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Isolation of Highly Effective, Super Nodulating and Competitive Rhizobium Strains of Egyptian Clover (*Trifolium alexandrinum* L.).

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ABSTRACT

Forty-eight root nodulating-clover *Rhizobium* strains isolated from 11 different Egyptian governorates were screened to select the high nitrogen fixing and well nodulated strains. Strains could be divided into three major groups based on variation of plant fresh weight between inoculated and non inoculated plants. The first group, high nitrogen fixing (47%), second moderate nitrogen fixing (33%) and third less nitrogen fixing (20%). The five effective strains for nodulating and nitrogen fixing were Rhiz 950, Rhiz 975, Rhiz 996, Rhiz 1017 and Rhiz1024. Inoculation with all the five representative strains resulted in duplicating the fresh weight of clover plants and NPK uptake than the control. These strains were mixed together with the same appropriate cell numbers (10^{-8} cells ml^{-1}) and were tested to select the most competitive strains for nodulating the common and widely cultivated clover cv. Meskawy under neutral and alkaline pH using the REP and RAPD markers. Under normal conditions, results of REP profiles indicated that strains Rhiz 950 and Rhiz1017 were the most competitive *Rhizobium* strains with 35.71% nodule occupancy, while under alkaline stress and based on RAPD primers, the alkaline tolerant Rhiz 1017 strain occupied 60% of the nodules, while the other four examined strains had equal competitiveness (10%). These findings indicated that strain Rhiz 1017 can be used as effective and competitive clover rhizobial inoculants in Egyptian soils.

Keywords: Clover symbionts, competitiveness, and rhizobial inoculation.

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INTRODUCTION

Competitiveness is the ability of *Rhizobium* strain to infect its legume host and form nodules in the presence of other specific rhizobial strains. Strains that dominate nodules are considered more competitive than other strains. The success of rhizobial inoculation under field conditions requires that the *Rhizobium* strains must be highly effective in nitrogen fixation and highly competitive against the indigenous rhizobial strains in the soil [1, 2, 3, 4]. To estimate competitiveness, it is urgently to identify the most completive strain which occupied the majority of nodulated roots. Authors have been used many different techniques to determine nodule occupancy such as fluorescent antibodies [5], antibiotic resistance [6], plasmid profiles [7] and specific gene probes [8, 9], *gus* gene coding for glucuronidase enzyme is a useful technique to mark rhizobial strain for studying the competitiveness [10, 11]. Nodules infected by rhizobial strains acquiring the *gus* gene or wild type stain blue and brown on respectively, when nodules incubated with x-gluc buffer [11].

Considerable efforts have been done to understand rhizobial competition and to know which biological factors can increase the competitiveness ability of rhizobial strains in the field [12]. One of the greatest difficulties in managing rhizobial competition is, in which phases of nodulation process competition can be occurred [13]. Some authors explained that the competitive ability of strains is due to the ability to survival under different environmental conditions [14], tolerance to antibiotic [15], chemotaxis [16], motility [17], soil textural and structural properties [18] and the efficiency to colonize roots and growth rate [19].

Other authors found that there are specific kinds of proteins such as rhizopine L-3-o- methyl-scyllo [20]; myo-inositol [21] and trifolitoxin [22] can play a prominent role in competition for nodule formation. Rhamnose plays a significant role in competition of *R. leguminosarum* bv. trifolii [23]. The aim goal of this study was to select high effective nitrogen fixing and competitive *Rhizobium* strains that can be used as inoculums for Egyptian clover in the newly reclaimed soils.

MATERIALS AND METHODS

Rhizobial isolation

Fort-eight rhizobial strains were isolated after surface sterilization of nodules collected from Egyptian clover as previously published by Shamseldin et al. [24].

