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Selection and characterization of inhibitor agents (bacteriocin like) produced by rhizobial strains associated to Medicago in western Algeria

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ABSTRACT: The effect of inhibitory substances (bacteriocins) produced by rhizobial strains isolated from Medicago nodules sampled from saline soils in the western region of Algeria and belonging to the genera Rhizobium, Sinorhizobium and Agrobacterium is performed. This study characterizes the inhibitory substances produced by these strains and determinates their activity spectrum against soil bacteria. The demonstration of their inhibitory effect permits the selection of two strongly inhibitory strains (Rhizobium sp. STM 1081, Rhizobium sp. STM 1823). These substances are characterized as bacteriostatic. The physicochemical characterization of these inhibitory substances reveals their thermolabile nature and resistance to acidic pH, but there are sensitive to basic pH, organic solvents and proteolytic enzymes. Analysis by SDS- PAGE electrophoresis of protein fractions indicates that the strain Rhizobium sp. STM 1823 has four bands with different molecular weight **(**46 kDa,44 KDa, 26 KDa and the 20 kDa)and the strain Rhizobium sp. STM 1081 shows a single band (28 KDa). The inhibitory effect of these substances against soil bacteria revealed a broad spectrum of activity**.**

Keywords: Bacteriocin; Competition; Inhibition; Rhizobia; Spectrum of activity.

INTRODUCTION

The Rhizobium-legume symbiosis is the most promising plant bacterium association so far known (Dommergues et Mangenot, 1970). Inoculated Rhizobium sp. strains often fail to compete with the indigenous soil rhizobia and do not increase nodulation (Hafeez et al., 2005). Thus the successful use of rhizobial inoculants requires the knowledge of factors affecting their effectiveness and competitiveness. One of the major factors reported to be affecting competition among rhizobia was bacteriocins (Oresnik et al., 1999).

Bacteriocins are all ribosomally synthesized proteinaceous compounds and are active against bacteria closely related to the producer bacteria (Tagg et al., 1976; Riley and Wertz, 2002). Several studies were reported on the bacteriocins action against phytopathogens (Osnat et al., 2005; Holtsmark et al., 2008). The potential of Gram-negative producing bacteriocins as mean of biological control in fighting plant pathogens has been investigated (McManus, 2002).

Root nodule bacteria have been shown to produce bacteriocins, they were grouped as small, medium or large based on their sizes and diffusion characteristics. Rhizobial bacteria produced mainly small bacteriocins which were found to be chloroform soluble,sensitive to heat and proteolytic enzymes with molecular mass less than 2000 Da (Schwinghamer and Brockwell, 1978;Gross and Vidaver, 1978; Schripsema et al., 1996)**.** The first description of bacteriocin production by a number of species within the economically important genus Rhizobium was published by Roslycky (1967). Many rhizobial species (bacteriocinogenic strains) produce bacteriocins, designated as rhizobiocins (Hirsch, 1979; Goel et al., 1999; Sridevi and Mallaiah, 2008).

Oresnik et al. (1999) found that the bacteriocins appear to play a major role in determining competitiveness for nodulation when assayed against some rhizobial strains, so the successful preparation of mixed inocula requires the knowledge of inocula strains bacteriocin production ability as well as their effect on the related rhizobia. In this study 15 strains belonging to the genera Rhizobium, Sinorhizobium, Agrobacterium were studied for revealing their interactions and their possible production of bacteriocins. Our investigation included partial purification and preliminary characterization of the bacteriocins like substance produce.

MATERIAL AND METHODS

Bacterial strains and culture media

Fifteen strains of rhizobia belonging to the genera (Agrobacterium, Rhizobium, Sinorhizobium) used in this work belong to the Biotechnology Laboratory of Rhizobia and Plant Breeding (LBRAP) at the University of Oran (Algeria) (Tab.1). Genetic characterization of these strains was performed at the Laboratory of Tropical and Mediterranean Symbiosis Institute (LSTM) by partial sequencing of 16S rADN (Merabet et al., 2007). Rhizobial strains were isolated from root nodules of Medicago sp., sampled in natura from saline soil regions. The bacterial medium used for rhizobial culture was YEMA (Yeast Extract Mannitol) (Vincent, 1977): Yeast extract 1g, Bergensen mineral solution **(**10M) [KCl1g; FeCl3 0,02g; $CaCl_2$12H2O 0,53g; Na2HPO4, 7H2O 40,5g; MgSO4 1g] 100ml, Mannitol 10g, Distilled water 900ml. pH was adjusted to 7. Media were used as broth, semisolid agar (0.6%) or solid (2% agar).

