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EVALUATION OF EFFICIENT *RHIZOBIUM* ISOLATES AS BIOLOGICAL CONTROL AGENTS OF *OROBANCHE FOETIDA* POIR. PARASITIZING *VICIA FABA* L. MINOR IN TUNISIA

M. BOURAOUI[1,2,*], Z. ABBES[2], N. ABDI[1], I. HMISSI[1] and B. SIFI[1]
[1] *Laboratoire des Sciences et techniques Agronomiques, Institut National de la Recherche Agronomique de Tunisie, Rue Hédi Karray, 2080 Ariana, Tunisia*
[2] *Laboratoire des Grandes Cultures, Institut National de la Recherche Agronomique de Tunisie, Rue Hédi Karray, 2080 Ariana, Tunisia*

Abstract

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Orobanche foetida is an important new agricultural biotic constraint which parasite grain legumes in Tunisia. Several broomrape control strategies have been developed, but without success. The present work aims to select the most efficient *Rhizobium* isolates for symbiotic nitrogen fixation and antagonist to *O. foetida* parasitism in faba bean. For this purpose, hydroponic co-culture and pots experiments were conducted using the commercial faba bean (Badï variety) and different *Rhizobium* isolates. Results showed that in hydroponic co-culture, *O. foetida* germination was significantly decreased (75%) only after inoculation with the *Rhizobium* isolate Mat. The percentage of reduction of tubercle number compared to the control reached 89% with this *Rhizobium* isolate tested. In pot experiments, only Bous.96 isolate reduced significantly the total tubercle number. The number of emerged parasites (stage 5) was significantly decreased with all *Rhizobium* isolates inoculation. A significant increase in faba bean shoot and root dry weight was recorded only with Mat isolate inoculation. No brownish radicals or necrosis in attached parasites was observed in the two trials. The two *Rhizobium* isolates (Mat and Bous.96) are potential candidates as inoculants production for plants growth promotion and chemical nitrogen fertilizer reduction. They would be a good tool to reduce parasitic infestation and to develop sustainable agriculture. The characterization of the resistance induced by these isolates against *O. foetida* and their use in field experiments is suggested.

Key words: Broomrape, *Orobanche foetida*, *Rhizobium*, faba bean, nitrogen fixation, bio-control

Introduction

Over 4000 species of angiosperms are able to parasite other plants. Unfortunately, for farmers, some of these species provide severe constraints to major crops (Parker and Riches, 1993; Rubiales, 2001; Joel et al., 2000; Fenández-Aparicio et al., 2006). By far, the most economically damaging are roots parasites of the genera *Striga* (witch weeds) and *Orobanche* (broomrapes). Witch weeds (*Striga* spp.) are very damaging in tropical Africa to cereals and legumes, which endangers food supplies in many developing countries. Parasitic species of the genus *Orobanche*, commonly called broomrapes, are among the worst weeds (Holm et al., 1997). They are responsible for severe losses to vegetable, legume, and sunflower crops, by their interference with water and mineral intake and by affecting photosynthate partitioning. The broomrapes (*Orobanche* spp.)

E-mail: mani.bouraoui@yahoo.fr; zouhaier.abbes@fst.rnu.tn; neilaabdi@yahoo.fr; imen.hmissi@yahoo.fr; sifi.bouaziz@iresa.agrinet.tn

are widespread in Mediterranean areas in Asia and Southern Europe, attacking dicotyledonous crops and depend entirely on their hosts for all their nutritional requirements (Fenández-Aparicio et al., 2006). In northern Africa, the economically important *Orobanche* species are *O. crenata*, *O. foetida* and *O. ramosa*. *O. crenata* and *O. foetida* mainly attack legumes, especially faba bean. *Orobanche crenata* (crenate broomrape) has threatened legume crops since antiquity, being of economic importance in faba bean (*Vicia faba*), pea (*Pisum sativum*), lentil (*Lens culinaris*), vetches (*Vicia spp.*), grass pea (*Lathyrus sativus*) and other grain and forage legumes (Rubiales et al., 2006). *Orobanche foetida* is widely distributed in natural habitats in the Western Mediterranean area (Portugal, Spain, Morocco, Algeria and Tunisia) parasitizing wild herbaceous leguminous plants (Pujadas Salva, 1999; Abbes et al., 2009). Nevertheless, *O. foetida* should be considered as an emergent important agricultural parasite in faba bean in Tunisia (Kharrat et al., 1992; Abbes et al., 2009) and on common vetch in Morocco (Rubiales et al., 2005). *O. foetida* seems to cause a serious damage on faba bean production in Tunisia. In Tunisia, this species is able to develop on certain cultivated species as broad beans (Kharrat et al., 1992) and can also be installed and develop on chickpea, lentils and vetch with variable levels of parasitism (Kharrat, 2002).