Plant nodulation assays

All the 48 rhizobial strains were examined for their capability of re-nodulting their clover host using plant infection technique. The nodulation assays were performed in sterilized Leonard jars with (1:1 V:V) of Patmos to Perlite as a medium for cultivation and N-free nutrient solution [25]. Patmos substituted vermiculite because clover seeds are very small and the use of vermiculite will lead to lose the seed to the end of jar where there is no enough oxygen for growth. Seeds of clover cultivar Meskawy were surface sterilized as described before by Shamseldin *et al*. [24] and soaked in sterile distilled water overnight in the refrigerator at 4ºC, then seeds were distributed on the surface of 1% agar plates and incubated for 3-4 days at 28 ºC for germination. After germination seedlings were transferred to Leonard jars and each unit was inoculated with 1 ml of rhizobial culture which containing about 10^8 cells ml^{-1}). Plants were cultivated in a controlled growth chamber with 15h of light at 25 ºC and 9h for darkness at 18 ºC, relative humidity of 75% and light intensity of 14 k Lux. Plants were thinned to three plants for each Leonard jar. After 25 days plants were removed to estimate the nodule number, nodule fresh weight and plant fresh weight.

REP and RAPD methods to select the most competitive rhizobial strains

Rep-PCR DNA fingerprints of isolates were obtained by using the Box1AR primer (5'-CTACGGCAAGGCGACGCTGACG-3') as previously described [26]. Gel images were captured with a FOTO/Analyst Archive electronic documentation system (Fotodyne Inc, Hartland, Wis.). For normalization of patterns, gel images were normalized using Bionumerics software (version 1.5; Applied Maths, Kortrijk, Belgium) in the presence of 1kb plus DNA ladder for comparison. Rep PCR was used to select the most effective *Rhizobium* strains under normal conditions.

Under alkaline stress (pH9), a specific primer of 20 nucleotides length (RPOl; 5'-AATTTTCAAGCGTCGTGCCA-3') which corresponds to the conserved domain sequence of *R. leguminosraum* sv. trifolii *nifHDK* promoter [9], was synthesized. It was used to differentiate among *Rhizobium* strains inside nodules and to select the most competitive strains after the DNA from nodule soup was incorporated with this specific primers and the PCR reaction was done according to the standard protocols and temperature program was as the following: 5 cycles of 30s denaturation at 92°C, 2 min annealing at 53°C and 90s, extension at 72°C; followed by 35 cycles at the same conditions and a final extension cycle for 5 min at 72°C. The PCR products were electrophorased on 1.5% agarose for 1h to obtain the DNA profiles. Data generated by both REP and RAPD primers was analyzed by using multivariate analysis of variance (MANOVA), a form of discriminate analysis accounting, for covariance structure.

Competition experiments

The five effective strains from plant infection test were selected and were grown in liquid YEM medium and their optical density was adjusted using sterile YEM broth to equal 1 OD at 660 nm, then they mixed together with an equal volume and cell density, and they used as one inoculum to inoculate sterilized clover seeds (cv. Meskawy) grown in Leonard jars. Seedlings of each Leonard jar unit were inoculated by adding 1 ml of rhizobial mix cultures (Rhiz 950, 975, 996, 1017 and 1024) at the mid of exponential growth phase with 10^8 cells ml^{-1}. To select the most competitive *Rhizobium* strains at normal conditions, each treatment was replicated three times. Plants were cultivated in growth chamber under the same conditions explained above. Plants were harvested 25 days after inoculation, and 56 nodules were randomly removed from roots and divided into to four parts and each part was loaded in one gel after the genome of nodule soup subjected with REP primers to know the DNA finger print of each strain. Each gel (14 profiles replicated three times and the best one in quality was showed in the manuscript). Nodule occupancy percents were estimated by accounting the repeated DNA profile from the gel and divided on the total numbers of profiles (14) and multiplied with 100. To estimate the most competitive strains under alkaline pH, the nutrient solution was buffered with 20 mM AMPD for a pH9 as mentioned by Shamseldin and Werner [27] and the nodule soup were subjected with specific RAPD primers for *Rhizobium leguminosarum* sv. trifolii and PCR to know the DNA fingerprint of each strain. Nodule occupancy percents were estimated as previously mentioned and 40 nodules were checked in three gels and the best gel in quality was included.

