodule senescence is determinant for enhanced nitrogen fixation and increased yields (Van de Velde et al. 2006; Mhadhbi et al. 2011).

The nitrogen fixation is a costly biochemical process that could stimulate photosynthesis due to the removal of C

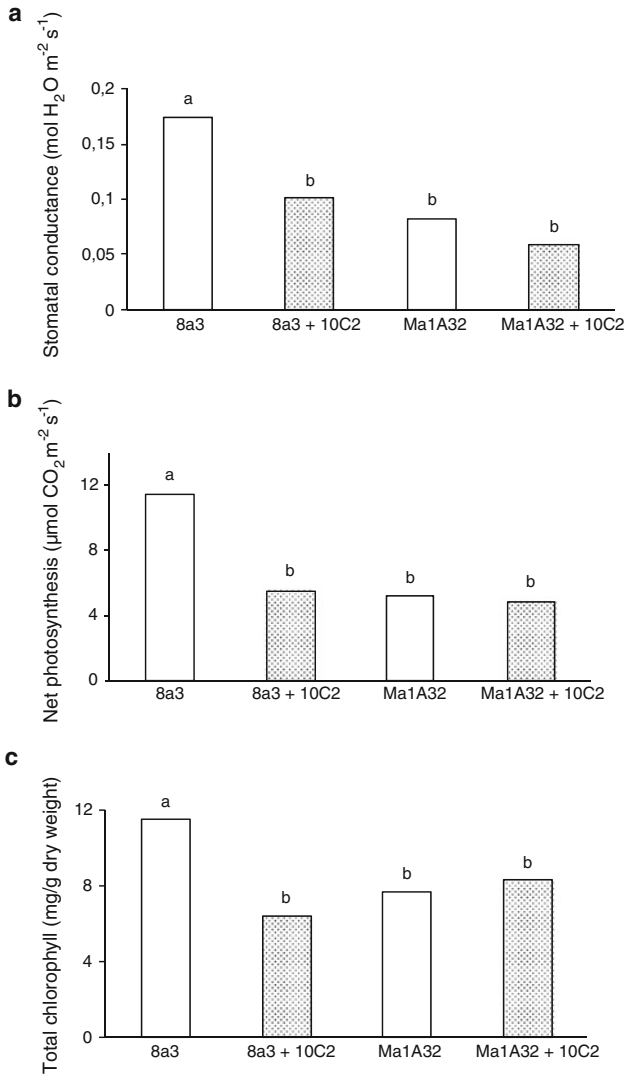

Fig. 3 Effect of co-inoculation of common bean with *Agrobacterium* sp. 10C2 and *R. gallicum* 8a3 or *R. etli* Ma1A32 on **a** stomatal conductance, **b** net photosynthesis and **c** total leaf chlorophyll content at 66 days after inoculation. Values with *identical letters* are not significantly different (LSD test, $P < 0.05$, $n = 4$)

sink limitation by nodule activity (Kaschuk et al. 2010, 2012). In our study, we found that *R. gallicum* 8a3 is more effective than *R. etli* Ma1A32, indicating that it should have larger C costs for nitrogen fixation (Skot et al. 1986; Kaschuk et al. 2012). In fact, nodules are powerful scavengers of photoassimilates (Harris et al. 1985; Ben Salah et al. 2009; Antolin et al. 2010), and legumes adapt their photosynthetic capacity to support the stronger carbon sinks created by faster rates of nitrogen fixation (Kaschuk et al. 2012).

In nodules colonized by both *R. gallicum* 8a3 and *Agrobacterium* sp.10C2, the significant decrease observed in the different parameters could be explained by competition of bacteria for nutrition and survival. Since the *Agrobacterium* strain is antagonistic versus strain 8a3, it would reduce the multiplication and proliferation of this strain along the infection thread leading to a reduced number of bacteroids and infected cells, and thereby a

