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Effect of Repeating Culture of Cowpea Plant on the Competition of Bacterial Strains Isolated from its Rhizosphere

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Abstract: The continual presence of the same plant hosts in natural plant communities should favour microorganisms below as well as above ground. Selection pressure imposed by root exudates may favour different populations of rhizosphere microorganisms to be more adapted to their rhizosphere during monoculture. The present study aims to investigate the effect of the repeating culture of cowpea plants in a short period on the rhizospheric bacterial enumeration and the competition ability of the strains isolated from the subsequent rhizospheric soils to colonize their rhizosphere. No significant differences were observed between the numbers of total bacteria obtained from the rhizospheric soils of the four cultures, indicating that the repeating culture of cowpea plants has no effect on the enumeration of rhizospheric bacteria. One stable mutant strain resistant to rifampicin ($1D1^R$) was used as a reference strain in order to compare the competition of the strains isolated from the rhizosphere of the 1st and 4th culture. The similarity of the mutant strain $1D1^R$ with its wild one were verified by the utilization of the 95 substrates of the Microplate $Biolog^R$ GN, growth curve and by the competition between the two clones in gnotobiotics conditions. Fifteen strains, strain by strain, from the rhizosphere population of the 1st or the 4th culture were confronted in competition with strain $1D1^R$. The distribution of strains in five classes of competition according to the indexes of competition CI1 (the ratio between the number of the CFU of the tested strains and the number of the CFU of the reference one) and CI2 (ratio between the number of the CFU of the tested strains and the number of the total CFU) were significantly different between the rhizosphere population of the 1st and 4th culture. The average of the competition indexes of the 15 strains of each population, 1st and 4th, were significantly different. The average values were always higher for the rhizosphere population of 4th than the 1st culture, whatever the index used for calculation. These results confirm that the strains obtained from the rhizosphere after the repeating culture of cowpea plants for four times have an ability of competition more than those obtained from the rhizosphere of the 1st culture.

Key words: Repeating culture, cowpea plants, competition, rhizosphere, rifampicin, mutant, $Biolog^R$ GN and gnotobiotics conditions.

INTRODUCTION

The composition and counts of microorganisms present in the rhizosphere of different plants may differ due to variations in the quantity and quality of compounds exuded by the different plants[1,7,9]. The rhizodeposition of easily available carbon makes the rhizosphere an area of high microbial activity[21,24]. Root exudates selectively influence the growth of bacteria and fungi that colonize the rhizosphere by altering the chemistry of soil in the vicinity of the plant roots and by serving as selective growth substrates for soil microorganisms. Consequently, different rhizosphere microbial communities are associated with different plants[15,17]. The root colonizing ability is an essential prerequisite for the success of rhizobacteria in serving useful functions. The improved understanding of the community diversity and population dynamics may be lead to develop a reliable strategy for selecting rhizosphere competent bacteria[11].

Any screening method is selective, therefore it is to be expected that only a part of the microorganisms will be detected. However, the important question is: Will the methods used select the most suitable and adaptable organisms for our purposes in the environment, in which they are meant to work? In the ecological approach such as biofertilization, biocontrol agent and phytormidiation, selected microorganisms should be able to function in the same environmental niche. Thus suitable places for isolation of such

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ecologically adapted microbes would be the surface of seeds and roots of the plants susceptible to do their function in question[22]. Obviously, in the absence of plants, selection for any purposes of inoculation alone will not say any thing about an organism's ability to colonize plant roots. Indeed, rhizosphere competence has been seen as an important issue for obtaining successful functioning of inoculated bacteria[12]. The introduced microorganisms in plant roots as boifertilizers, biocontrol agents[26], and plant growth promoters have generally shown a progressive decline in population size leading to limit their effectiveness[4]. So, the inoculant bacteria must be able to establish themselves in the rhizosphere at population densities sufficient to produce beneficial effects. Therefore, efficient inoculant bacteria should survive in the rhizosphere, make use of nutrients exuded by the plant roots, proliferate, be able to efficiently colonize the entire root system and highly demonstrate rhizosphere competence with indigenous microorganisms.

