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Full Length Research Paper

Growth promotion and protection against *Orobanche foetida* of chickpea (*Cicer aerietinum*) by two *Rhizobium* strains under greenhouse conditions

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Fetid broomrape (*Orobanche foetida* Poir.) is a chlorophyll lacking holoparasite that subsists on the roots of plants and causes significant damage to the culture of leguminous plants particularly chickpea (*Cicer aerietinum* L.). The investigation was done about potential of *Rhizobium* strains for biological control of *O. foetida* using a commercial chickpea cultivar (Béja 1) and different *Rhizobium* strains. Firstly, benefit of bacterial inoculation on plant growth and efficiency in N-incorporation were demonstrated with four isolates, Azm, Bj, Sd.N2 and Sd.N1. *Rhizobium* strains were investigated for their ability to control *O. foetida* using pot and Petri-dish experiments. Inoculation of chickpeas with two (Azm and Bj) of the *Rhizobium* strains induced a significant decrease in *O. foetida* seed germination and in the number of tubercles on chickpea roots. Furthermore, other symptoms, including the non-penetration of the germ tube of germinated seeds into chickpea roots followed by radical browning and death of the parasite, were observed in the presence of these inoculated chickpea plants. The hypothesis that roots secrete toxic compounds related to *Rhizobium* inoculation is discussed.

Key words: Biological control, *Rhizobium* strains, *Orobanche foetida*, chickpea, necrotic symptoms.

INTRODUCTION

Chickpea (*Cicer arietinum* L.) is one of the most popular vegetable in many regions of the world. Chickpea also known as Bengal gram is an important pulse crop, hence a major source of protein in human diet. In Tunisia, because of biotic and abiotic constraints, the cultivated area and production is instable and decreases. The presence of *Orobanche* spp. in some chickpea growing areas is considered as a limiting factor to the expansion of the crop. *Orobanche* spp. are root parasitic flowering plants devoid of chlorophyll that cause important yield losses in several crops especially in food and feed legumes. In the Mediterranean region and Middle East, *Orobanche* spp. infests about 16 million hectares arable land (Sauerborn, 1991). In Tunisia, yield losses may reach 50 to 80 % in medium to high levels of soil infestation (Kharrat et al., 1994; 2002). Chickpea is a host of three different species of broomrapes, namely crenate broomrape (*Orobanche crenata* Forsk.), fetid broomrape (*O. foetida* Poir.) and Egyptian broomrape (*Phelipanche aegyptiaca* (Pers.). The crop suffers only little damage in the traditional spring sowing, but there is concern that the continued spread of the practice of winter sowing might lead to an outbreak of broomrape infection in chickpea (Rubiales et al., 2003). *Orobanche* is considered as an important agricultural parasite in chickpea in the Beja region of Tunisia (Kharrat et al., 1992). *O. foetida* is known as a weed of faba bean (*Vicia faba* L.) and chickpea

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Table 1. *Rhizobium* strains collected from different localities used in experiments.

Strain	Locality	year
Azm	Nabeul - Tunisia	2002
Bj	Beja – Tunisia	1992
DMS	CIRAD Montpellier, France	1989
Pch 43	ICARDA -Syria	1988
35T	INRA Montpellier-France	1989
SOM	SOM, Morocco	1988
Kor	Nabeul - Tunisia	2002
Sd.N2	Beja – Tunisia	2002
Sd.N1	Beja – Tunisia	2002
Bkh1	El kef – Tunisia	2002
Bouf3	Sousse - Tunisia	2002
Test2	Béja – Tunisia	1992
Mor2	Ariana – Tunisia	1992
Mor1	Ariana – Tunisia	1992
Mat	Bizerte - Tunisia	2002
Rah2	Nabeul - Tunisia	2002
MBou1	Bizerte - Tunisia	1992
Rai3	Jendouba -Tunisia	2002
Isol	Beja – Tunisia	2002
Bouf2	Sousse – Tunisia	1998

in Tunisia, but the species is common in native habitats in other North African countries and Spain. The plant has unbranched stems that bear red or purple flowers that release an unpleasant smell. *P. aegyptiaca* parasitizes faba bean, chickpea and lentil (Mabrouk and Belhadj, 2012) and also many other crops belonging to various families, including *Asteraceae, Brassicaceae, Cucurbitaceae, Fabaceae*, and *Solanaceae*. In Tunisia, this species causes yield losses from 66 to 90% (Abbes et al., 2007).