Nodulation and symbiotic effectiveness assays in pot experiments

The five effective and competitive *Rhizobium* strains selected based upon the results of plant infection technique and competition trials were re-examined in pot experiments as inoculants for clover plant for long cultivation period to estimate their symbiotic effectiveness without to rely on nitrogen fertilizer. Pots of two kilogram capacity were filled with buildings sand and vermiculite and perlite 1:1:1. Pots were cultivated with germinated seedlings of clover cvs. Meskay (after sterilization) and arranged in completely randomized design with six replicates in the green house experiment of the National Research Center for 50 days (from 20 September to 10 of November 2013). Cultures of the selected five effective *Rhizobium* strains were used to inoculate pots with 5 ml with cell density of 10^8 ml^{-1}. All treatments were fertilized with starter dose of P and K that required for giving minimal growth (30kg fed^{-1}K and 60kg fed^{-1} P), no nitrogen fertilizer was added to the pots during the whole duration experiment. These small quantities of P and K were added to pots after one month of rhizobial inoculation to avoid affecting on nodulation process. Plants were thinned after two weeks of inoculation to keep about 20 plants pot^{-1}. After 50 days of inoculation plants were harvested and shoot fresh weight, shoot dry weight, nodule dry weight, nodulation index and NPK were estimated. NPK were measured in the unit of analysis and consultants at NRC in Egypt using the standard applied method in the unit of soil science analysis.

RESULTS AND DISCUSSION

Screening to select the well nodulated and high nitrogen fixing *Rhizobium* strains

Fort-eight rhizobial strains were isolated from randomly collected roots of Egyptian clover after surface sterilization. All of these strains were examined in plant infection test for selecting the strains that able to re nodulate clover host and high effective for nitrogen fixing. All the examined strains were able to re-nodulate and fix nitrogen with clover host, except strain Rhiz 902 which failed to form any nodules on the legume host. Ten strains were gave high record of nodule numbers that ranged from 13 to 31 nodules $plant^{-1}$

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(Table 1). These strains were 887, 888, 910, 913, 916, 952, 996, 1002, 1017 and 1032. The forming of nodules reflected positively on the fresh weight of plants, however strains that gave high record of fresh weight of plants were different than those gave high record of nodule numbers. These strains were 884, 913, 916, 935, 950, 959, 965, 970, 971, 975, 1007, and 1032 which gave plants fresh weight higher than the uninoculated plant (435 mg plant^{-1}) by about 31.3%, 17.8%, 22.9%, 49.2%, 20.5%, 29.5%, 35.7%, 36.9%, 31.6%, 29.9%, 39.8% and 32.8% on respectively. A bout 20% of strains were less effective for nitrogen fixing as they produced plant fresh weight equal or lower than the control. These strains were Rhiz 893, 902, 925, 932, 937, 957, 977, 1028, 1030 and 1040 on respectively.

Table 1: Nodulation parameters of EWBC with rhizobial strains after 21 days of inoculation.

Treatments	Nodule numbers	Nodules fresh weight mg plant^{-1}	Plant fresh weight mg plant^{-1}
Control	0.00	0.00	435
Rhiz 884	9	58	633
Rhiz 886	8	33	445
Rhiz 887	13	28	517
Rhiz 888	13	35	527
Rhiz 891	10	41	437
Rhiz 893	4	9	275
Rhiz 895	10	40	507
Rhiz 897	8	36	527
Rhiz 902	0.0	0.0	315
Rhiz 907	12	54	529
Rhiz 910	14	26	439
Rhiz 913	18	20	529
Rhiz 916	31	41	564
Rhiz 919	10	47	558
Rhiz 925	7	12	301
Rhiz 928	11	18	339
Rhiz 931	10	16	495
Rhiz 932	9	17	271
Rhiz 935	9	17	857
Rhiz 937	9	14	274
Rhiz 938	9	14	374
Rhiz 941	9	18	459
Rhiz 945	11	44	576
Rhiz 948	10	25	478
Rhiz 950	7	46	547
Rhiz 952	13	54	454
Rhiz 956	11	31	530
Rhiz 957	5	11	384
Rhiz 959	10	18	617
Rhiz 961	11	20	541
Rhiz 965	11	25	677
Rhiz 970	9	21	689
Rhiz 971	12	60	636
Rhiz 975	11	45	621
Rhiz 977	12	37	255
Rhiz 982	11	60	360
Rhiz 985	8	25	520
Rhiz 996	14	69	554
Rhiz 1002	17	62	502
Rhiz 1007	6	12	723
Rhiz 1010	8	12	310
Rhiz 1017	13	38	402
Rhiz 1024	10	26	504
Rhiz 1026	8	47	534
Rhiz 1028	5	13	270
Rhiz 1030	5	16	205
Rhiz 1032	15	50	647
Rhiz 1034	13	126	612
Rhiz 1037	13	52	547
Rhiz 1040	7	12	289
LSD at 0.5	**1.6**	**4.8**	**65**