The continual presence of the same plant hosts in natural plant communities should favour microorganisms below as well as above ground. Selection pressure imposed by root exudates may favour different populations of rhizosphere microorganisms to be more adapted to their rhizosphere during monoculture. Mohamed[18], studied the role of the repeating culture of soybean plants in a short period for four cultures in increasing the competition of the strains isolated from its rhizosphere. He found a significant change in bacterial enumeration, metabolic and genetic diversity. Furthermore, rhizospheric strains isolated after the 4th culture were more competitive than those isolated from the 1st one.

The present study aims to investigate the effect of the repeating culture of cowpea plants on: (1) the rhizospheric bacterial enumeration and (2) the competition ability of the strains isolated from the subsequent rhizospheric soils to colonize their rhizosphere.

MATERIALS AND METHODS

Soil and Plant: The soil studied was a sandy loam (coarse sand 41.11%, fine sand 26.37%, silt 27.17% and clay 5.35%; 1.6% organic matter; 16% $CaCO_3$, pH 8.1) recovered from 0-15 cm layer at the experimental farm of the Faculty of Environmental Agricultural Sciences, El-Arish, Suez Canal University, North Sinai Governorate, Egypt. Cowpea seeds (*Vigna unguiculata* L. Walp. cv. Dokki 331), were sterilized in hypochloride solution (70% active chlorine) for 20 min, rinsed several times with sterile water. Sixteen seeds were sowed in pots containing 2kg of this soil to have finally eight plants per pot which considered as a replicate. Three replicates were done. Soil moisture was maintained at 80% of water holding capacity. Soil adhering to the roots was recovered after six weeks for counting, isolation and competition study of the bacteria. The process of cultivation for six weeks and bacterial counts and isolation was repeated for four times.

Soil Rhizosphere Recovery: The plants were lifted out from the pots and root systems of eight plants (considered as a replicate) were transferred to a 1l Erlenmeyer flask containing 100 ml sterile distilled water. The flask was shaken for 10 min on a rotary shaker and the 100 ml soil suspension was considered as rhizosphere soil suspension.

Colony Forming Unit (CFU) Counts and Strain Isolation: Serial dilutions of the rhizospheric soil suspension were plated on Tryptone Soybean Agar (TSA) medium diluted at 1/10. Three plates of each appropriate dilution were incubated at 25°C. After 5 days, bacterial Colony Forming Unites (CFU) were counted. Variance analysis for the obtained CFU of the four subsequent rhizospheres were performed using StatView Version 5 (SAS Institute Inc., USA) with the Fisher's PLSD test at the probability level of 5%.

Ninety five colonies of each replicate were always isolated from the same dilution and grown in microplates in 1/10 Tryptone Soybean Broth (TSB). The microplates were incubated at 25°C for five days. Afterwards, the microplates were conserved in -20°C for further study.

Obtaining the Reference Strain (Selection and Validation of the Reference Strain): In order to test the competition of the strains isolated from the 1st and the 4th rhizosphere, a reference strain should be selected. In this context, spontaneous mutant clones of the rifampicin were obtained by the following procedure. Twenty five strains were isolated from each rhizosphere (first culture, R1st and fourth culture, R4th). The strains were purified by plating at 1/10 TSA. Approximately 10^8-10^9 CFU/plate of each strain was plated on 1/10 TSA supplemented with 100 mg l^{-1} rifampicin and incubated at 25°C for 5 days. Three dishes per strain were plated, one was plated on 1/10 TSA without rifampicin in order to compare the growth of mutants with the wilds ones. Two to four clones per strains, represented the same morphology of the colony of the wild strain were isolated, inoculated into 1/10 TSB supplemented with 100 mg l^{-1} rifampicin and incubated at 25°C for 5 days.

Stability of Mutants Clones: In order to test the stability of each clone, five cultures in 1/10 TSB without rifampicin were made in sterilized microplate with 95 wells. At the 5th culture, serial dilutions were

plated on 1/10 TSA with or without rifampicin and incubated at 25°C for 5 days. The counts of CFU were recorded for each clone cultivated with or without rifampicin.

Variance analysis for the recorded numbers of each clone were performed using StatView Version 5 (SAS Institute Inc., USA) with the Fisher's PLSD test at the probability level of 5%.