There are difficulties in controlling broomrape parasitism. These difficulties are due to the production of a large number of seeds that can remain dormant in the soil for many years. Broomrape seeds germinate only if stimulated by host root exudates. Once a seed is stimulated, it produces a germ tube that grows in the direction of the root host plant. The germ tube develops a haustorium when it meets the host plant, penetrates the root, and forms a tubercle. The underground tubercle formation and development is the most damaging phase during which the parasite withdraws water, nutrients, and photosynthates from the host. By the time, the parasite emerges from the soil, most of the damage to the host plant has already been occurred (Vurro et al., 2006).

Some strategies of broomrape control have been developed, from cultural practices to chemical control (Parker, 1991; Rubiales et al., 2003; Abbes et al., 2010) but all without unequivocal success, being not feasible, uneconomic, hard to achieve or resulting in incomplete protection. A resort to the biological control against the broomrape by antagonistic agents constitutes an alternative method to control this parasite and to fill the limits of the traditional methods. Indeed, in recent years, the interest of biological control against plant diseases has been stimulated to develop sustainable agriculture and make farmers aware of the danger of pesticide use. Moreover, the biological control can carry a solution to problems, which are partially regulated by other methods of control. The main bio-control components are virulent insects and fungal pathogens, or fungal toxin (Andolfi et al., 2005; El-Kassas et al., 2005). Antagonism of Rhizobacteria with *Striga* is known and results from a negative effect on parasite germination (Bouillant et al., 1997). In contrast, information concerning antagonistic bacteria to *Orobanche* was successfully related by some investigators. Recently, Zermane et al. (2004) identified some *Pseudomonas* and *Ralstonia* strains as natural antagonist to *Orobanche* and Mabrouk et al. (2007a, b) identified some *Rhizobium* strains antagonistic to *O. crenata* in pea. The present work aims, for the first time, to select the most efficient and antagonistic *Rhizobium* strains to control *O. foetida* parasitism in faba bean. For this purpose, we have isolated and selected ten *Rhizobium leguminosarum* strains from faba bean roots in *Orobanche* free areas. The choice of the efficient *Rhizobium leguminosarum* strains was based on the estimation of the number of nodules formed on faba bean and N-incorporation in greenhouse experiments. Their respective antagonistic effect towards *O. foetida* was estimated during both early and later stages of parasite development in both Petri dish and pot experiments.

Materials and Methods

Bacterial strains and growth conditions

Ten *Rhizobium Leguminosarum* strains were isolated from faba bean roots (Table 1). These strains were grown at 28°C (Vincent, 1970) on a yeast extract mannitol medium containing 0.1% yeast extract (w/v) and 1%mannitol (w/v). Stocks of strains were prepared on yeast extract-mannitol agar and kept at 4°C as source cultures. A culture was prepared every six months to have stocks of younger generations.

Table 1
Reference and origin of *Rhizobium* strains

Number	Strain Name	Reference	Origin
1	Beja1	Bj1	Beja,Tunisia
2	Beja2	Bj2	Beja,Tunisia
3	Beja3	Bj3	Beja,Tunisia
4	Bouselem.96	Bous.96	Bouselem,Tunisia
5	Fr.481	Fr.481	ICARDA
6	Fernena	Fern.	Fernena,Tunisia
7	Korba.92	Korba	Korba,Tunisia
8	Mateur	Mat	Mateur,Tunisia
9	SOM	SOM	Maroc,Tunisia
10	Testour	Test	Testour,Tunisia

Plant materials

The faba bean (Badï variety) used in this work is known for its high productivity in Orobanche-free soils and its susceptibility to *O. foetida* and *O. crenata* (JORT, 2004; Abbes et al., 2007; Abbes et al., 2011). Faba bean seeds were surface-sterilized with 6.7% calcium hypochlorite for 15 min and then rinsed three times with sterile distilled-water. Sterilized seeds were placed in Petri dishes on a sterile filter paper imbibed with sterile distilled-water and allowed to germinate at 21±2°C in the dark for seven days.

