GLOBAL JOURNAL OF BIOLOGY, AGRICULTURE & HEALTH SCIENCES
(**Published By**: Global Institute for Research & Education)
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Effect of the Inoculation of Chickpea by Rhizobia on Growth Promotion and Protection against *Orobanche Crenata*

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Abstract

Broomrape (*Orobanche crenata* Forsk.) is a chlorophyll lacking holoparasite that subsists on the roots of plants and causes significant damage to the culture of leguminous plants and, in particular, to chickpea (*Cicer aerietinum* L.). Here, we investigated the potential of some *Rhizobium* strains for biological control of *O. crenata* using a commercial chickpea cultivar (Amdoun) and different *Rhizobium* strains. Firstly, benefit of bacterial inoculation on plant growth and efficiency in N-incorporation were demonstrated with four isolates, Pch. Azm, Pch. Bj1, Pch. Bj2 and Pch. Bj3. *Rhizobium* strains were investigated for their ability to control *O. crenata* using pot and Petri-dish experiments. Inoculation of chickpeas with two *Rhizobium* strains (Pch. Azm and Pch. Bj1) induced a significant decrease in *O. crenata* seed germination and in the number of tubercles on chickpea roots. Furthermore, broomrape necrosis was observed both before and after parasite attachment to inoculated chickpea roots. The hypothesis that roots secrete toxic compounds related to *Rhizobium* inoculation is discussed.

Key words: biological control; *Rhizobium* strains; *Orobanche crenata*; chickpea; necrotic symptoms

Introduction

Chickpea (*Cicer arietinum L.)* is one of the most popular vegetables in many regions of the world. Pulses are important sources of protein for vegetarian population. Chickpea commonly known as gram is an important pulse crop. In Tunisia, the cultivated area and production have significant instability and decrease, the chickpea crop was affected by biotic and abiotic constraints. 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.) that suffers little damage in the traditional spring sowing, but there is concern that the continued spread of the practice of winter sowings might lead to an outbreak of broomrape infection in chickpea (Rubiales et al., 2003). Orobanche is considered an important agricultural parasite in chickpea in Beja region of Tunisia (Kharrat et al., 1992). The main Orobanche species in Tunisia include *O. crenata, O. foetida* and *O. ramosa* (Kharrat & Halila, 1994).The estimated levels of Orobanche incidence was indicated that about 5 000 ha out of 70 000ha planted to food legumes might have Orobanche infestation and Yield losses are approximate from 20 to 80%.

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., 2007a). 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. crenata*. Firstly, compatibility with chickpea was checked by estimating nodulation-related impact on chickpea growth and N-incorporation in glasshouse experiments. Secondly, their respective antagonistic activity towards crenate broomrape was estimated during development of parasite in both pot and Petri dish experiments.

Material and Methods

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

Strains	Localities	years
Pch. Azm	Nabeul – Tunisie	2009
Pch. Bj1	Beja – Tunisie	2007
Pch. DMS	CIRAD Montpellier	1989
Pch. 43	ICARDA –Syria	1988
Pch. 35T	INRA Montpellier	1989
Pch. SOM	Maroc	2000
Pch. Kor	Nabeul – Tunisie	2010
Pch. Bj2	Beja – Tunisie	2009

Strains	Localities	years
Pch. Bj3	Beja – Tunisie	2008
Pch. Bouf3	Sousse – Tunisie	2002
Pch. Bj4	Béja – Tunisie	1992
Pch. Mat	Bizerte – Tunisie	2002
Pch. Rah2	Nabeul – Tunisie	2002
Pch. MBou1	Bizerte – Tunisie	1992
Pch. Bj5	Beja – Tunisie	2002
Pch. Bouf2	Sousse – Tunisie	1998

Bacterial strains and growth conditions

Sixteen rhizobia strains were collected from different localities (Table 1). All strains were isolated from chickpea roots harvested from Orobanche-free crops. 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.

Seeds sterilisation and germination

Chickpea (cv. Amdoun) 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 sterile water and allowed to germinate at 28°C in the dark for 7 days.