Several control strategies have been proposed and employed, but none have provided complete protection (Rubiales et al., 2003). *Orobanche* spp. is not usually amenable to control by persistent selective herbicides, since herbicides cannot differentiate between crops and these parasites (Joel et al., 1995). The main bio-control components are virulent insects and fungal pathogens, or fungal toxins (Andolfi et al., 2005; El-Kassas et al., 2005). For a more integrated *Orobanche* management program, a combination of agronomic practices, chemical and biocontrol approaches would be more suitable. In earlier studies, Arfaoui et al. (2005) showed that *Rhizobium* strains can be used as potential biocontrol agents for sustainable agricultural development. Recently, it was shown that symbiosis with some *Rhizobium leguminosarum* strains could induce in pea both better development and lower susceptibility to *O. crenata* (Mabrouk et al., 2007).

In this study, we have initiated a research program aiming at identifying some *Rhizobium* strains which display both compatibility with chickpea and antagonism to *O. foetida*. Firstly, compatibility with chickpea was checked by estimating nodulation-related impact on chickpea growth and N-incorporation in greenhouse experiments. Secondly, their respective antagonistic activity towards fetid broomrape was estimated during development of parasite in both Petri dish and pot experiments.

MATERIALS AND METHODS

Rhizobium bacterial and growth conditions

Rhizobium isolates were obtained from nodules of 50 days old chickpea plants using the crushed nodule method (Vincent, 1970). All isolates were purified and tested for their ability to form nodules on chickpea as previously described (Beck et al., 1993). Forty-two isolates were collected from different localities in Tunisia (Table 1). These strains were grown at 28°C (Vincent, 1970) on a yeast extract mannitol medium containing 0.08% yeast extract (w/v) and 1% mannitol (w/v). Stocks of strains were prepared on yeast extract mannitol agar and kept at −70°C (under 30% of glycerol) for long-term storage and at 4°C as source cultures. A culture was repeated every six months to have stocks of younger generations.

Plant materials

Chickpea (cv. Beja1) seeds were surface-sterilized with 10% calcium hypochlorite for 30 min and then rinsed three times with sterile water. Seeds were placed in Petri-dishes on a sterile filter paper imbibed with H_2O and allowed to germinate at 28°C in the dark for 5 days.

O. foetida Poir. seeds were collected from flowering spikes in infested faba bean fields from Beja (Tunisia) in spring 2003. Washed seeds were surface-sterilized in 10% sodium hypochlorite and rinsed six times with sterile distilled water.

Evaluation of plant growth promotion responses to inoculation

These experiments were performed in greenhouse at the National Agronomic Research Institute, Ariana in Tunisia. Chickpeas were grown under natural light, keeping the minimum temperature above 20°C. Following germination in Petri-dishes, crop seedlings were transferred to plastic pots (1 L) containing 1 kg of sterilized sand, with N-free nutrient solution and inoculated 5 ml of the selected strain (10^7 *Rhizobium* ml^{-1}). Controls were non-inoculated seedlings grown on an irrigated N-free nutrient solution. Shoots were harvested after 45 days and their dry weights recorded after drying in an oven (70°C, 72 h). Nodules were separated from roots for counting and weighing. Shoots were analyzed by Kjeldahl digestion (Parkinson and Allen, 1975) to determinate total shoot N. Phosphorous uptake by chickpea plants was measured according to Nitro-vanadomolibdate method (Fleury and Leclerc, 1943).

Evaluation of *Rhizobium* strains as biological control agents of broomrape (*Orobanche foetida*)

Petri-dishes experiments

The ability of *Rhizobium* strains to control *O. foetida* was studied in Petri-dishes assay (Rubiales et al., 2003). Chickpea seeds were germinated in Petri-dishes on wet glass-fiber filter paper and kept in

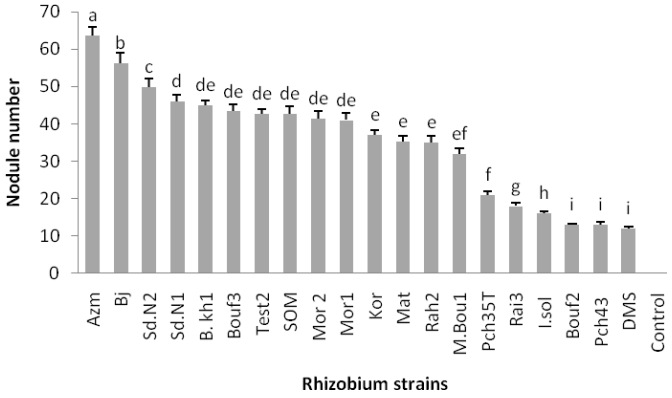

Figure 1. The influence of *Rhizobium* isolates on nodule number in chickpea. The figure shows means of five replicates with standard deviations. Different letters indicate significant differences between treatments according to the Fisher's protected LSD test at $P < 0.05$.