O. foetida seeds were collected from flowering spikes in infected faba bean fields from Béja (Tunisia) in 2003. Washed seeds were sterilized in 6.7% calcium hypochlorite for 5 min and rinsed five times with sterile distilled-water.

Seeds and *Rhizobium* strains were provided by legume program, field crops laboratory of National Agronomic Research Institute in Tunisia (INRAT).

Evaluation of plant growth promotion responses to inoculation

These experiments were performed in greenhouse at the National Agronomic Research Institute of Tunisia (INRAT). Plants were grown under natural light, keeping the temperature above 25±3°C and in humidity above 78%. Following germination in Petri-dishes, seedlings were transferred to plastic growing pots containing sterilized gravel, with N-free nutriments solution and inoculated with 10 ml of the selected *Rhizobium* strains. Controls were non-inoculated seedlings grown on an irrigated N-free nutriments solution. Eleven Treatments were realized with four replicates each one. Shoots were harvested after 60 days and their dry weights were recorded after being dried in an oven at 70°C during 72h. Shoots were also analyzed by Kjeldahl digestion (Parkinson and Allen, 1975) to determine total shoot N. Nodules were separated from roots for evaluating their number and weight.

Evaluation of *Rhizobium* strains as biological control agent of *O. foetida*

Hydroponic co-culture

The hydroponic co-culture technique was used to evaluate the underground development of root parasitic weeds such as germination, appressorium and haustorium formation and further growth stages since such evaluation is impossible in pot experiments. All the steps of the hydroponic co-culture were performed under sterile conditions. Faba bean and Orobanche seeds was surface sterilized as described above. Five perforations were made in the Plastic Petri dishes (120 x120 x17 mm, Greiner) which is filled with sterilized sand moistened with 50 ml of water and then covered with a water-imbibed fiber glass filter paper: the big one was made in the highest board, to allow the shoot out of the dish, and the others were made on the opposite sides to allow root feeding in culture medium. Sterilized Orobanche seeds (15 mg) were spread between the dish cover and a fiberglass filter paper. Petri dishes were closed and vertically stored in a sterile polypropylene tray containing sterile distilled water. The hole was placed in darkness at 21°C for 10 days. After this pre-conditioning step, faba bean seeds were pre-germinated and imbibed in the inoculums, containing the corresponding strains for 2h, and placed on the fiber glass filter paper in Petri dishes. To this, 5ml of the selected bacterial culture was added. Four replicates for every strain were done and every four dishes was placed separately from the others to be sure that there is no contamination by the other strains. Seven days later, 5ml of the selected bacterial culture was added to improve the presence of the *Rhizobium* strains in the hydroponic co-culture system. This co-culture system was kept in the greenhouse at a temperature above 25±3°C and in humidity above 78%. *O. foetida* seed germination was

evaluated 45 days after inoculation (DAI) by using a binocular microscope. Four squares of 1 cm^2 near infested faba bean roots per Petri dish were observed and the number of germinated seeds counted and expressed as percentage of total seeds. In addition, tubercle formation was counted.

Pot experiments

Five *Rhizobium* strains (Mat., Bj1, SOM, Bous.96, Fern.) was tested in a pot experiment with 10 replicates per treatments. The experiments were performed in greenhouse at the National Agronomic Research Institute in Tunisia (INRAT). Plastic pots with a capacity of 2l were filled with sterile soil as support artificially infested with 30 mg of *O. foetida* seeds. Before transplantation in pots, faba bean seeds were pre-germinated and imbibed in the inoculums, containing the corresponding strains for 2h. Plants were grown under natural light, keeping the temperature above 25±3°C and in humidity above 78%. At maturity stage (120 days after transplantation), roots of infected plants were gently removed from the substrate, washed with water, and the Orobanche attachments were carefully harvested. The harvested Orobanche samples were sorted according to their developmental stage (Labrousse et al., 2001). The S2, S3 and S4 stages correspond to small tubercles without root development, to growing tubercles with crown roots without shoot formation and to the tubercles carrying underground growing shoots, respectively. The first developmental stage S1 that corresponds to the parasite attachment to the host root was not observed now of sampling. The dry weights of root system as well as the dry matter of the aboveground host plant, the number and dry weight of nodule and the number and dry weight of tubercle were recorded.