Table 1.Rhizobial strains used in this study, their molecular characterization based on partial 16S rDNA characterization and associated plant host.

Code	Partial 16S rDNA caracterisation	Plant host
STM 1823	Rhizobium sp.	Medicago ciliaris
STM 1081	Rhizobium sp.	Medicago ciliaris
STM 1082	Agrobacterium sp.	Medicago ciliaris
STM 2058	Sinorhizobium medicae	Medicago ciliaris
STM 3468	Sinorhizobium medicae	Medigago sp.
STM 2216	Rhizobium sp.	Medigago sp.
STM 1825	Rhizobium sp.	Medicago ciliaris
STM 26B	Rhizobium sp.	Medicago polymorpha
STM 26B1	Rhizobium sp.	Medicago sp.
STM 81	Sinorhizobium medicae	Medicago ciliaris
STM 2210	Sinorhizobium medicae	Medicago sp.
STM 2233	Sinorhizobium medicae	Medicago ciliaris
STM 1066	Sinorhizobium meliloti	Medicago sp.
STM 1819	Rhizobium sp.	Medicago polymorpha
STM 1820	Rhizobium sp.	Medicago sp.

Nodulation test

This test was performed to verify the ability of conserved strains to renodulate their host plants. In this work, Medicago sativa seeds were used. The seeds were scarified and disinfected with sulfuric acid ($H_2SO_4$96%), for 30 seconds to 1 minute. This was followed by a thorough rinsing (6-10 rinses) with sterile distilled water. The seed was then allowed to swell for 5 minutes in sterile distilled water. Seeds then were disposed aseptically in Petri dishes containing water agar 1% and placed inverted in the dark at a temperature of 28°C (Tillard and Drevon, 1988). Germination was observed after 3 days. Seed germination rate of Medicago sativa obtained with this scarification method was of 98% after 48 h of incubation. Seedlings were transferred aseptically in to Gibson tubes filled with a nutrient solution (Bertrand, 1997) free from nitrogen in triplicate. After 3 to7 days, seedlings were inoculated with 2 ml of bacterial suspension of each strain at log phase in order to identify the strains infectivity. Non inoculated plants served as negative controls. The prepared tubes were kept at room temperature. All plants were watered twice a week by aseptically plant nutrient solution alternating with sterile distilled water (Rome et al., 1996).

Screening inter-bacterial inhibitions

The identification of cross-bacterial inhibition was achieved by two methods:
A direct-method (according to Fleming et al., 1975 and Tagg et al., 1976).
An indirect-method (according to Barefoot and Klaenhammer, 1983).

DIRECT METHOD

Inhibitory/indicator strains couples selection of both direct methods was used on solid medium. The fifteen rhizobial strains were tested, assuming that each strain can be inhibitor or indicator, 225 combinations were performed for each technique.

Fleming et al. (1975) technique

The strains which were considered as inhibitor were inoculated by touch on YEMA plate and incubated at 28 °C for 24 hours. Strains considered as indicator were cultivated until reaching log phase, 0.5 µl of the bacterial culture was mixed with 100 ml of YEMA broth, then the mixture was incubated at 28 °C. After 24 to 48 hours of incubation, 0.5 ml of each bacterial suspension was inoculated into 8 ml of YEMA semi solid medium

(0.6% agar) and poured onto the first inoculated plate. Results were reported after 24 to 72 hours of incubation. The presence of a clear zone around strain point inoculation indicated its inhibitory effect.

Tagg et al. (1976) technique

A volume of 1 ml of the strain which was considered as indicator was mixed with 20 ml of YEMA. After solidification, the bacterial strains which were tested for their inhibitory effect were inoculated by touch onto the solid media and incubated at 28 °C for 24 to 72 hours. Inhibitor and indicator strains were used for further experimentation.

Identification of inhibitory agent nature

The inhibition of indicator strain growth may be due to the organic acids production, the phage presence or the bacteriocins (Moreno et al, 1999; Torodov and Dicks, 2005).