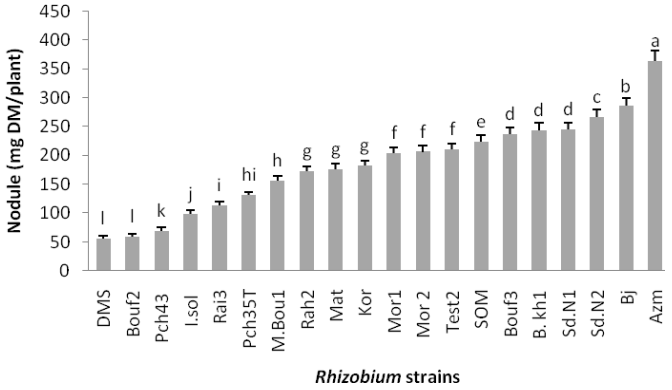

Figure 2. The influence of *Rhizobium* isolates on nodule dry matter in chickpea. The figure shows means of five replicates with standard deviations. Different letters indicate significant differences between treatments according to the Fisher's protected LSD test at $P < 0.05$.

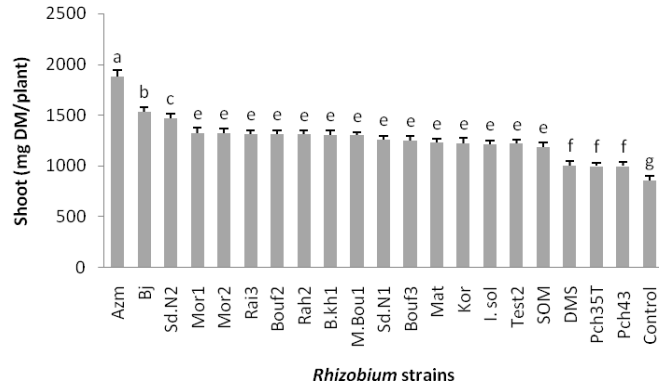

Figure 3. The influence of *Rhizobium* isolates on shoot dry matter in chickpea. The figure shows means of five replicates with standard deviations. Different letters indicate significant differences between treatments according to the Fisher's protected LSD test at $P < 0.05$.

the dark at 20°C for 7 days. When the radical reached 4 to 5 cm length, plant were transferred to new dish (9 cm diameter) using perlite and glass-fiber paper (Whatmann GF/A) as a substrate. *O. foetida* seeds (8 mg) were previously spread on the paper, after being disinfected with sodium hypochlorite (10% for 20 min) and placed in darkness at 20°C for 15 days. Dishes were sealed with parafilm, covered with aluminium foil to exclude light and were placed vertically, the germinating host plant up-wards, in trays with nutrient solution (Vadez et al., 1996). Test plants were maintained in a growth chamber at 20°C with a 14 h photoperiod. *O. foetida* seed germination was evaluated 45 days after transplanting, by using a stereoscopic microscope (x 20). Five hundred seeds located close (< 3 mm) to the chickpea roots were observed. Seeds were considered to be germinated when the germ tube was at least 0.1 mm long; the number of germinated seeds were counted and expressed as percentage of the total. In addition, tubercle formation was evaluated for 45 days after transplanting.

Pot experiments

Twenty *Mesorhizobium ciceri* strains were tested in a pot experiment with five replicates per treatment. Plastic pots (1 L) were filled up to 2/3 of their height with a mixture of local field soil and sand (1:1, v/v). The soil was heated at 120°C for 4 h to destroy weed seeds. *O. foetida* seeds (75 mg) were mixed with the soil. Three chickpea seeds were sown in each pot and 5 ml of inoculum was added. Two control treatments were used: Chickpea only and chickpea with *O. foetida* seeds. Seventeen days after sowing, plants were thinned to one plant per pot and the pots were irrigated twice by week. Experiments were terminated when the host plants in the control treatments stopped growing due to *O. foetida* infection. Roots were taken out of plastic pots and washed. The numbers of healthy and diseased Orobanche shoots were recorded. Root dry weight as well as the dry matter of the above ground host plant were analyzed.