Statistical analysis

Data are means ± confidence limits. The statistical model for co-culture and pot experiments involved a completely randomized design with five replicates, in which the Rhizobium strain treatment was the unique fixed factor. The experiments were repeated twice. Since the results of the two experiments were similar, they were pooled for statistical analysis. Results were analyzed using the SPSS 15.0 software (Windows edition). Mean comparisons were made using Duncan's multiple-range classification test at P = 0.05.

Results

Evaluation of plant growth promotion responses to rhizobia inoculation

The various isolates of *Rhizobium* tested showed specificity to the plants of faba bean translated by the formation of nodules with indefinite growth. A significant variation in the nodule number and dry matter (Table 2) was observed between the different tested *Rhizo-*

Table 2

Effect of inoculation with *Rhizobium* strains on shoot and root DM, nodulation efficiency and total N content in faba bean

Treatments	Shoot, mgDM/Pl	Root, mgDM/Pl	Nodules number/ Pl	Nodules, mgDM/Pl	Nitrogen, %
Control	780 ±87^{a}	500±56^{a}	0^{a}	0^{a}	1.37±0.88^{a}
Bous.96	1620±664^{ab}	480±271^{a}	29.33±0.88^{b}	70±1^{b}	3.01±0.06^{b}
Korba92	1710±621^{ab}	520±201^{a}	46.67±21.40^{b}	60±11^{b}	2.98±0.17^{b}
Bj2	1810±10^{ab}	500±132^{a}	31±4.35^{b}	70±6^{b}	2.99±0.29^{b}
Bj1	1880±387^{ab}	770±221^{a}	42±14.46^{b}	90±21^{bc}	2.68±0.35^{b}
Fr.481	1970±192^{ab}	640±164^{a}	38.33±3.38^{b}	80±8^{bc}	3.12±0.32^{b}
Test.	2070±221^{b}	790±126^{a}	47±10.14^{b}	90±12^{bc}	3.21±0.37^{b}
Mat.	2140±252^{b}	820±257^{a}	51.33±12.38^{b}	90±1^{bc}	3.25±0.15^{b}
Bj3	2220±130^{b}	700±29^{a}	37.67±8.83^{b}	90±7^{bc}	2.36±0.34^{b}
Fern.	2280±192^{b}	600±97^{a}	49.67±7.17^{b}	80±11^{bc}	2.64±0.30^{b}
SOM	2500±415^{b}	870±214^{a}	26.67±3.75^{ab}	120±2^{c}	3.21±0.38^{b}

Data with the same letter per column are not significantly different (P<0.05, Duncan test).

bium strains. The nodule number was ranged from 27 nodules/Pl (with SOM strain) to 51 nodules/Pl (with Mat strain) corresponding to a weight of 120mg/Pl and 90 mg/Pl, respectively. These results showed no correlation between the number and the weight of nodule per plant. Nitrogen concentration in shoot showed an important and significant variation (Table 2) among inoculated plants and the control. The highest concentration (3.25%) was registered following the inoculation by Mat strain. Concerning shoot and root dry matter (DM), a significant difference was observed between inoculated plants with different strains and non-inoculated control (Table 2). The shoot DM ranged from 780 mg/Pl at the non-inoculated control to 2500 mg/Pl with plants inoculated by the strain SOM. Whereas, root DM passes from 500mg/Pl in control to 870 mg/Pl with plants inoculated by the strain SOM. These results show that SOM, Test, Mat, Bj1 and Bous.96 are efficient and conferred to faba bean nodulation, nitrogen-fixing capacity and shoot growth higher than the non-inoculated control and the other strains.

Evaluation of *Rhizobium* strains as biological control against *O. foetida*

Effect of Rhizobium strains on underground stages of O. foetida

The germination of *O. foetida in vitro* showed a significant decrease (75%) only after inoculation with the *Rhizobium* strain Mat (Figure 1). The other strains have not any effect or reduced insignificantly the germination percentage of broomrapes. No brownish radicals were observed during the essay. The germ tube and its attachment were healthy. Similarly, the number of tubercles formed on faba bean's roots inoculated with bacteria was significantly reduced compared to the non-inoculated control (Figure 2). The percentage of reduction compared to the control reached 89% with the Mat strain. For the other strains reduction, it ranged from 61% to 86%. Compared to the non-inoculated control, the tubercle formation was delayed by 22 days after inoculation with Test strain and by 14 days after inoculation with the other *Rhizobium* strains. No necrotic tubercles were observed in the case of inoculated faba bean plants.