Growth Curves Similarity Between Mutants and Wild Strains: Growth curves for the stables mutants clones and their wild type strains were compared by inoculating the same number of each strain or its mutant clone in a sterilized 95 well microplate, each well contains 150 µl 1/10 TSB. This was achieved by adjusting the OD at 590nm by a Microplate reader, Model Stat Fax-2100, Awareness Technology Inc. USA. Each culture was repeated three times. Optical densities were measured 0, 18, 23, 27, 42, 47, 51, 66 and 71 hours after inoculation and initial OD was subtracted from the subsequent readings. Growth curves were achieved between the obtained OD and the time of measurement.

Competition in Soil with the Indigenous Bacteria: In order to select one reference strain, the mutants which have the same growth curves as their wild strains were tested for their competition with indigenous bacteria. Pre germinated cowpea sterilized grains were cultivated in tubes 22 mm in diameter containing 8 g of coarse sand at the bottom of the tube and 24 g of the same soil described above humidified with nutritive solution contains salts concentrations illustrated in Mohamed and El Tantawy[19]. Each grain was inoculated with 10^6 CFU of each mutant. Humidity was maintained at 80% of water holding capacity. Total CFU and CFU of each mutant in four repetitions were recorded in the rhizospheric soil 14 days after planting. Existence of indigenous rifampicin mutants was also verified by counting total rhizospheric bacteria on 1/10 TSA +100 mg rifampicin in a non inoculated soils.

Test of the Competition Similarity Between the Mutant Reference Strain and the Correspondent Wild Strain in Gnotobiotic Conditions: Selected mutant and its correspondent wild strain were tested for their competition in the cowpea rhizosphere. A mixture of the mutant clone and its wild one containing 5 x 10^6 ml^{-1} for each one were used for inoculating cowpea grains. Pre germinated and sterilized grains were inoculated with this mixture to give 10^6 CFU/grain. The grains were cultivated in sterilized tubes 22 mm in diameter containing 40 g of sterilized coarse sand humidified with a the sterilized nutritive solution mentioned before. Each set was replicated three times. Fourteen days after planting, roots were lifted out of sands and root system was then transferred to a bottle containing 10 ml sterilized distilled water. The samples were shaken for 10 min and serial dilution were plated on 1/10 TSA with or without 100 mg rifampicin. CFU of the mutant and wild strain were counted five days after incubation at 25°C. Roots were dried for 3 days in an oven at 80°C in order to determine their dry weight. Variance analysis for the obtained numbers of the mutant and its wild strain were performed using StatView Version 5 (SAS Institute Inc., USA) with the Fisher's PLSD test at the probability level of 5%.

Metabolic Characterization (Biolog GN) of the Reference Mutant Comparing with its Correspondent Wild Strain: Metabolic profiles of the mutant reference strain in compared with wild type one were realized in three replicates using BIOLOG GN microplates following the recommendations of the producer (Biolog Inc., Hayward, Ca). The microplates were inoculated with 150 µl containing the same number of the mutant or its wild one and incubated at 25°C for 168 hours. Color development was measured after 0, 24, 48, 72, 96, 120 and 168 hours, at 590nm using a Microplate reader Model Stat Fax-2100, Awareness Technology Inc. USA. Initial ODs were measured immediately after inoculation and were subtracted from subsequent readings. Average Well Colour Development (AWCD) was determined for each plate[3,18]. The maximum AWCD was compared by PLSD test of Fisher at a probability of 5%.

Competition Strain by Strain with the Reference One in Gnotobiotic Conditions: In order to test if the strains isolated from the rhizosphere of 4th cowpea culture ($R4^{th}$) are more competitive than the isolated ones from the 1st culture ($R1^{st}$), 15 strains from each population were randomly selected and tested strain by strain with the reference one in gnotobiotic conditions. One standard curve for each of the tested strains and for the reference one was achieved in order to inoculate the same number of the tested and reference strain. Strains were grown in 1/10 TSB at 25°C for five days. Serial dilutions were made and appropriate dilutions were plated on 1/10 TSA and incubated at 25°C. Five days after, CFU were recorded. ODs at 590 nm were measured for strains dilutions and standard curves were made between ODs and CFU ml^{-1}[18].