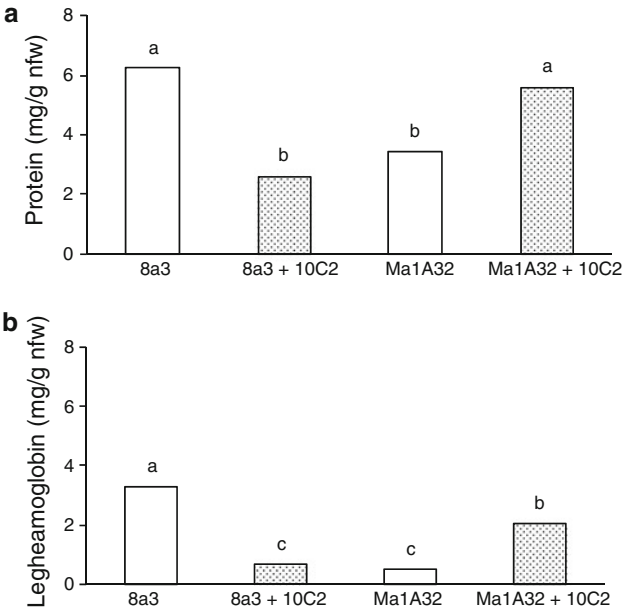

Fig. 4 Effect of co-inoculation of common bean with *Agrobacterium* sp. 10C2 and *R. gallicum* 8a3 or *R. etli* Ma1A32 on **a** nodule protein content and **b** leghaemoglobin content at 77 days after inoculation. Values with *identical letters* are not significantly different (LSD test, $P < 0.05$, $n = 3$)

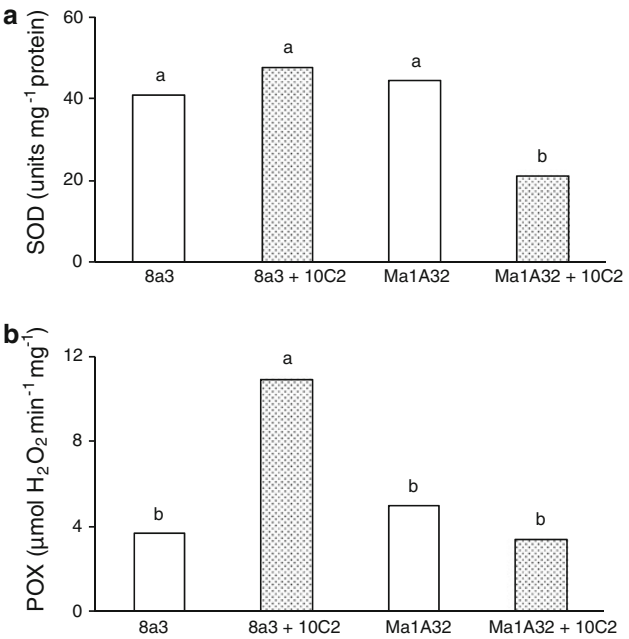

Fig. 5 Effect of co-inoculation of common bean with *Agrobacterium* sp. 10C2 and *R. gallicum* 8a3 or *R. etli* Ma1A32 on **a** superoxide dismutase and **b** guaiacol peroxidise in common bean nodules at 77 days after inoculation. Values with *identical letters* are not significantly different (LSD test, $P < 0.05$, $n = 3$)

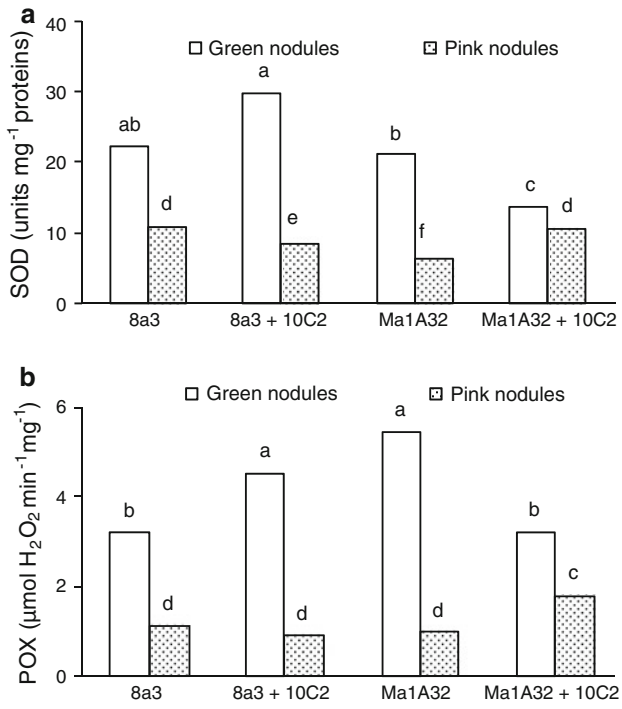

Fig. 6 Effect of co-inoculation of common bean with *Agrobacterium* sp. 10C2 and *R. gallicum* 8a3 or *R. etli* Ma1A32 on **a** superoxide dismutase and **b** guaiacol peroxidase in *pink* and *green* nodules of common bean at 48 days after inoculation. Values with *identical letters* are not significantly different (LSD test, $P < 0.05$, $n = 3$)

reduced nodule efficiency. However, in the case of the resistant *R. etli*, no apparent effect on nitrogen fixation was scored till 66 dai, and hence no effect was also scored on the photosynthetic parameters since legumes adapt their photosynthetic activity according to the efficiency of nitrogenase activity.