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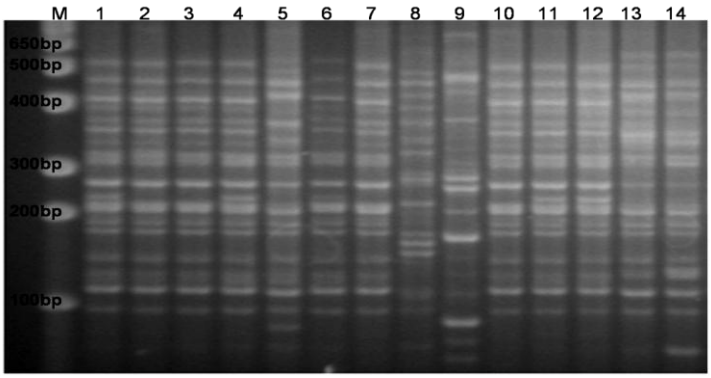

Figure 1: Competitiveness estimation for five representative *Rhizobium* strains from clover nodulated roots grown in pot experiment under normal conditions based on REP-PCR fingerprint of crushed nodules soup. M is 1 kb ladders; lanes (1, 4, 6, 11 and 12) are REP patters of Rhiz1017; lanes (2,3,5,7 and 10) are REP patterns by Rhiz 950; lanes 13 and 14 strain Rhiz 996. Lane 8 for Rhiz 975 and lane 9 for Rhiz 1024.

Selection of the most competitive *Rhizobium* strains using REP and RAPD markers

Five representative strains were chosen to select the most competitive strains using the REP and RAPD markers, among these strains two of them were highly reported to form nodules on clover roots (Rhiz 996 and 1017), two were noted to give the highest record of plant fresh weight (Rhiz 950 and 975) and one Rhiz1024 gave normal nodulation and fresh weight. The selection of these strains were done also based on their phenotypic characteristics such as resistant to salt, alkaline and temperature stress, strain Rhiz950 was resistant to 3% NaCl, Rhiz1017 was resistant to pH9 and high temperature 42 ºC [24]. Fifty six nodules which examined in four gels were removed randomly and were used to estimate the nodule competitiveness using REP-PCR. The four gels of REP PCR for analyzing the competitive strains were identical, while results in Figure 1 showed the best results and high resolution of gel REP-PCR (14 from 56) and repeated profile of DNA finger print was used to indicate for the most competitive *Rhizobium* strains. Under normal conditions, both of strain Rhiz1017 and Rhiz 950 were occupied five REP profiles (1,4,6,11 and 12) and (2,3,5,7 and 10) on respectively, followed by strain Rhiz 996 that occupied two REP profiles 13 and 14, while strain Rhiz 975 and Rhiz1024 each occupied only one REP profile (Table 2). Results of REP profiles indicated that strains Rhiz950 and Rhiz1017 were the most competitive *Rhizobium* strains with 35.71% for each one followed by strain Rhiz996 with 14.3% competitiveness. Strain Rhiz 975 and Rhiz1024 were having similar competitiveness percent (7.14%). Under alkaline stress and using the RAPD primers (Figure 2), the alkaline tolerant strain Rhiz 1017 occupied 60% of the nodules of nodule occupancy, while the other four examined strains had equal competitiveness (10%).