A mixture of each tested strain and the reference one containing 5 x 10^6 ml^{-1} of each one were used for inoculating cowpea grains. Inoculation and cultivation in gnotobiotic conditions, plating on TSA 1/10 and incubation were done as described before. The total CFU and the CFU of the reference one were counted five days after incubation at 25°C. CFU of reference strain (Growth on 1/10 TSA containing 100 mg rifampicin) was subtracted from the total CFU to give the CFU of each tested strain. The CFU were expressed per g dry root.

The competition index was calculated for each strain in two manners: (1) by calculating the ratio between the number of the CFU of the tested strains and the number of the CFU of the reference one, (CI1) and (2) by calculating the ratio between the number of the CFU of the tested strains and the number of the total CFU (CI2)[18]. The strains were regrouped in classes of competition according to these two indexes. The distribution of strains of P1 and P4 in classes of competition were compared by the StatXact program (version 3 pour Windows, Cytel Software Corporation). The χ^2 test was used to compare the distribution of strains in the classes of competition for the two populations[16,18].

RESULTS AND DISCUSSION

Total Bacterial Counts in the Subsequent Rhizospheres: Results of the total rhizospheric bacterial counts and their probabilities values are given in Table (1). The numbers of total bacterial recovered from the four rhizospheric soils ranged between $3.8x10^9$ to $4.2x10^9$ CFU g^{-1} dry soil. No significant differences were observed between the numbers of total bacteria obtained from the four rhizospheric soils, indicating that the repeating culture of cowpea plants has no effect on the enumeration of rhizospheric bacteria.

Obtaining the Reference Strain (Selection and Validation of the Reference Strain): Obtaining strains resistant to rifampicin was applied on 25 strains of the rhizosphere population $R1^{st}$ and $R4^{th}$. Only 13 and 12 clones from the populations of the $R1^{st}$ and $R4^{th}$, respectively, were stables. From these 25 stables mutants, the counts of seven clones represented the strains (1D1, 1G1, 3C1, 4B1, 3F4, 7F4 and 8B4) were not significantly different in the TSA medium with or without rifampicin since their probability values were 0.8237, 0.6825, 0.9763, 0.9333, 0.8571, 7049 and 0.5923, respectively. The growth curves of five from the seven mutants (represented the strains 1D1, 1G1, 3F4, 7F4 and 8B4) in the liquid medium were as those of their wild strains, Fig. 1a, b. Moreover, the maximum growth were not significantly different between these five mutants and their wild type strains since their probability values were 0.9821, 0.7813, 0.2432, 0.9434, 0.3355 for the strains 1D1, 1G1, 3F4, 7F4 and 8B4, respectively.

The five stables mutants were evaluated in competition with the soil indigenous bacteria, Table 2, column 1 and 2. The strain 8B4 was the highest competitive one since it represents more than 3% of the total rhizospheric bacteria suggesting that this strain has a high competitive ability with the indigenous bacteria. In the contrary, the strains 1G1 and 7F4 represent only 0.02 and 0.01% of the total rhizospheric bacteria, indicating a very low ability of competition. Any of these three strains could not be retained as a reference strain since they may eliminate the differences between the tested strains. The strain 1D1 was a less competitive than 3F4 but it was in the intermediate between the five tested strains. Therefore, the strain 1D1 was retained as a reference strain.

The utilization of the 95 substrates of the Microplate Biolog^R GN by the strain 1D1 and its mutant $1D1^R$ was compared. The average well color development (AWCD) for the strain 1D1 and its mutant $1D1^R$ was identical, Fig. 2. The maximum AWCD of the mutant reference clone $1D1^R$ and its correspondent one was not significantly different (P = 0.1427). By the same manner, the utilization of each substrate 168 hours after incubation by the mutant reference clone $1D1^R$ or its correspondent one was not different, Fig. 3.

The wild strain 1D1 was tested in competition with its mutant $1D1^R$ (reference strain) in gnotobiotics conditions. The percentages of CFU of the wild 1D1/(wild + mutant 1D1) was 53% where it was 47% for the mutant one. However, the competition between the reference mutant strain and its correspondent one was not significantly different (P = 0.4407).

Competitive Ability of Strains Isolated from the First and Fourth Soil Rhizosphere of Cowpea Culture: Fifteen strains of each population ($R1^{st}$ and $R4^{th}$) were confronted strain by strain with the strain $1D1^R$. The competition indexes CI1 (the ratio between the number of the CFU of the tested strains and the number of the CFU of the reference one) and CI2 (the ratio between the number of the CFU of the tested strains and the number of the total CFU) were calculated for each tested strain.