Inhibitions due to acid production

Inhibitory activity was detected by the organic acids by modified Spelhaug and Harlander (1989) method: buffered solid medium B-glycerophosphate 1% was replaced by YEMA medium buffered with a sodium phosphate buffer (Na/Na_2, 0.1 M, pH 7) and YEMA medium non buffered was used as control. Couples inhibitor / indicator strains were inoculated according to Fleming et al. (1975) on both media. After incubation, the inhibition diameter zones were measured.

Bacteriophage revelation

A fragment of agar from inhibition zone was thoroughly homogenized and suspended in 1 ml of sterile YEM medium containing 50 µl of chloroform based on modified Lewis et al. (1991) method. After stirring, the preparation was centrifuged at 6000 rpm/15 min. Then 300 µl of supernatant was added to 7 ml of soft agar containing 500 µl of a young culture serving as indicatory strain. The mixture was poured on the solid medium surface. Plates were incubation at 28 °C for 24-72, lysis area was observed.

INDIRECT METHOD

The method of Barefoot and Klaenhammer (1983) was based on the diffusion of the antimicrobial substance supernatant produced by inhibitory strain against the tested strain. A volume of 200 ml of a 48 hours culture at 28 °C was centrifuged (12000 rpm, for 15 minutes). The supernatant was concentrated by dialysis and filtered on a 0,22 µm pore membrane (Millipore Minisart. SM 16584, Sartorius). A volume of 0.2 ml of the indicator strain was inoculated into 7 ml of semi solid YEMA medium and poured onto a Petri dish. After solidifying, wells were filled with 100 µl of concentrated supernatant. Following incubation the presence of the inhibitor agent was indicated by the appearance of inhibition zones. Two strongly inhibitory strains were selected for the following steps of this work (Rhizobium sp. STM 1823 and Rhizobium sp. STM 1081).

Concentration estimation and partial purification of the inhibitory agent

The partial purification of the two highly inhibitory strains supernatant was performed by addition of solid ammonium sulfate $(NH_4)\ 2SO_4$ in graded concentrations to the supernatant until complete saturation from 20 to 80 % (w/v). The mixture was stirred in the cold for 3 hours. After centrifugation at 12000 rpm/min for 30 min, the precipitate was resuspended in a volume of buffer ($KH_2PO_4PO_4/NaH_2$, 0.1 M, pH 7) equal to one tenth of the initial supernatant volume used. The ammonium sulfate was then removed by dialysis in phosphate buffer ($KH_2PO_4/Na_2H_2PO_4$, 0.1 M, pH 7) overnight (the suspension obtained was designated as protein fraction) and the activity of each fraction was tested by Barefoot and Klaenhammer (1983) method. Protein concentration quantification was done by standard Bradford (1976) method. Bovine serum albumin (BSA) was used to construct the standard curve.

Antibacterial activity determination of the inhibitory agent in liquid medium

This method consisted in carrying out in a microtiter plate (96 wells) with a dilution series containing ½ small volume fraction for both selected inhibitory strains as described by Daba et al, (1993). The inhibitory activity was expressed in arbitrary units per ml (AU/ml) and calculated by the following formula: (1000/125) (1 /D), D: represents the highest dilution inhibiting the growth of the indicator strain.

Physicochemical characterization of the inhibitory agent

Temperature effect

A volume of 200 µl of both fractions was treated for 5, 10, 15, and 20 minutes in a water bath at 100 °C and120°C for 15 minutes (autoclaving). The same volume was set to 4 C° and-15 °C for one week. A volume of

50 µl of each treated fraction was deposited in a well made in the soft agar containing the indicator strain. After incubation, the diameter of the inhibition zone was measured.

pH effect

The effect of pH was studied by adjusting both fractions at different pH values 2, 3, 5, 10, 12, and 14 by NaOH (1N) or HCl (1N). A volume of 50 µl was tested for its antibacterial activity in the presence of the indicator strain by the well method.

Organic solvent effect

A volume of 100 µl of both fractions was separately added to 50 µl of chloroform, and 50 µl of cold acetone. After homogenization, the preparations were tested for their inhibitory activity according to the well method.