Statistical analysis

The greenhouse cultures were conducted during two consecutive years. Similar results were obtained in both experiments. In all the experiments, five plants were grown per treatment. Consequently, the data are means ± confidence limits ($n = 5$, $\alpha = 0.05$, Student's t test). In addition, data were analyzed by multifactorial analysis of variance (ANOVA, SPSS 12.0 for Windows) and significant differences among treatments were considered at the $P < 0.05$ level.

RESULTS

Evaluation of plant growth promotion responses to inoculation

A remarkable variation in nodule number and nodule biomass was found between strains (Figures 1 and 2). The mean nodule number per plant varied from 12 with DMS strain to 63.67 with Azm, which is the more infective strain (Figure 1). Significant differences in shoot were observed between inoculated plants with different strains and non inoculated controls (Figure 3). Shoot growth was significantly increased by 218.5 % ($p < 0.05$) when the substrate was inoculated with Azm compared to the control. The highest values of controlled growth parameter were obtained with plants growing in soil inoculated with

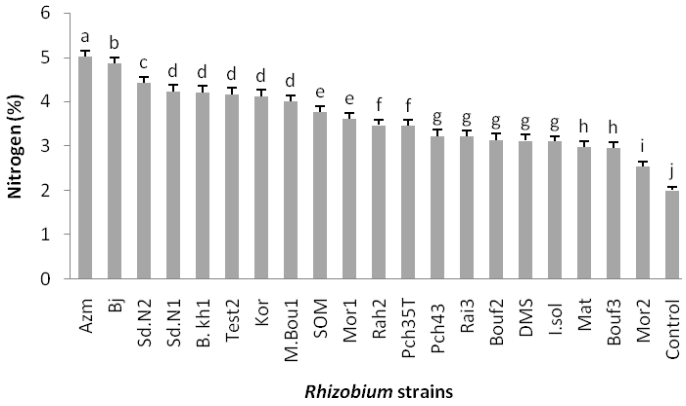

Figure 4. The influence of *Rhizobium* isolates on total N content in chickpea. The figure shows means of five replicates with standard deviations. Different letters indicate significant differences between treatments according to the Fisher's protected LSD test at $P < 0.05$.

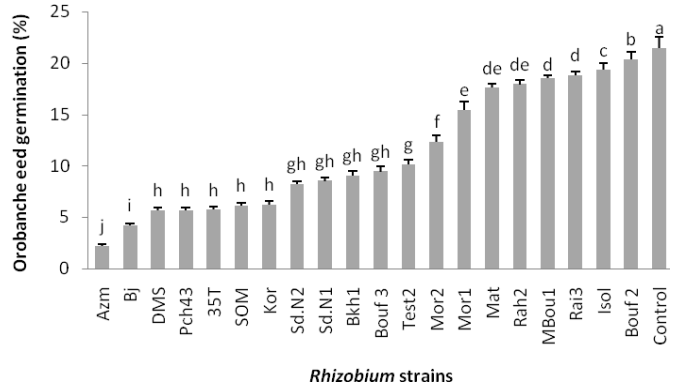

Figure 5. Percentage of germination of *O. foetida* seeds on chickpea inoculated or not with different *Rhizobium* strains. The figure shows means of five replicates with standard deviations. Different letters indicate significant differences between treatments according to the Fisher's protected LSD test at $P < 0.05$.

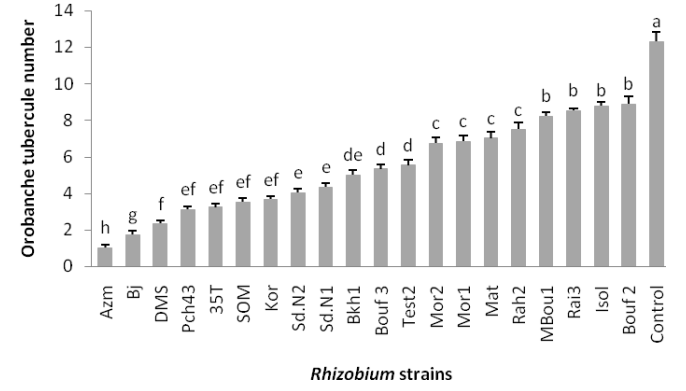

Figure 6. Tubercle numbers on chickpea inoculated or not with different *Rhizobium* strains. The figure shows means of five replicates with standard deviations. Different letters indicate significant differences between treatments according to the Fisher's protected LSD test at $P < 0.05$.