The validity of the test gnotobiotic were confirmed by comparing the order of the five strains used for selecting one reference strain (1D1, 1G1, 3F4, 7F4 and 8B4) in soil non sterile conditions (competition against the total rhizospheric microflora) and in conditions gnotobiotics (competition against the reference strain $1D1^R$). The five strains have the same order of competition with the two techniques (Table 2).

The distribution of strains in five classes of competition according to the indexes of competition CI1 and CI2 were significantly different between the population of the $R1^{st}$ and $R4^{th}$ culture according to the c^2 test, Table 3, see also Fig. 4. More than fifty percent of the tested strains of the $R1^{st}$ belonged to the class of the lowest competition (<1), according to the index CI1. By the contrary, only 6.6% of the tested strains of the $R4^{th}$ belonged in this class indicating that the strains delivered from the $R1^{st}$ were less

Table 1: Effect of repeating culture of cowpea plants on the enumeration of rhizospheric bacteria (values of the probability (P) from the PLSD Fisher test at a level of significance of 5%).

Cowpea culture cycles	Total bacterial counts (CFU g^{-1} dry soil)	Cowpea culture cycles	Values of probabilities
1st	$3.8x10^9$	1st and 2nd	0.7999
2nd	$4.1x10^9$	1st and 3rd	0.9288
3rd	$3.9x10^9$	1st and 4th	0.7621
4th	$4.2x10^9$	2nd and 3rd	0.8694
Probability value	0.9874	2nd and 4th	0.9605
		3rd and 4th	0.8307

Table 2: Orders of tested strains in non-gnotobiotic conditions (for selecting a reference one) and in gnotobiotic conditions.

Strain order (non-gnotobiotic conditions)	Ratio of CFU (tested strain/total) %	Strain order (gnotobiotic conditions)	Ratio of CFU (tested strain/tested and reference strains) %
8B4	3.14	8B4	87
3F4	0.21	3F4	79
1D1	0.14	1D1	53
1G1	0.02	1G1	49
7F4	0.01	7F4	33

Table 3: The distribution of strains of P1 and P4 in classes of competition (Program StatXact was used to achieve this analysis using the χ^2 test).

Classes of CFU (tested strains/reference strain, CI1)	S 1st	S 4th	Classes of CFU (tested strains/total, CI2)	S 1st	S 4th
<1	8	1	<0.1	0	0
1-2	2	2	0.11-0.3	5	0
2-3	3	8	0.31-0.5	3	1
3-5	2	3	0.51-0.7	4	6
>5	0	1	>0.71	3	8
Probability value	0.0419	Probability value		0.0311	

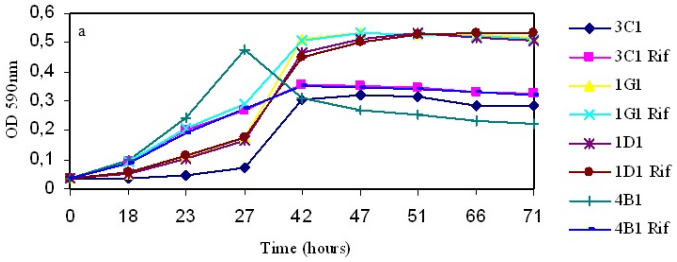

Fig. 1a: Growth curves of the seventh stable mutants compared with their correspondent ones: Stable strains of the first culture.

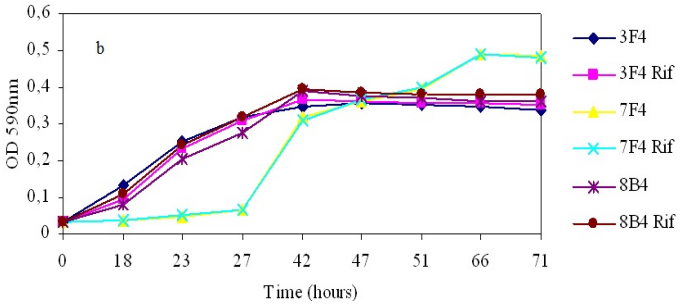

Fig. 1b: Growth curves of the seventh stable mutants compared with their correspondent ones. Stable strains of the fourth culture.