Enzymes effect

A volume of 120 µl of both fractions was mixed with 25 µl for each type of enzyme (trypsin, proteinase K, lysozyme at a concentration of 1mg/ml). All these enzyme solutions were prepared in phosphate buffer K/Na_2 0.01M, pH 7 and filter sterilized. The mixture (Enzyme/supernatant) was incubated for 1 hour at 28 °C (Gross et Vidaver, 1978). After incubation, the solutions were heated for 5 minutes at 100 °C. The sensitivity of antibacterial substance to a given enzyme was determined by determining the residual activity by measuring the diameter of inhibition zone.

Mode of action of the inhibitory agent

The bactericidal or bacteriostatic effect of inhibitors on the sensitive bacteria was determined by the method of Toba et al. (1991).Very small fragments were removed from inhibition zones agar obtained by the indirect method, under sterile conditions using a loop. These fragments were then streaked on the surface of a solid YEMA medium and incubated at 28 °C for 48 hours.

SDS-PAGE

Polyacrylamide Gel Electrophoresis in the presence of 10% Sodium Dodecyl Sulphate (SDS) was performed. Electrophoresis was conducted at a constant current of 30 mA for 12h at 30 °C. The gel was stained with comassie blue. Referring to different molecular weight markers: bovine serum albumin (67 kDa), trypsin (25 kDa) and casein (45 kDa).

Activity spectrum of the inhibitory agent

To reveal spectrum of action of the two strongest inhibitory strains, they were tested on three strains, isolated from soil sampled around root legumes of Lotus sp., Retama sp., Ononis sp., localized in three sites at Ain Témouchent (Algeria). A final bulk of 100 g of soil was used. Bacteria were isolated, purified, phenotypically and biochemically (API E and API NE) characterized. Soil bacteria were used as indicators, bacterial suspension was mixed with YEMA, after solidification, active supernatants of both inhibitory strains (Rhizobium sp.STM 1823 and Rhizobium sp.STM 1081) were poured on the wells, inhibition zones were reported after 24 to 48 h at 30°C.

Efficiency test

The strains considered as inhibitor were tested for their efficiency on Medicago sativa plants. The experimentation was conducted as described in nodulation test above in triplicate, plants were harvested after 90 days of incubation at room temperature. Roots and shoots were allowed to dry two days at 60°C, then dry weight were reported for inoculated and unoculated plants which served as negative control. Statistical analyzes were performed by SPSS (Statistical Package for the Social Sciences), analysis of one variance with the Fisher test at the 5% level.

RESULTS

All the tested strains renodulated Medicago sativa plants; proven their belonging to BNL (Bacteria Nodulating Bacteria) group.

Interbacterial inhibition revelation

From the 450 interactions conducted by using both techniques of direct methods, 27 couples showed an inhibitor/indicator effect. By Fleming et al. (1975) technique, we obtained six inhibitory strains namely: STM 81, STM 2216, STM 1082, STM 1081, STM 1823 and STM 2058. The strains STM 1081 (Rhizobium sp.) showed the largest spectrum of inhibition with four associated sensitive strains (Tab. 2). However, the Tagg et

al. (1976) technique revealed more inhibitory strains (08), it shared four strains with the first technique: STM 81, STM 1082, STM 1081, STM 1823 and four others: STM 2233, STM 26B, STM 1825 and STM 1066. Nevertheless, these inhibitory strains showed a lesser activity spectrum compared to STM 2233 (Rhizobium sp.).The nature of the inhibiting agent assay was conducted on the 27 inhibitor/indicator couples. The organic acid production was responsible for 7% of inhibition action by comparison between buffered and non buffered YEMA, this acidic inhibition concerned ten inhibitor/indicator couples out of the 27 previously used then two inhibitory strains were discarded: STM 81 and STM 1082. Whereas 93% of inhibitions were due to another action, on which the indirect method was conducted. On the other hand, there was no revelation of phage activity for all the studied couples.