O. crenata seeds were collected from flowering spikes in infested faba bean fields in Nabeul (Tunisia) in 2010. Seeds were surface-sterilized in 10 % sodium hypochlorite and rinsed with sterile distilled water. Seed viability was estimated at 75% using the 2,3,5-triphenyl tetrazolium chloride (TTC) test (Aalders & Pieters, 1985).

Symbiotic traits

These experiments were performed in glasshouse at the National Institute of Agronomy, Toulouse in France. Chickpea plants were grown under natural light, keeping the minimum temperature above 20°C. Following germination in Petri-dishes, chickpea seedlings were transferred to plastic pots (1L) containing a sterilized mixture of local field soil and sand (1:1 V/V). Inoculations were performed directly following transfer of chickpea seedlings into culture pots by addition of 5 mL of bacterial suspension (10^7 cells mL^{-1}). Plants were supplied weekly with N-free nutrient solution (Vadez et al., 1996) and water. The beneficial effect of rhizobia on chickpea development in Orobanche-free soil was estimated from 45 day old. Shoot dry matter (DM) was weighed after drying in an oven (70°C, 3 days) and total N was analysed by Kjeldahl digestion (Parkinson & Allen, 1975). In addition, nodules were separated from chickpea roots for counting and weighing after drying (DM).

The impact of rhizobia on chickpea infection by *O. crenata*

Co-culture in Petri-dishes

Co-culture was performed according to Mabrouk et al., (2007b). Chickpea seedlings (7–10 day old) and sterilized *O. crenata* seeds (8mg) were transferred to Petri dishes (120 X 120 X 17 mm, Greiner). Three perforations were made, in the two opposite borders of the Petri dish, one to hold the chickpea shoot out of the dish and the others to allow plant root feeding in culture medium. To this, 3 mL of selected bacterial culture (10^7 cells mL^{-1}) was added. Dishes were sealed with parafilm, covered with aluminium foil to exclude light and were placed vertically, the germinating host plant upwards, 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. crenata* seeds germination was evaluated 45 days after transplanting, by using a stereoscopic microscope (x20). Four areas per Petri dish, each containing 200 seeds located close (<10 mm) to the pea roots, were observed and the number of germinated seeds counted and expressed as percentage of total seeds. Germination rates were expressed by taking account of the viability of the seed lot used for experiments (75% see above). In addition tubercle formation was evaluated 45 days after transplanting. Non-inoculated pea seedlings growing in contact with Orobanche served as controls.

Pots experiments

Twenty *Mesorhizobium ciceri* strains were tested in a pot experiment with six replicates per treatment. Chickpea seedlings were transferred in plastic pots (1 L) containing a mixture of local field soil and sand (1:1, v/v) infested by *O. crenata* seeds (8 mg Kg^{-1}).

Four sets of pot cultures were managed simultaneously: (i) chickpea plants growing in Orobanche-free soil (control 1), (ii) chickpea plants growing in infested soil (control 2), (iii) chickpea plants growing in Orobanche-free soil inoculated with the selected isolates, and (iv) chickpea plants growing in infested soil inoculated under the same conditions. Inoculations were performed directly following transfer of chickpea seedlings into culture pots by addition of 3 mL of bacterial suspension (10^7 cells mL^{-1}). Experiments were terminated when the host plants in the control (ii) treatments stopped growing due to *O. crenata* infection. Chickpea roots were gently harvested, washed with water, and necrotic and total tubercles per plant counted.

Statistical Analysis

In all the experiments, six plants were grown per treatment. Consequently, the data are means ± confidence limits (n=6, $\alpha = 0.05$, 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

Impact of different rhizobial strains on shoot growth and N incorporation in chickpea

Inoculation of Chickpea plants by diffrent *Rhizobium* strains showed significant differences in nodule number and DM by 45 DAI (Table 1). Indeed, both Azm and bj inoculated plants displayed high nodule number and DM. in contrast, few nodules were observed on chickpea roots inoculated with the isolates, Pch. DMS, Pch. 43 and Pch. Bouf2. Concerning shoot DM, significant differences were observed between inoculated plants with different strains and non inoculated controls (Table 2). Shoot growth was significantly increased by twofold when the chickpea plants were inoculated with Pch.Azm and Pch. Bj1 compared to the control. Nitrogen concentration in shoot showed an important variation among plants inoculated with efficient *Rhizobium* strains. The highest concentrations were found with Pch. Azm, Pch. Bj1, Pch. Bj2 and Pch. Bj3 strains (Table 2). These strains conferred to chickpea nodulation, nitrogen fixing capacity and shoot growth higher than those attained with the other strains tested.