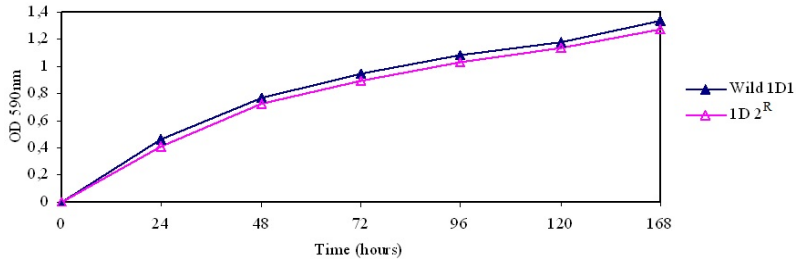

Fig. 2: Comparison of the AWCD between the mutant reference strain ($1D1^R$) and its correspondent wild (1D1). (Average of three replicates).

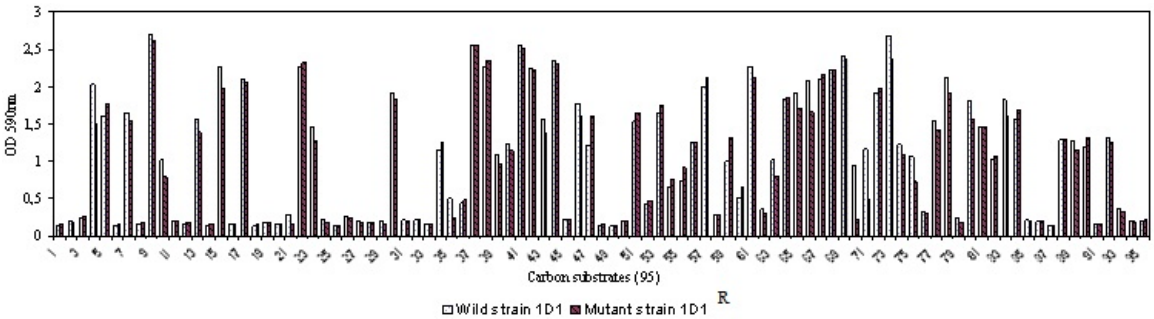

Fig. 3: Comparison of the utilization of each of the 95 carbon substrates of the GN microplate 168 hours after incubation between the mutant reference strain ($1D1^R$) and its correspondent wild (1D1). (Average of three replicates).

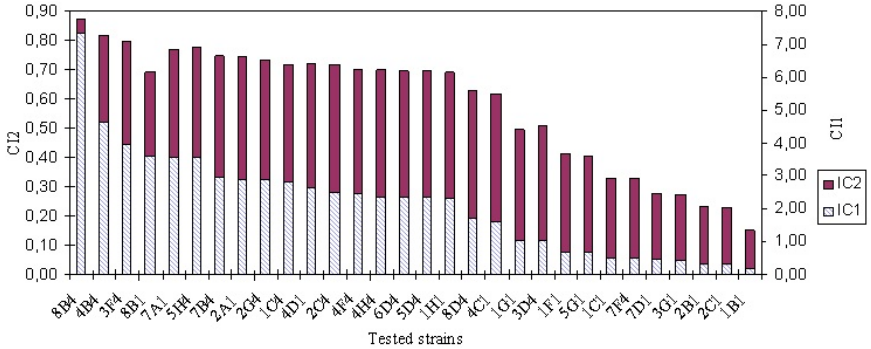

Fig. 4: The thirty tested strains in competition (each alone) with the reference strain, 1D1. The index CI1 express the ratio between the CFU of tested strain and CFU of reference one, and CI2 express the ratio between the CFU of tested strain and CFU of total (CFU of tested strain and reference one, 1D1).

competitive then those issued from the $R4^{th}$ one. According to the same index, while only 33% of the tested strains of the 1st rhizosphere have an index value of more than 2, this percentage increased to reach to 80% for the strains of the 4th rhizosphere. This indicates again that the strains delivered from the 4th rhizosphere have a high ability of competition when compared with those of the 1st rhizosphere. The same trend was observed when considering the CI2 index with a probability value more than which was obtained when considering the CI1 index.