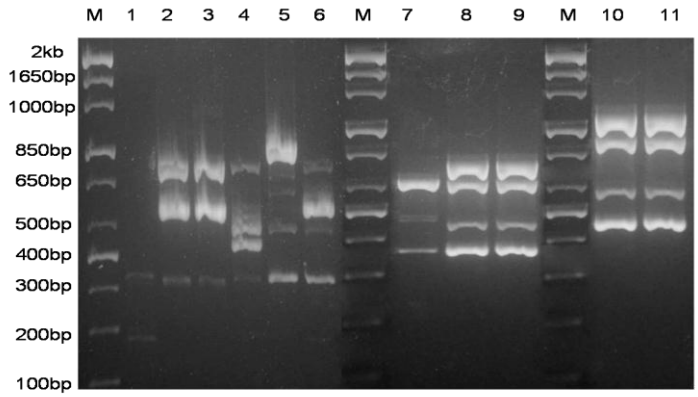

Figure 2: RAPD-PCR to select the most competitive strains under alkaline pH9, M is 1kb plus DNA ladder, lane 1 is empty, lanes 2, 3, 8, 9, 10 and 11 strain Rhiz 1017, each strain had only one pattern of the rest.

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Table 2: Nodule occupancy of *Rhizobium* strains nodulated Egyptian Clover estimated by REP and RAPD PCR at neutral pH7 and pH9, 25 days after inoculation.

Strains used	% of Nodule occupancy at neutral pH7 (REP PCR)	% of Nodule occupancy at alkaline pH9 (RAPD PCR)	Host cultivar of clover
Rhiz 950	35.71%	20%	Meskawi
Rhiz 975	7.14%	10%	Meskawi
Rhiz 996	14.3%	10%	Meskawi
Rhiz 1017	35.71%	50%	Meskawi
Rhiz 1024	7.14%	10%	Meskawi

Symbiotic performance of *Rhizobium* strains in pot experiments

To estimate the symbiotic effectiveness for long time cultivation without nitrogen fertilizers applications, the five effective and competitive *Rhizobium* strains were selected to examine their symbiotic efforts to increase the growth of Egyptian clover. Plants from pot experiments were harvested after 50 days of inoculation. Results in Table 3 show the shoot fresh and dry weight, nodule dry weight and nodulation index. The best strain for shoot dry weight was Rhiz 950 (33.49g plant^{-1}) followed by Rhiz 1017 (28.83g plant^{-1}) compared to the control. All the inoculated strains were greatly increased the dry weight of plant by about 100% more than the control. Both strains Rhiz950 and Rhiz1017 were the best strains for forming nodules. The nodulation index was 0.02g /g plant except with strain Rhiz1017 (0.03g/ g plant). Results in Figure 3 show the remarkable production of shoot fresh weight produced by inoculation with *Rhizobium* strains Rhiz 950, Rhiz1017 and Rhiz975 compared to control. Most of nodules produced by inoculated strains were formed on the main roots and a few number of them were occupied the lateral roots (Figure 4). By checking the nodules crushed soup we could easily recognized that all the tested strains were effectively due to the red color appearance of nodule pigments, indicating on the presence of leghemoglobin on white papers (Figure 5). No green nodules were observed at all.

Table 3: Nodulation parameters of the selected five effective and competitive *Rhizobium* strains in pot experiments after growing for 50 days.

Treatments	Shoot fresh weight g pot^{-1}	Shoot dry Weight g pot $^{-1}$	Nodule dry weight mg pot^{-1}	Nodulation index
Control	22.92d	11.96d	0.00e	……
Rhiz 950	107.3a	33.49a	750a	0.02
Rhiz 975	62.70b	26.30b	532c	0.02
Rhiz 996	64.52b	25.88b	550c	0.02
Rhiz 1017	103.6a	28.83ab	720b	0.03
Rhiz 1024	54.76c	22.50bc	490d	0.02
L.S.D. at 0.05	7.95	4.2	18.5	

Nodulation index= Nodule dry weight ÷ Shoot dry weight

Figure 3: Variations in shoot fresh weight as a result of clover inoculation with *Rhizobium* strains Rhiz 950 (A); Rhiz1017 (B) and Rhiz 975 (C) against control on the left side for each pair.