Table 2: impact of some *Rhizobium* strains on nodulation efficiency: nodule number, shoot DM and total N content in chickpea.

Treatments	Nodule number/plante	Shoot (g DM/plant)	Nitrogen (%)
Control	0	0.87 ± 0.16	1.80 ± 0.12
Inoculated with Pch. Azm	63.30 ± 2.4^0	2.02 ± 0.18	4.98 ± 0.28
Inoculated with Pch. Bj1	55.45 ± 1.98	1.98 ± 0.25	4.80 ± 0.17
Inoculated with Pch. DMS	6.28 ± 1.52	1.35 ± 0.22	3.20 ± 0.24
Inoculated with Pch. 43	4.80 ± 2.10	1.21 ± 0.13	1.41 ± 0.41
Inoculated with Pch. 35T	22.70 ± 1.78	0.91 ± 0.18	2.51 ± 0.54
Inoculated with Pch. SOM	24.17 ± 3.85	0.80 ± 0.15	1.90 ± 0.78
Inoculated with Pch. Kor	10.18 ± 4.15	1.03 ± 0.19	2.37 ± 0.28
Inoculated with Pch. Bj2	49.80 ± 2.78	1.81 ± 0.14	4.27 ± 0.45
Inoculated with Pch. Bj3	47.21 ± 5.85	1.70 ± 0.22	4.11 ± 0.48
Inoculated with Pch. Bouf 3	23.20 ± 3.25	1.07 ± 0.21	3.21 ± 0.21
Inoculated with Pch. Bj4	23.10 ± 4.52	1.21 ± 0.20	2.10 ± 0.44
Inoculated with Pch. Mat	39.70 ± 2.10	1.51 ± 0.16	2.90 ± 0.14
Inoculated with Pch. Rah2	38.60 ± 1.98	1.10 ± 0.20	1.70 ± 0.33
Inoculated with Pch. MBou	36.89 ± 2.22	0.55 ± 0.11	2.20 ± 0.27
Inoculated with Pch. Bj5	18.70 ± 1.25	0.48 ± 0.14	2.30 ± 0.28
Inoculated with Pch. Bouf 2	6.70 ± 2.21	0.87 ± 0.16	1.80 ± 0.15

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

Effect of Rhizobium *strains on underground stages of* O. crenata *in Petri dish experiements.*

In vitro germination of *O. crenata* seeds was significantly decreased by 80% and 90% after the inoculation with *Rhizobium* strains Pch. Bj1 and Pch. Azm respectively (Fig. 1). The number of tubercles formed on chickpea roots inoculated with bacteria was significantly reduced compared to the non inoculated control (Fig. 2). Tubercles were rarely observed in the roots inoculated with the tow bacteria (1–2 tubercles/plant).