Effect of Rhizobium strains on O. foetida development in pots

In the pots experiments, faba bean inoculated with *Rhizobium* strains (Bous.96, SOM, Bj1, Fern, Mat) show a variation in the total tubercle number on faba bean's roots compared to the non-inoculated control (Table 3). Only the Bous.96 reduced significantly the total tubercle number. The total tubercle DM on the faba bean's roots inoculated by the used *Rhizobium* strains does not differ significantly. Similarly, a significant difference was observed when the number of underground attachments was considered (Figure 3). The number of emerged parasites (stage 5) was significantly decreased with all *Rhizobium* strains compared to the control. No

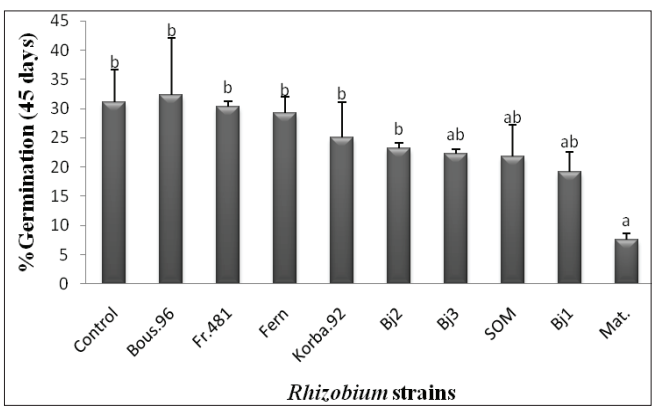

Fig. 1. Effect of Rhizobium strains on germination of *O. foetida* in faba bean after 45 days of the inoculation hydroponic co-culture
Data are means ± confidence limits

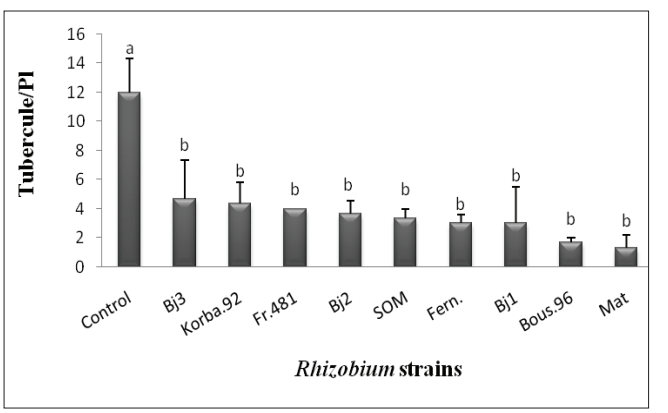

Fig. 2. Effect of Rhizobium strains on tubercle number of *O. foetida* hydroponic co-culture
Data are means ± confidence limits

necrosis of attached tubercles was observed upon infection by *O. foetida* on faba bean roots.

Concerning faba bean shoot and root DM, only Mat strain induced a significant increase compared to the control. The other strains decreased root and shoot DM, despite their beneficial effect in reducing parasitism by *O. foetida* (Table 3).

Discussion

Several authors (Fyson et al., 1981; Gallacher and Sprent, 1978) described the specific shape of nodule of broad beans. Allen and Allen (1981) and Zakhia and De Lajudie (2001) showed that *Rhizobium leguminosarum* induce the formation of fixing nitrogen nodule when they are in symbiosis with broad beans. Variation in shoot and root DM and the nitrogen concentration in shoot after inoculation by the different *Rhizobium* strains confirm the results reported by Dobbelaere et al. (2003), which showed that an inoculation, precociously and positively, affects the plant development and its nitrogen fixation efficiency. Difference in strain effectiveness can be associated with compatibility with host plant controlled by a complex interaction mechanism (Hirsh et al., 2001; Denarié et al., 1992). In our study, higher nitrogen fixation efficiency was observed with all *Rhizobium* strains especially Mat strains.