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Figure 4: Heavy nodulation of Egyptian clover by *Rhizobium* strains Rhiz 950 and Rhiz 1017 on respectively on main clover roots.

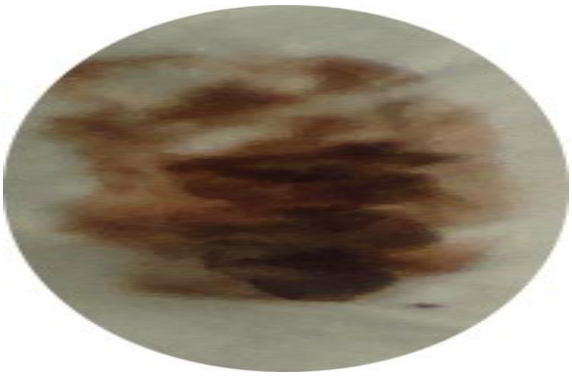

Figure 5: Red color pigments of clover nodules which indicated on the presence of leghemoglobin.

Parameters of NPK

Inoculation with the selected strains increased significantly the percent of nitrogen than the control by 20%, 16.7%, 24.3%, 33.3% and 28.2% (Table 4), while inoculation and addition of phosphorus 60kg fed^{-1} raised the phosphorus percent by 24%, 40.6%, 40.6%, 56.8% and 34.5 with the examined strains on respectively. The same trend of results was obtained with potassium content which increased due to rhizobial inoculation and K application 30kg fed^{-1} than the control by 24%, 25.8%, 45.2%, 39.5% and 35.2%. The best strain for fixing nitrogen was Rhiz 1017 followed by strain Rhiz 1024 which gave 33.35% and 28.2% over the un-inoculated plants indicating on their highly effectiveness.

Table 4: NPK parameters obtained clover plants inoculated with the selected five effective *Rhizobium* strains in pot experiments after 50 days.

Treatment	% Nitrogen	% Phosphorus	% Potassium
Control	2.8±0.23	0.19±0.06	4.6±0.1
Rhiz 950	3.5±0.45	0.25±0.07	6.1±0.4
Rhiz 975	3.4±0.51	0.32±0.09	6.2±0.2
Rhiz 996	3.7±0.31	0.32±0.05	8.4±0.1
Rhiz 1017	4.2±0.17	0.44±0.14	7.6±0.1
Rhiz 1024	3.9±0.61	0.29±0.06	7.1±0.2

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DISCUSSION

Strains were identified as *Rhizobium* strains which more closest to *R. etli* with different lineages based on the use of several molecular biology markers such as 16S *rRNA*, *atpD*, *recA* as chromosomal genes and both of *nodC* and *nifH* as symbiotic plasmid-located genes as previously published by Shamseldin *et al*. [24]. Contribution of nitrogen fixation in agriculture can be improved by inoculation of legume crops with efficient and more competitive rhizobial strains which can outcompete against indigenous and less effective strains [28]. Consequently, 48 clover rhizobial strains were screened in this study to test their symbiotic effectiveness and to select the most competitive strains that can be used as inoculants in alkaline soils as major problem in Egyptian soils. Results of plant infection technique (Table 1) indicated that there was variation in symbiotic effectiveness among strains which reflected on the plant health as over production in shoot fresh weight compared to control. About 27% of the examined strains were reported to be highly effective in nitrogen fixing with clover as they gave plant fresh weight over than the control by 17.8% to 49.2%, while a bout 20% of the examined strains were very less effective as they produced plant fresh weight equal to or less than control. Variation of symbiotic effectiveness within the same group of *Rhizobium* strains has been previously reported by Bottomley and Jenkins [29] and Valdiva *et al*. [30]. This variation in N_2 fixation capacity of some rhizobial strains may be correlated to change in their genetic structure contributing to reduce their symbiotic capacity as a result of long exposure under environmental stresses in the soil, several authors have been reported the variation of symbiotic effectiveness of rhizobial strains [29, 31, 32].