Effect of Rhizobium strains on O. crenata development in pot experiements

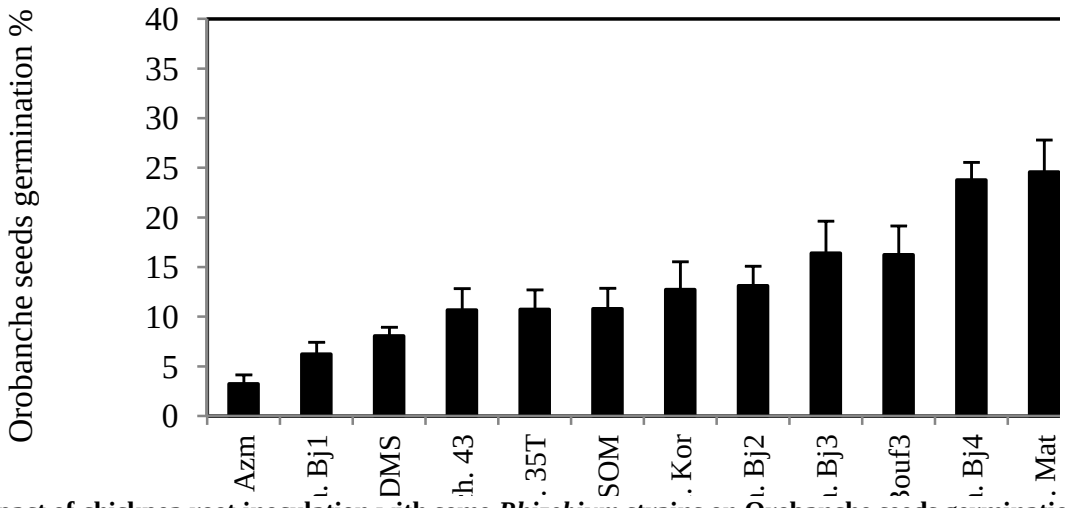

Figure 1: Impact of chickpea root inoculation with some *Rhizobium* strains on Orobanche seeds germination in Petri dish assays.

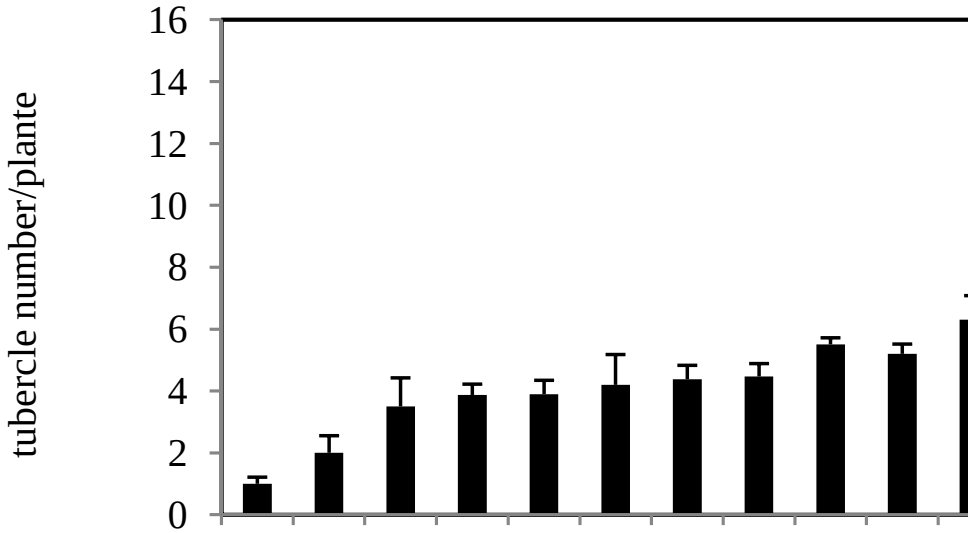

Figure 2: Tubercles number on chickpea roots growing in Petri dish assays and inoculated or not with different *Rhizobium* strains.

In the pot experiments chickpea inoculated with *Rhizobium* strains (Pch. Azm and Pch. Bj1) resulted in decrease of the number of tubercles on chickpea roots (Table 3). However, the number of tubercles formed did not differ statistically between control and chickpea inoculated with *Rhizobium* strain Pch. Bouf2 (Table 3). The total *Orobanche* dry matter per pot was significantly reduced in chickpea inoculated with different *Rhizobium* strains. Using Pch. Azm and Pch. Bj strains chickpea dry matter increased (Table 3). In greenhouse *Rhizobium* strains caused necrotic symptoms on *O. crenata* shoots (data not shown). Whereas, no symptoms occurred on the not inoculated plants.