Table 2. Inhibition zones revealed by the direct method (Fleming et al, 1975; Tagg et al., 1976)

Inhibitor strains (Fleming et al., 1975)	Indicator strains	Diameter zone (mm)	Inhibitor strains (Tagg et al., 1976)	Indicator strains	Diameter zone (mm)
STM 81	STM 1066	20	STM 1082	STM 1066	7
	STM 1819	7	STM 2233	STM 1819	8
	STM 26B1	11		STM 1820	5
				STM 3468	6
STM 2216	STM 1081	30			
STM 1082	STM 2233	18	STM 1081	STM 1819	8
	STM 2058	10		STM 1082	15
STM 1081	STM 2058	27	STM 1823	STM 1819	10
	STM 1820	13		STM 1820	12
	STM 3468	18			
	STM 26B1	11			
			STM 26B	STM 2058	3
STM 1823	STM 2058	10			
	STM 3468	5	STM 1825	STM 1820	10
	STM 26B1	5	STM 81	STM 3468	9
			STM 1066	STM 1823	6
STM 2058	STM 26B	14			
	STM 2210	13			

The inhibition effect intra-and inter-genus between rhizobial strains showed that Rhizobium genus had the higher number of inhibitory strains (06) with an important diameter zone (> 30 mm) and the largest activity spectrum than those observed for the two other species (Sinorhizobium and Agrobacterium). We observed that Rhizobium species had the higher inhibitory effect on species of same genus, at the contrary Sinohizobium inhibited principally Rhizobium species. While, Agrobacterium inhibited only Sinohizobium species (Fig.1).

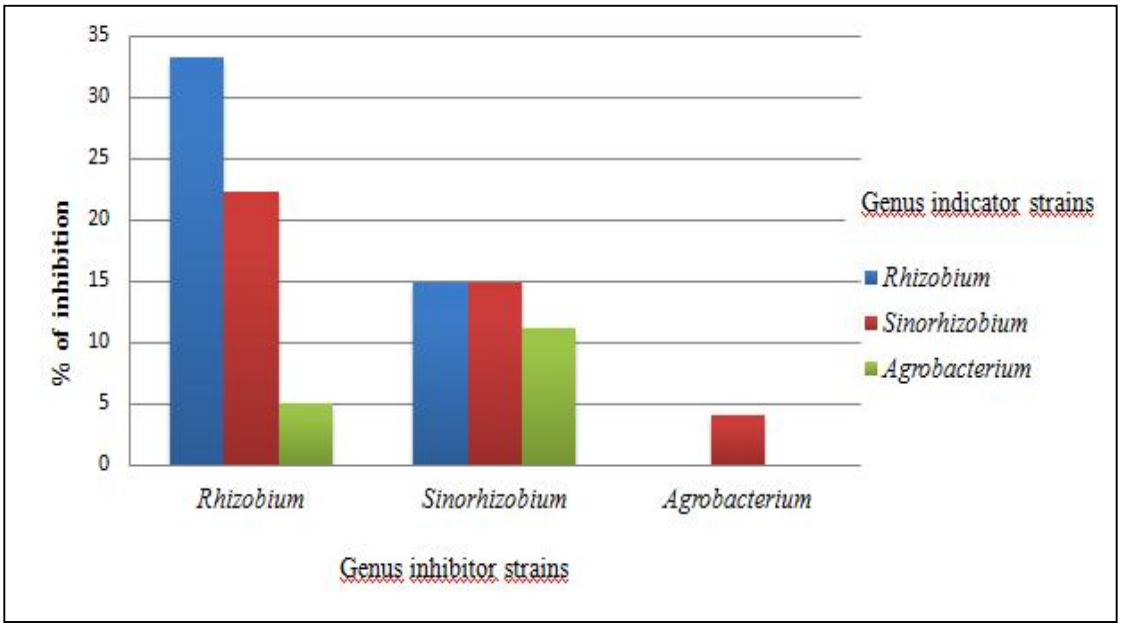

Figure1. Inhibition interaction between different rhizobial genus

The indirect method results revealed the expression of the inhibitory activity of concentrated supernatants by dialysis and were shown in Fig.2. Two strains: Rhizobium sp. STM 1823 (15 mm of inhibition zone) and Rhizobium sp. STM 1081(18 mm of inhibition zone) were identified as potent inhibitors, probably as bacteriocinogenes and were used for further experimentation.