In response to microbial invaders, plants produce reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2) and superoxide anion radical (O_2^{-}.) (Aver'yanov 1993; Iwano et al. 2002). These ROS play the role of antibacterial agents, by facilitating the formation of cell wall barriers and inducing hypersensitive response and signal intermediates, which mediates the activation of defence mechanisms (De Gara et al. 2003) such as PR proteins (Yang et al. 1997), salicylic acid biosynthesis (Leon et al. 1995) and systemic acquired resistance (Ryals et al. 1996). To combat or limit the toxicity of ROS, legume nodules display many enzymatic mechanisms (Matamoros et al. 2003; Munns 2005). In order to investigate the nodule metabolism protection, the antioxidant enzymes SOD and POX were assessed. Results showed that *Agrobacterium* sp. 10C2 induced a higher stimulation of POX activity in the case of the sensitive strain, while a significant decrease in the SOD activity was observed in the case of the resistant strain. In addition, SOD and POX activities were higher in green nodules than in pink nodules. Thus, the difference in antioxidant enzymes between both strains is mainly due to differences in proportions of green and pink nodules, indicating differences in senescence level. The increase in POX activity in nodules is considered as indicator of nodule senescence (Sheokand and Swaraj 1996; Hernandez-Jeménez et al. 2002). POX was also reported for its role in cell wall reinforcing in legume pathogen interactions, where it assures some physiological processes linked to cell growth control and stress tolerance via lignification and suberization, cross-linking of cell wall components and synthesis of phytoalexins (Almagro et al. 2009; Djebali et al. 2007, 2011).

The SOD activity was negatively correlated with the proportion of fully active nodules. This means that the increase in pink nodules indicates a less stressing state and consequently a needless antioxidant system mobilization for protection, which explains the slowdown of SOD activity within these nodules. By contrast, SOD stimulation in nodules was reported only in case of severe stressful states (Hernadez-Jimenez et al. 2002; Mhadhbi et al. 2004, 2008).

Our results showed that the variation in nitrogen fixation capacity was correlated with the variation of SOD and POX activities in pink nodules. Following inoculation with the *Agrobacterium* strain, these enzyme activities in pink nodules increased with the resistant strain and decreased with the sensitive one. The expression level of these enzymes seems to be in relation to symbiotic efficiency, and probably they could also play a role in the adaptation against *Agrobacterium* sp. 10C2 in the case of the resistant strain. These two enzymes are reported for their role in protecting nodules against natural or stress-induced senescence (Chen et al. 2004; Mhadhbi et al. 2011), which explains the lasting of a higher nitrogen fixation with the resistant strain after 66 dai. In addition, Chen et al. (2004) also suggested that both enzymes may take part in the process in which plants react against microcystins produced by some species of cyanobacteria.

Conclusion

This study showed that the endophytic *Agrobacterium* sp.10C2 has for the most parameters analysed two contrasting effects according to the rhizobial strain. In the case of the sensitive strain *R. gallicum* 8a3, *Agrobacterium* induced a significant decrease in the proportion of pink nodules, probably by an antibiosis effect leading to a reduction in infected cells and thereby a decrease in total soluble proteins, leghaemoglobin content, photosynthesis and nitrogen fixation capacity. In this case, the *Agrobacterium* strain is regarded as a plant pathogen, and the nodule reacts by increasing POX activity, which assures some physiological processes linked to cell growth control and stress tolerance via lignification and suberization.

However, in the case of the resistant strain *R etli* Ma1A32, the total number of nodules and the proportion of pink nodules were increased, probably through neoformation of nodules, and thereby, total soluble proteins, leghaemoglobin content, biomass production and nitrogen fixation are enhanced. The *Agrobacterium* strain is regarded in this case as a plant growth–promoting rhizobacterium. It would be interesting to go further into this study by conducting histochemical analyses in order to investigate the effect of this *Agrobacterium* strain on nodule ultrastructure.

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