The competition average of the 15 strains of each population $R1^{st}$ and $R4^{th}$ were significantly different whatever the index of competition. The average values of the CI1 were 1.419 and 2.894 for the population of $R1^{st}$ and $R4^{th}$, respectively, with a probability value of 0.0088. By the same, the average values of the CI2 were 0.469 and 0.696 for the population of $R1^{st}$ and $R4^{th}$, respectively, with a probability value of 0.0017. These results always confirm that the strains obtained from the rhizosphere after the repeating culture of cowpea plants for four times have an ability of competition more than those obtained from rhizosphere of the 1st culture.

Discussion: Data obtained from the enumeration of total bacteria in the rhizosphere clearly indicate that the rhizosphere has a high microbial activity since the

counts of total rhizospheric bacteria were more than 10^9 CFU g^{-1} dry soil. These results are in agreement with those of Mohamed[18] and Söderberg *et al.*[25] who found that the rhizosphere bacterial community differed from the bulk soil community using Biolog GN microtitre plates. In opposition with the weak microbial activity in bulk soil, the root rhizosphere are the place of an intense microbial life[10]. Repeating culture of cowpea plants in intensive culture (8 plants per 2kg soil) and in a short time (6 weeks) has no obvious effect on the enumeration of total rhizospheric bacteria. However, the important question is: Will the strains isolated from the rhizosphere of the 4th culture compete for rhizosphere colonization as those isolated from the rhizosphere of the 1st culture or more?

To verified this strategy of selection competitive strains, one reference strain marked for their resistant to rifampicin used to test the competition of the strains isolated from the rhizosphere of the 1st and 4th culture. Resistant to rifampicin was used because it is mediated by a mutation in β subunit of RNA polymerase[20], unusual among soil bacteria. The chromosomal nature of the mutation affords greater stability than occurs with plasmid-borne markers and is also advantageous since the mutation is not transferable[4]. This technique is simple, rapid, sensitive, inexpensive and has been used successfully with samples from various environments[14,18].

Fifteen strains from each population (R1st and R4th) were confronted, each alone, with the selected reference strain, $1D1^R$. The competition indexes CI1 (the ratio between the number of the CFU of the tested strains and the number of the CFU of the reference one) and CI2 (the ratio between the number of the CFU of the tested strains and the number of the total CFU) were calculated for each tested strain. The gnotobiotic test used in the present study is valid, since the order of the five strains used for selecting one reference strain in non sterile soils (competition against the total rhizospheric microflora) and in gnotobiotics conditions (competition against the reference strain $1D1^R$) is the same. This test was used before by Mohamed[18], and is more simple and rapid than the test of Delorme[8], since she obtained a marked mutant for each tested strain.

In the test used in the current study, the competition of the whole 15 strains isolated from the rhizosphere of the 4th culture was more than those of the 1st one. This result confirms the initial hypothesis, that the repeated culture may enrich the rhizosphere with more competitive strains. The distribution of strains in five classes of competition for the strains of the two populations was different. Most of tested strains of the 4th culture belonged in the classes with high competition index value suggesting that rhizospheric strains were more adapted to their rhizosphere after repeating culture of cowpea plants for four times.

The continued monoculture of wheat caused a shift in the rhizospheric population toward both higher numbers and a higher percentage of wheat rhizosphere-inhabiting fluorescent *Pseudomonas spp*. inhibitory in vitro to *G graminis* var. *tritici*. Moreover, this repeated monoculture convert the soil from conducive to suppressive to take-all[2,5,6,23]. The decline in take-all is associated with changes in the rhizosphere microflora, including a build up of *Pseudomonas* species that are specifically antagonistic to *G. graminis* and the increase of other microorganisms that are suppressive through competition for substrate[13]. Recently, Mohamed[18] found that the repeating culture of soybean plants caused an increase or a tendency of increasing the competition of the strains isolated from the rhizosphere of the repeated culture.

From the overall results, the repeating culture of cowpea plants has no effect on the enumeration of its rhizospheric bacteria. However, the rhizospheric strains of the 4th culture have an ability of competition for colonization more than those of the 1st culture, suggesting that the obtained strains after the repeating culture were more adapted to their rhizosphere.

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