Azm strain compared to the soil without inoculation and the other treatments. Nitrogen concentration in shoot showed an important variation among plants inoculated with efficient *Rhizobium* strains. The highest concentrations were found with Azm, Bj, Sd.N2 and Sd.N1 strains (Figure 4). These strains conferred to chickpea nodulation, nitrogen fixing capacity and shoot growth higher than those attained with the other strains tested.

Evaluation of *Rhizobium* strains for biological control of broomrape (*O. foetida*)

Effect of Rhizobium strains on underground stages of O. foetida

In vitro germination of *O. foetida* seeds was significantly decreased by 80 and 89% after the inoculation with *Rhizobium* strains Bj and Azm respectively (Figure 5). The number of tubercles formed on chickpea roots inoculated with bacteria was significantly reduced compared to the non inoculated control (Figure 6). Tubercles were rarely observed in the roots inoculated with the two bacteria (0 to 2 tubercles).

Effect of Rhizobium strains on O. foetida development in pots

In the pot experiments chickpea inoculated with *Rhizobium* strains (Azm and Bj) resulted in decrease of the number of tubercles on chickpea roots (Figure 7). However, the number of tubercles formed did not differ statistically between control and chickpea inoculated with *Rhizobium* strain Bouf2 (Table 2). The total *Orobanche* dry matter per pot was significantly reduced in chickpea inoculated with different *Rhizobium* strains. Using Azm and Bj strains chickpea dry matter increased (Table 2). In greenhouse *Rhizobium* strains caused necrotic symptoms on *O. foetida* shoots. Whereas, no symptoms occurred on the not inoculated plants.

DISCUSSION

Variations of shoot dry matter (DM), nodule number and nodule DM with the inoculated *Rhizobium* strains confirmed the observations by L'taief et al. (2007) and Karasu et al. (2009) on chickpea-rhizobia symbiosis. Bacterial partner influence on symbiosis performance was mentioned in several reports; hence, Aouani et al. (1997) grouped rhizobial strains according to their effectiveness on common bean cultivar. Differences in strain effectiveness can be associated with compatibility with host plant controlled by a complex interaction mechanism (Hirsch et al., 2001). Our results demonstrate that the Azm, Bj, Sd.N2 and Sd.N1 *Rhizobium* strains had higher N2 fixation efficiency than the other strains assayed. The inoculation of the legumes with *Rhizobium* has often been found to increase symbiotic properties,