The validity of rhizobial inoculation in the field is commonly restricted by competition between native and introduced rhizobial strains [11]. Different trials are successfully used to increase competitive ability of rhizobial strains in the field such as selection of strains with high motility [33], improve chemical or physical soil prosperities through management agricultural practices [34]; Selecting a legume hosts cultivar more compatible with its micro-symbiont [35]; Selection of strains tolerant to a particular environmental stresses [36, 11]; engineered rhizobial strains capable of producing bacteriocins antimicrobial [37,22] and Manipulating rhizobia for selective substrate utilization to deliver a nodulation advantage [38]. In this study we used the strategy of selecting strains with high advantageous to survive under salt and alkaline stress [24].

De Bruijn [39] noted the suitability of using Rep-PCR fingerprint to differentiate among rhizobial strains belonging to different symbiovars, therefore the two DNA markers REP and RAPD were suggested to be used for selecting the most competitive rhizobial strains as previously noted by Moawad *et al*. [40] and Duodu *et al*. [41]. The most competitive strains were estimated after random collection of 56 nodules and surface sterilized and the nodule soup were subjected to identify the DNA fingerprint using the REP-based PCR technique. The REP PCR technique was useful to select the most competitive *Rhizobium* strain [40, 41], but it was not allowed to estimate the double nodule occupancy using this technique compared to use *gus* marker [11]. Both the salt resistant strain Rhiz 950 and alkaline tolerant strain Rhiz 1017 were equaled for its competitive ability at normal conditions. Each strain had 35.71% of nodule occupancy, while strains Rhiz 1024 and Rhiz 975 occupied 7.14%. At alkaline pH9, the competitive ability of alkaline tolerant strains Rhiz1017 increased to 60% of nodule occupancy. These results are consistent with those obtained by Shamseldin and Werner [11] who found that the salt resistant strain EBRI 26 nodulating common bean could occupy 66% of nodule occupancy at normal conditions, while its competitive ability increased to 80% at alkaline pH8. The same trend of results was observed by Moawad *et al*. [40] who noted that clover *Rhizobium* strains could occupy from 52-79% of nodules against native rhizobia. Our results are similar also to results obtained by Duodu *et al*. [41] who found that *Rhizobium leguminosarum* sv. trifolii strain 20-15 occupied 50% of nodules against other strain to nodulte white clover. The salt tolerant strain Rhiz 950 gave nodule occupancy (20%) better than the salt sensitive strains under alkaline stress. A field trial is supporting our results that alkaline tolerant strain Rhiz 1017 gave promising results in saline soil than the salt resistant strain (unpublished data)

To test the symbiotic effectiveness of the five effective strains selected based upon plant infection and competition experiments for long duration (55 days) revealed that these five strains were able to supply the clover plants with their total nitrogen requirements without to rely on nitrogen fertilizer during the whole cultivation period and this clearly shown from the difference of green growth between inoculated and non inoculated plants (Figure 3). All of these five strains were duplicated the plant dry weight compared to the control, such results were agreement with those obtained by

Marek-Kozaczuk *et al*. [42] who noted that the inoculation of clover with *R. leguminosarum* sv. trifolii combined with *Pseudomonas fluorescens* strains contributed to gave over production in plant dry weight by 180% than control. Smilar results were also obtained by Elkoca *et al*. [43] who noted that inoculation of chickpea could increase the shoot dry weight by 19.7-54.3%. The heavy nodulation by Rhiz 950 and Rhiz1017 were noticed on the top of main roots (Figure 4), such observations were previously reported by Beatie *et al*. [44]. Inoculation with all the examined five selected *Rhizobium* strains increased significantly the N% by 16.7% to 33.3%. These results are congruent with those obtained previously by Elkoca *et al*. [43] who noted that inoculation of chickpea with *Rhizobium* strains contributed to give higher nitrogen uptake than uninoculated plants.

CONCLUSIONS

The results highlighted that the variation of symbiotic effectiveness among 48 root nodulating clover bacteria was found and concluded that five strains are highly nodulating and nitrogen fixing, in addition to produced two competitive strains under neutral or alkaline soil which can be used as biofertilizer in the newly reclaimed Egyptian soils.

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