Table 3: impact of some *Rhizobium* strains on chickpea growth and tubercles formation on root plants in pot experiments

Treatments	Shoot (g DM/plant)	Root (g DM/plant)	Total number of tubercles/plant	*Orobanche* dry matter
Control	0.98 ± 0.11	0.44 ± 0.02	6.33 ± 0.24	0.78 ± 0.13
Inoculated with Pch. Azm	4.02 ± 0.18	2.10 ± 0.08	1.05 ± 0.05	0.25 ± 0.01
Inoculated with Pch. Bj1	3.07 ± 0.12	1.91 ± 0.07	1.75 ± 0.11	0.31 ± 0.01
Inoculated with Pch. DMS	2.75 ± 0.11	1.19 ± 0.04	4.25 ± 0.10	0.57 ± 0.08
Inoculated with Pch. 43	2.24 ± 0.03	1.18 ± 0.03	3.25 ± 0.15	0.51 ± 0.09
Inoculated with Pch. 35T	1.81 ± 0.08	1.07 ± 0.04	4.75 ± 0.12	0.48 ± 0.04
Inoculated with Pch. SOM	1.60 ± 0.05	1.05 ± 0.04	4.23 ± 0.19	0.52 ± 0.03
Inoculated with Pch. Kor	2.06 ± 0.09	1.30 ± 0.05	4.75 ± 0.21	0.57 ± 0.06
Inoculated with Pch. Bj2	1.62 ± 0.07	0.92 ± 0.04	4.33 ± 0.19	0.51 ± 0.07
Inoculated with Pch. Bj3	2.12 ± 0.10	1.14 ± 0.05	3.75 ± 0.17	0.47 ± 0.02
Inoculated with Pch. Bouf 3	2.14 ± 0.10	1.09 ± 0.04	5.33 ± 0.24	0.60 ± 0.03
Inoculated with Pch. Bj4	2.02 ± 0.10	1.02 ± 0.05	5.45 ± 0.26	0.65 ± 0.02
Inoculated with Pch. Mat	2.03 ± 0.16	1.09 ± 0.04	5.33 ± 0.18	0.55 ± 0.02
Inoculated with Pch. Rah2	2.19 ± 0.10	0.98 ± 0.03	5.75 ± 0.19	0.51 ± 0.02
Inoculated with Pch. MBou	1.02 ± 0.01	0.88 ± 0.04	5.33 ± 0.18	0.49 ± 0.02
Inoculated with Pch. Bj5	0.94 ± 0.04	0.81 ± 0.04	5.75 ± 0.20	0.42 ± 0.01
Inoculated with Pch. Bouf 2	1.24 ± 0.06	0.94 ± 0.04	6.07 ± 0.18	0.45 ± 0.01

Discussion

Variations of shoot DM, nodule number and nodule DM with the inoculated *Rhizobium* strains confirm 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 (Hirsh et al., 2001). Our results demonstrate that the Pch. Azm, Pch. Bj1, Pch. Bj2 and Pch. Bj3 *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, plant biomass and yields under greenhouse or field conditions (Sindhu et al., 1992).

In addition to compatibility with chickpea, inoculation with Pch. Azm and Pch. Bj1 rhizobia significantly decreases chickpea susceptibility to the parasite *O. crenata*. *In vitro* germination of *O. crenata* 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). Nevertheless, our data may support the hypothesis that inoculation of roots with Pch. Azm and Pch. Bj1 *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 Pch.Azm- and Pch. Bj1-inoculated plants. Nevertheless, the mechanism involved in this hypothetical inoculation-mediated decrease in stimulant production requires clarification. A later resistance was observed in Pch. Azm and Pch. Bj1-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 found to inhibit germination of *O. crenata* seeds due to the production of the macrocyclic trichothecene, verrucarin A. Recently it has been demonstrated that some *Rhizobium leguminosarum* strains decrease *O. crenata* infections in peas by inducing systemic resistance (Mabrouk el al., 2007a).

We conclude that two *Rhizobium* strains (Pch. Azm and Pch. Bj1) protect efficiently their chickpea partners against *O. crenata*. This is based on the observations that, in the presence of the symbionts, chickpeas grew better, while both parasite germination and development were reduced. Under natural conditions, these *Rhizobium* strains could have a major impact on both parasite germination and chickpea growth. The Pch. Azm and Pch. Bj1 *Rhizobium* strains are potential candidates as inoculants for growth promotion and fertilizer reduction. At the same time, they would also be a good tool to reduce parasitic infestation. This treatment could provide a double benefit for *O. crenata* contaminated and nutrient deficient soils.

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