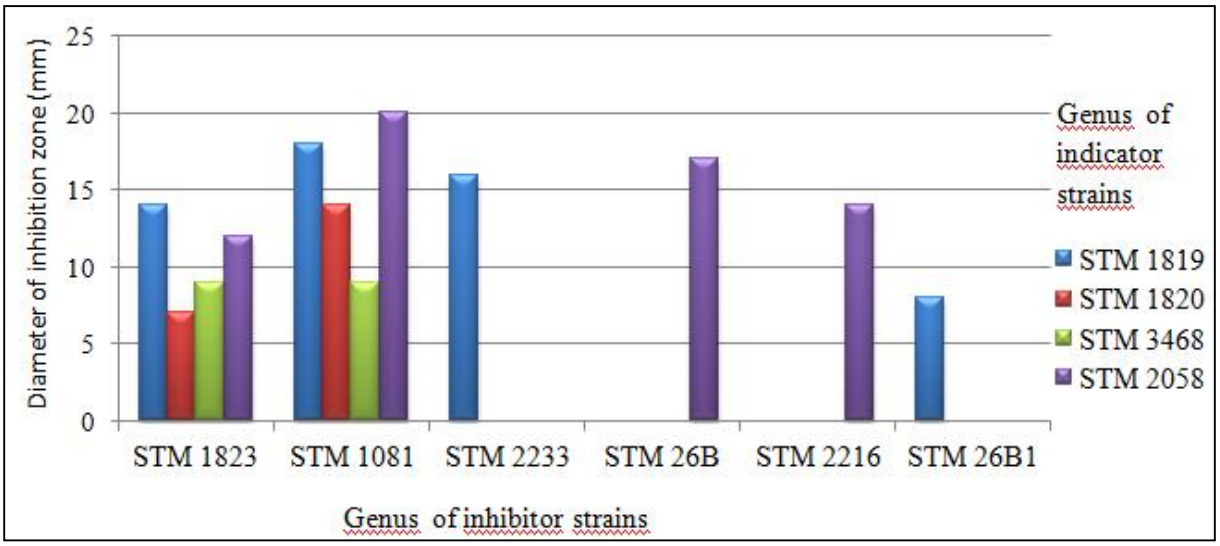

Figure2. Inhibitory activity of concentrated supernatants by dialysis on four indicator strains

Partial purification of the inhibitory substances showed that they were precipitated with 75% saturated ammonium sulfate (fig.3).This has led to the use of partially purified fractions of these inhibitory strains Rhizobium sp. STM 1823 (F1823) and Rhizobium sp. STM 1081(F1081).Their activity didn't depend on the quantity of the produced protein (Tab.3).

Figure3. Inhibition of growth of the indicator strain by the fraction of Rhizobium sp.STM 1823 (F1823) according to the method of Barefoot and Klaenhammer (1983)

Table 3.Concentration and partial purification of F 1823 and F 1081 produced by Rhizobium sp. STM 1823 and Rhizobium sp. 1081 strains

Purification stages	Diameter of inhibition zone	Activity (AU/ml)[1]	Protein concentration (mg ml^{-1})
Fraction F 1823	18	100	8,20
Fraction F 1801	20	210	7,80

Inhibitory agent nature characterization

Physico-chemical tests showed that both F1823 and F1081preserved their antibacterial activity in the pH range from 2 to 7 and maintained full stability after 7 days storage at 4°C and-15°C. They were also active after incubation for 15 min or 20 min at 100°C, whereas no activity was detected at 121°C.The inhibitory activity of Rhizobium sp. Strains STM 1823 (F1823) and STM 1081 (F1081) were sensitive to organic solvents. Since bacteriocins were proteinaceous substances, by definition, they must be sensitive to at least one proteolytic enzyme. Therefore, the sensitivity of the protease was an essential criterion for their characterization. In our case, lysozyme, trypsin or proteinase K had an effect on both F1801and F1823 activities. The results suggest

that Rhizobium sp. STM (F1823) and Rhizobium sp. STM (F1081) produce as mall compound, antimicrobial protein (bacteriocin like).
The two antibacterial agents F1823 and F1081 were bacteriostatic towards the target strain. Analysis by SDS-PAGE of the isolated protein from the strain Rhizobium sp. (F1081) showed the presence of a single band of 28 kDa molecular weight, while the strain of Rhizobium sp. (F1823) had four bands of different molecular weight: 46 kDa, 44 KDa, 26 KDa and 20 kDa.
The characterization of the soil bacteria using API gallery 20NE and API 20E showed he affiliation of three bacteria to Vibrio sp. (AM1), Clostridium sp. (AM2) and Enterobacter sp. (AM 3). F1081 and F1823 fractions showed inhibitory effect (inhibition zone diameter >15mm) against the three isolated soil bacteria.
The effect of inoculation on Medicago sativa growth varied depending on the bacterial strains. The obtained results showed that the two strains have improved favorably shoot and root development compared to unoculated controls (Fig.4).