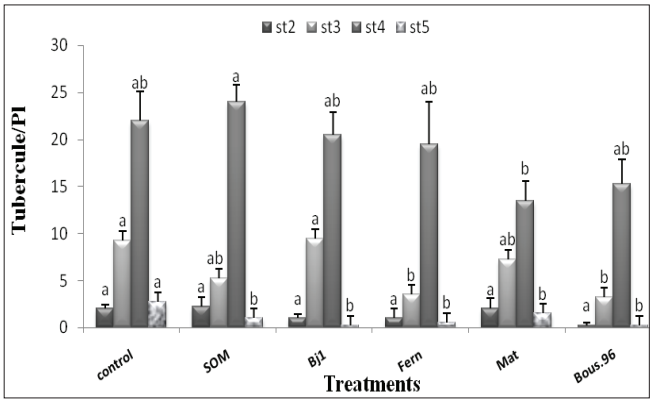

Fig. 3. Effect of *Rhizobium* strains on underground stages development of *O. foetida* (Pots experiments)
Data are means ± confidence limits

The Germination of *O. foetida* seeds *in vitro* decreased significantly after inoculation by Mat *Rhizobium* strain. Mabrouk et al. (2007a, b) obtained similar Results on peas where inoculation by *Rhizobium* strains decreased significantly the germination of *O. crenata*. In the same way, Bouillant et al. (1997) and Miche et al. (2000) related that the inoculation of the ground by isolated bacteria reduced significantly the *Striga* seeds germination. This reduction in seed germination can be explained by the induction of host defense mechanisms in faba bean in response to *Rhizobium* strains inoculation. This decrease can be related for example to the reduction of the stimulant activity of the root exudates through the enrichment of bean plants by nitrogen resulting from the nodules formation. Cechin and Press (1993) indicated that nitrogen enrichment of the culture media induces a reduction in the active production of root exudates by Sorgho plants infested by *Striga*. The same finding was observed in the pathosystem red Clover -*O. minor* (Yoneyama et al., 2001). Nevertheless, the mechanism involved in this hypothetical

Table 3
Evaluation of *Rhizobium* strains as biological control agent of *O. foetida* in pots experiments. Control corresponds to non-inoculated faba bean and infested by broomrapes

Treatments	Shoot, mgDM/Pl	Root, mgDM/Pl	Tubercle number/Pl	Tubercle, mgDM/Pl
Control	1113±85^{ab}	790±56^{ab}	35.75±3.54^{a}	1758±184^{a}
SOM	763±67^{b}	393±46^{d}	32.5±3.18^{ab}	1443±253^{a}
Bj1	875±126^{b}	627±37^{bc}	31.25±3.66^{ab}	1808±138^{a}
Fern	973±112^{ab}	557±44^{cd}	25.5±6.51^{ab}	1558±235^{a}
Mat	1283±189^{b}	845±88^{a}	24.75±3.07^{ab}	1758±543^{a}
Bous.96	758±42^{b}	683±88^{abc}	18.5±2.96^{b}	1648±280^{a}

Data with the same letter per column are not significantly different (P<0.05, Duncan test).

inoculation-mediated decrease in stimulant production requires clarification.

The number of *O. foetida* tubercles/Pl was significantly reduced after the inoculation by the different *Rhizobium* strains. This reduction can be explained by the decrease in the percentage of *O. foetida* germination in the case of Mat strain. For the other *Rhizobium* strains, the decrease in tubercle number was not correlated with the germination percentage, suggesting that bacteria induced probably another mechanism of defense to the host plant against *O. foetida* parasitism. Pot experiment showed also the beneficial effect of *Rhizobium* strains in controlling *O. foetida* parasitism especially Mat strain. Similar results were observed in the antagonism *Rhizobium-O. crenata* in peas (Mabrouk et al., 2007a).

In the two experiments (Petri dish and pot experiments), no necrosis of germinated seeds nor attached tubercles were observed. In contrast, Mabrouk et al. (2007a, b, c) showed that in pea, some nodulating strains of *Rhizobium leguminosarum* were inducing resistance to *O. crenata* through induction of necrosis of attached parasites. This was mainly achieved in host plant roots by increasing the peroxidase and phenylalanine ammonia lyase activities and the production of phenolic compounds, which possess some inhibitory activities on Orobanche seed germination and parasite attachment.

Conclusion

According to the results presented here, we conclude that the inoculation by the compatible *Rhizobium* strains (Mat and Bous.96) can protect faba bean against *O. foetida*. The Mat and Bous.96 *Rhizobium* strains are potential candidates as inoculants for growth promotion and fertilizer reduction. At the same time, they would be a good tool to reduce parasitic infestation. Further study to characterize the resistance induced by *R. leguminosarum* against broomrape and the use of these strains in field experiments is suggested. This will help farmer to use the efficient *Rhizobium* strains and antagonist to the parasitism of broomrape in faba bean. The use of the *Rhizobium* strains with high potential for fixing atmospheric N will protect the environment, develop sustainable agriculture and increase the productivity of the faba bean.

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