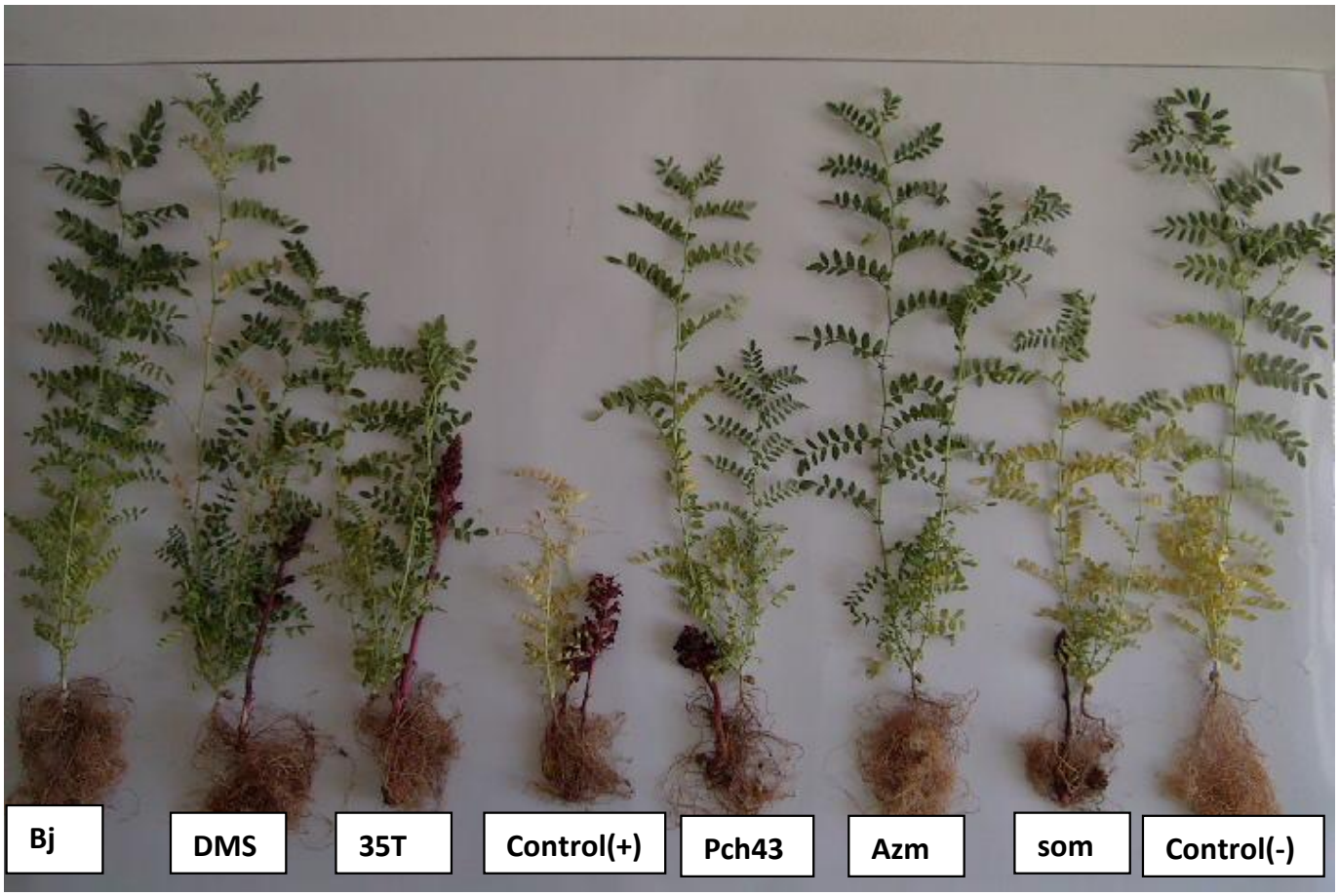

Figure 7. Effect of *Rhizobium* strains on *O. foetida* development in pots.

plant biomass and yields under greenhouse or field conditions (Sindhu et al., 2010). Karasu et al. (2009) observed that inoculation of chickpea seeds with *R. ciceri* isolate had a significant effect on seed yield, plant height, first pod height, number of pods per plant, number of seeds per plant, harvest index and 1,000 seed weight. But, nitrogen doses (applied at 0, 30, 60, 90, and 120 kg ha^{-1} level as ammonium nitrate) had no significant effect on yield and yield components. Local population genotype as crop material gave the highest yield (2,149.1 kg ha^{-1}) among three chickpea genotypes used.

Sindhu et al. (1992) compared the potential of N fixed by *Rhizobium* strains in chickpea using non-nodulating genotype PM233 derived from normal nodulating genotype ICC640. The N fixed by the *Rhizobium* strains Ca534 and Ca219 in parent cultivar gave the plant dry weights more than those obtained by applying urea (80 kg N ha^{-1}) in the non-nodulating mutant PM233, suggesting that in chickpea effective symbiosis with rhizobia provides more than 80 kg N ha^{-1}. In addition to compatibility with chickpea, inoculation with Azm and Bj rhizobia significantly decreases chickpea susceptibility to the parasite *O. foetida*. *In vitro* germination of *O. foetida* seeds decreased significantly after inoculation with both *Rhizobium* strains. Similarly, some bacterial isolates obtained from soil collected from sorghum fields were known to be antagonistic to the root-parasitic weed *Striga*, reducing seed germination of the parasite when inoculated onto sorghum roots (Bouillant et al., 1997). Another *Rhizobium* Morn1 induced some increase of growth of chickpeas, but did not inhibit *O. foetida* germination or tubercle formation on chickpea roots.