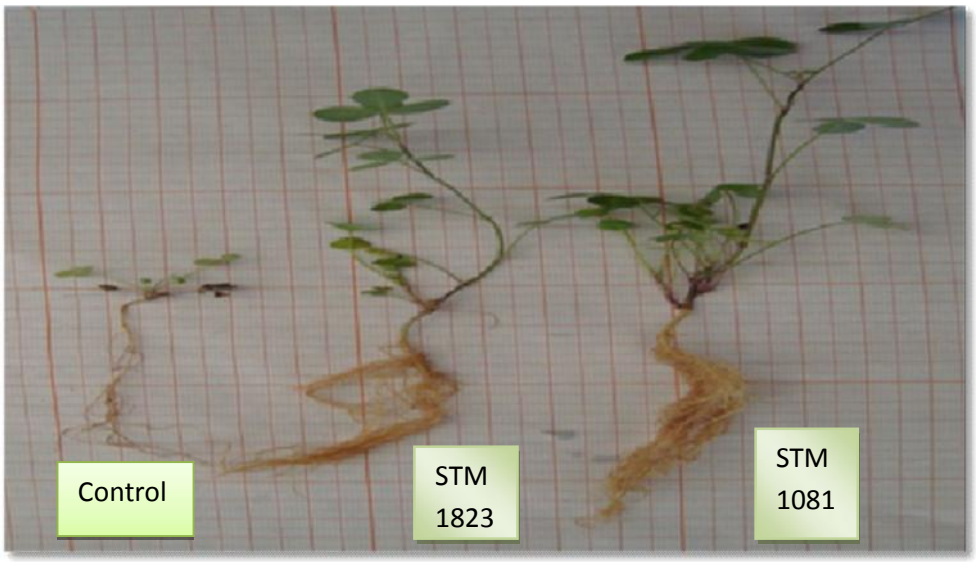

Figure 4.Medicago sativa plant aspect inoculated with Rhizobium sp. STM 1823 and Rhizobium sp.STM 1081 compared to unoculated control after three months of incubation

Inoculation had a significant effect on root and shoot dry weight with $p < 0.05$, for both strains. Rhizobium sp. STM 1081 was more efficient than Rhizobium sp. STM 1823.

DISCUSSION

Research on bacteriocins has expanded during the last decades, for their use as biocontrol of many plant pathogens (Holtsmark et al., 2008) and enhancing inocula efficiency. The two direct techniques (Fleming et al., 1975) and (Tagg et al.,1976) showed that there was no self-inhibition which was a characteristic of bacteriocin, this result was in agreement with those obtained by Hardy (1975); Nirmala and Gaur (2000) ;Sridevi and Mallaiah (2008).The lack of self-inhibition in rhizobia was explained by the existence of a strain "immunity" against its own inhibitory substance. This "immunity" was one of the most important criteria used to describe the bacteriocinogenicity in bacteria (Vidaver, 1983; Herbinet al, 1997). Inhibitions intra-and inter-strain rhizobia showed that inhibitions caused by strains of the genus Rhizobium were more frequent than those observed for other genus. This result is explained by the fact that different strains of Rhizobium had abroad spectrum of activity and inhibiting agent can be structurally and functionally variable (Wijffelman et al, 1983; Oresnik et al., 1999). However, it was reported that the inhibitions caused by other types of rhizobia could be reduced because strain sensitivity depended on the species and even serotype within the same species (Nirmala and Gaur, 2000). Among the six inhibitory substances concentrated by dialysis, two strains proved to be highly inhibitory, F1823 and F1081 excreted from Rhizobium sp.STM 1823 and Rhizobium sp.STM 1081 respectively, they showed an inhibition zone of 18 mm 25 mm respectively in the presence of the target strain. The inhibitory substances were precipitated with 75% saturation ammonium sulfate. The same results were obtained by Hafeez et al. (2005) for the precipitation of bacteriocins produced by Rhizobium leguminosarum bv. viciae. This has led to the use of partially purified fractions of these inhibitory strains Rhizobium sp. STM 1823 (F1823) and Rhizobium sp. STM1081 (F1081).

The physicochemical characterization of inhibitory substances showed that the two inhibitory fractions (F1823) and (F1081) were thermolabile. Similar observations were made for the bacteriocins that have been produced by Rhizobium Trifolli. (Schwinghamer,1975). Both inhibitor substances were not affected by acidic pH but were sensitive to alkaline pH. The bacteriocins were generally stable in acidic or neutral pH (Leroy and De Vuyst, 1999). The effect of pH on the bacteriocin activity was related to electrostatic interactions to ensure the adsorption between the hydrophilic surfaces and the bacteriocin and the sensitive bacterium (Abee et al., 1994). The work of Yang et al. (1992) reported that the adsorption of several bacteriocins was in function of pH and the higher the pH dropped, the greater the concentration of the bacteriocins in the bacterial wall increased. The organic solvents (chloroform and acetone) affected negatively both inhibitory fractions (F1081) and (F1823),the obtained inhibition diameter in acetone presence decreased the inhibition zone diameter from 18 mm to15 mm for F1081 and from 18 mm to 10 mm for F1823. There was a total disappearance of inhibitory activity in chloroform presence for both fractions. These results have been reported by several authors (Hirsch, 1980 and Van Brussel, 1985), these authors found that bacteriocins produced by rhizobia had a low molecular weight and were soluble in chloroform. The inhibitory activity of both Rhizobium sp. Strain (Rhizobium sp.1823 and Rhizobium sp.1081) lost their inhibitory activity when they were associated with proteolytic enzymes. Similar observations were made by Gross and Vidaver (1978), this finding confirmed its proteinaceous nature. Some of the characteristics of the selected antibacterial substances in our study exhibited properties similar to those of bacteriocins of several rhizobia (Wijffelman et al., 1983; Leroy and De Vuyst, 1999). Hirsch (1979) has described two types of bacteriocins produced by Rhizobium leguminosarum; these were designated as small and medium bacteriocins. The small bacteriocin was heat labile and resistant to proteolytic enzymes, whereas the medium one was also heat labile but sensitive to proteolytic enzymes. On another hand, a study conducted by Schwinghamer and Brockwell (1978) have described the production of bacteriocin by Rhizobium trifolii which was sensitive to heat and proteolytic enzymes. At present, the proteinaceous nature was confirmed for medium bacteriocins and the precursor gene was isolated (Rodelas et al., 1998; Wisniewski, 2002). In addition, van Brussel et al. (1985) have reported that the small bacteriocins produced by rhizobia were chloroform soluble. Both inhibitors Rhizobium sp. STM 1823 and Rhizobium sp. STM 1081 had bacteriostatic activity. The same results were obtained by Gross and Vidaver (1978).

Both bacterioncin like substances produced by Rhizobium sp. STM 1823 and Rhizobium sp. STM 1081 inhibited the three soil bacteria Gram positive Clostridium sp. (AM2) and Gram negative Vibrio sp. (AM1) and Enterobactere sp. (AM3). This finding was not in agreement with the data generally indicating that bacteriocins inhibited bacterial genera related to the producing bacteria (Tagg et al., 1976; Nettles and Barefoot, 1993). Moreover, the spectrum of activity of bacteriocins may not be restricted to species taxonomically close or even didn'toccupy the same ecological niche. From a practical point of view, the activity spectrum of a bacteriocin may be larger or smaller depending on the environmental conditions (Klaenhammer, 1993). A similar result was obtained by Smaoui (2010) for the new bacteriocin produced by BacTN635 (Lactobacillus plantarum sp.TN635) with a very broad spectrum of action against Gram- negative and Gram positif and even against filamentous and unicellular fungi (Fusarium sp. and Condida tropicalis R2).

The results of this study showed that the production of bacteriocins may play an important role in-interspecific competition. In this study, the broad spectrum activity of Rhizobium sp. STM 1823 and Rhizobium sp. STM 1081 against Rhizobium sp. And soil bacteria, in regards to their efficiency, they could help to improve legume inoculants potency, competitivity and survival after inoculation.

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