Our data may support the hypothesis that inoculation of roots with Azm and Bj *Rhizobium* strains enhances a host defense mechanism in chickpeas. A decrease of stimulant production by inoculated chickpeas could explain the reduced proportion of parasite germination observed in the presence of Azm- and Bj-inoculated plants. The mechanism involved in this hypothetical inoculation-mediated decrease in stimulant production requires clarification. A later resistance was observed in Azm and Bj-inoculated chickpeas, corresponding to browning of attached tubercles. This was obvious in pot experiments as well as in Petri-dish co-cultures similarly, necrosis of Orobanche seedlings was observed for various Orobanche species confronted to resistant hosts (Goldwasser et al., 1997). Similarly, El-Kassas et al. (2005) reported that *Myrothecium verrucaria* isolated from faba bean roots has been

Table 2. Effect of *Rhizobium* strains on *O. foetida* development in pots. The Table shows means of five replicates with standard deviations. Different letters indicate significant differences between treatments according to the Fisher's protected LSD test at $P < 0.05$.

Treatment	Shoot (g DM/plant)	Root (g DM/plant)	Total number of tubercles/plant	*Orobanche* dry matter
Control	0.58 ± 0.01^{j}	0.44 ± 0.02^{k}	6.33 ± 0.24^{a}	0.78 ± 0.13^{a}
Inoculated with Azm	4.02 ± 0.18^{a}	2.10 ± 0.08^{a}	1.05 ± 0.05^{g}	0.25 ± 0.01^{f}
Inoculated with Bj	3.07 ± 0.12^{b}	1.91 ± 0.07^{b}	2.75 ± 0.11^{f}	0.31 ± 0.01^{e}
Inoculated with DMS	2.75 ± 0.11^{c}	1.19 ± 0.04^{d}	2.25 ± 0.10^{f}	0.37 ± 0.08^{e}
Inoculated with Pch43	2.24 ± 0.03^{d}	1.18 ± 0.03^{d}	3.25 ± 0.15^{e}	0.45 ± 0.09^{c}
Inoculated with 35T	1.81 ± 0.08^{e}	1.07 ± 0.04^{f}	2.75 ± 0.12^{f}	0.32 ± 0.04^{e}
Inoculated with SOM	1.60 ± 0.05^{fg}	1.05 ± 0.04^{f}	4.23 ± 0.19^{d}	0.52 ± 0.03^{bc}
Inoculated with Kor	2.06 ± 0.09^{d}	1.30 ± 0.05^{c}	4.75 ± 0.21^{c}	0.57 ± 0.06^{bc}
Inoculated withSd.N2	1.62 ± 0.07^{fg}	0.92 ± 0.04^{h}	4.33 ± 0.19^{d}	0.51 ± 0.07^{bc}
Inoculated with Sd.N1	2.12 ± 0.10^{d}	1.14 ± 0.05e	3.75 ± 0.17^{e}	0.47 ± 0.02^{c}
Inoculated with Bkh1	1.73 ± 0.07^{f}	0.94 ± 0.04^{h}	4.07 ± 0.19^{d}	0.56 ± 0.02^{bc}
Inoculated with Bouf 3	2.14 ± 0.10^{d}	1.09 ± 0.04^{f}	5.33 ± 0.24^{b}	0.60 ± 0.03^{b}
Inoculated with Test2	2.02 ± 0.10^{d}	1.02 ± 0.05^{g}	5.45 ± 0.26^{ab}	0.65 ± 0.02^{ab}
Inoculated with Mor2	1.92 ± 0.08^{f}	1.31 ± 0.06^{c}	5.23 ± 0.26^{b}	0.62 ± 0.03^{b}
Inoculated with Mor1	2.77 ± 0.13^{c}	1.19 ± 0.05^{d}	5.75 ± 0.25^{ab}	0.68 ± 0.03^{ab}
Inoculated with Mat	3.03 ± 0.16^{b}	1.09 ± 0.04^{f}	5.33 ± 0.18^{b}	0.55 ± 0.02^{bc}
Inoculated with Rah2	2.19 ± 0.10^{d}	0.98 ± 0.03^{h}	5.75 ± 0.19^{ab}	0.51 ± 0.02^{bc}

suggesting the central role of the phenylpropanoid/ isoflavonoid pathways in the elicited defence (Mabrouk et al., 2007b; 2007c; 2010).

We conclude that two *Rhizobium* strains (Azm and Bj) protect efficiently their chickpea partners against *O. foetida*. This is based on the observations that, in the presence of the symbionts, chickpeas grew better, preventing parasite attachment and growth of installed tubercles. This treatment could provide a double benefit for *O. foetida* contaminated and nutrient deficient soils. Nevertheless, further studies are needed to better characterize the mechanisms involved in Rhizobium-inoculated chickpea resistance to *O. foetida* before such *Rhizobium* strains are used as biocontrol agents in chickpea fields.

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