Egypt. J. Microbiol. 37, No. 3, pp . 213 - 231 (2002)

Response of Faba Bean to Inoculation with Fungicide Auxotrophic Mutants Induced in *Rhizobium Leguminosarum* Biover Viceae

K.A., Zaied[1], A.M. El-Adl[1] , M. A. Nassef[2] and A.A. Zehry[1]
[1]Department of Genetics, Faculty of Agricultural, Mansoura University and [2]Water , Soil and Environmental Research Institute, Agricultural, Res earch Center , Giza, Egypt .

THE DEVELOPMENT of nitrogen-fixing nodules is a complex process involving the expression of symbiotic genes in both the legume host and the microsymbiont. The symbiotic process has been studied here in faba bean using fungicide-auxotrophic mutants induced in *Rhizobium* . The results revealed that some auxotrophic mutants-induced had significantly effect to increase nodule development, dry matter yield, and also nodule dry weight, in contrast, some others resulted in fewer nodules development. Many of biochemical mutants-induced revealed significant stimulation in chlorophyll formation. This is due to their ability to establish an effective symbiotic relationship. Seed treatment by some biochemical mutants had a better effect on seed yield per plant. Auxotrophic mutant AMV_{10} was superior to the wild type strain in terms of increasing grain yield to 118.48%. Inoculated plant, under the field conditions, accumulated nitrogen in the range between 55.84 to 216.31 mg/plant, while uninoculated ones accumulated nitrogen between 3.53 to 77.82 mg/plant.

Keywords: Chlorophyll formation, Nodule development, Nodulation index, Nitrogen accumulation , *Rhizobium,* seed yield, Rhizobium mutants, Fungicide - induced mutants

Rhizobia is gram-negative bacteria that form nodules on legumes. In this association, the bacteria fix atmospheric nitrogen that is then assimilated by the

Corresponding author : Khalifa A. Zaied
E- mail : khalazaied @ mans . edu .eg

plant. The genetic information controlling symbiotic activity in the fast-growing rhizobia is encoded in plasmids (Hombrecker *et al.,* 1981). A symbiotic (Sym) plasmid has been defined as one that determines the plant species specificity for nodulation and contains the nitrogenase enzyme structural genes (*nif* genes) (Hooykaas *et al.,* 1982). The formation of nitrogen-fixing nodules on roots of legumes by rhizobia is a complex developmental process that involves constant communication between the partners. Products of the phenylpropanoid biosynthetic pathways, flavonoids (Rolfe, 1988), have been widely identified in legumious plants as being involved in the initial signaling steps between plant and rhizobia, including chemotaxis towards a potential host (Kape *et al.*,1991) and induction of the bacterial nodulation (*nod*) genes which are necessary to elicit a corresponding signal to the plant from the bacterium (Dahiya,1991). Most types of flavonoid compounds are found systemically in a wide variety of plants (Harborne, 1984) with the exception of the isoflavonoids which are almost exclusively found in the legumes (Harborne, 1971). Fungicidal treatments of faba bean is found effective in improving germination and emergence of the crop (Sundaresh and Hiremath, 1982). However, results on the effect of seed dressing chemicals on symbiosis of *Rhizobium* are controversial (Sundaresh and Hiremath, 1982). It may be because of the incompatibility of certain chemicals with the rhizobia, variation in techniques, soil and environmental conditions existing at the time of experimentation. The present investigations were undertaken to study the effect of inoculation with rhizobia fungicide-auxotrophic mutants induced on root nodulation, nitrogen fixation and vegetative growth parameters of faba bean through symbiotic relationship with rhizobia.

Material and Methods

Bacteria and growth conditions

Wild type isolates of *Rhizobium leguminosarum* biovar *viceae* were isolated from the root nodules of faba bean at El-Dakahlia and El-Behaira Governorates, according to the method described previously by Somasegaran and Hoben (1985). The isolates of wild type and fungicide-auxotrophic mutants induced are listed in Table 1. All of these isolates were evaluated for their efficiency in nodulation and nitrogen fixation with their legume host. Cells were grown as previously described by Karanja and Wood (1988) on yeast extract mannitol medium at 28°C. Yeast extract mannitol agar medium (YEMA medium "79") (Allen, 1959) was used for growing and for maintaining cultures according to Vincent (1970).

TABLE 1. Wild type isolates and mutants induced used in this study.

Isolate No.	Phenotype	Reference	Designation
1	WT	El-Behaira Governorate	EBW1
2	WT	El-Dakhlia Governorate (Shirbin Center)	EDSW2
3	WT	,, ,, (El-Mansoura Center)	EDMW3
4 (Strain No. 207)	WT	Agric. Res. Center (ARC), Egypt	ARCW4
5 (Strain No. 481)	WT	Agric. Utilization Res., USA	AURW5
6	AM	20 ppm Carboxin (Vitavax)	AMV6
7	AM	20 ppm Carboxin	AMV7
8	AM	40 ppm Carboxin	AMV8
9	AM	40 ppm Carboxin	AMV9
10	AM	60 ppm Carboxin	AMV10
11	AM	80 ppm Carboxin	AMV11
12	AM	100 ppm Carboxin	AMV12
13	AM	100 ppm Carboxin	AMV13
14	AM	100 ppm Carboxin	AMV14
15	AM	80 ppm Benomyl (Benlate)	AMB15
16	AM	100 ppm Benomyl	AMB16
17	AM	100 ppm Benomyl	AMB17
18	AM	2 ppm Dithane	AMD18
19	AM	2 ppm Dithane	AMD19
20	AM	8 ppm Dithane	AMD20
21	AM	10 ppm Dithane	AMD21
22	AM	4 ppm Dithane	AMD22
23	AM	8 ppm Dithane	AMD23
24	AM	10 ppm Dithane	AMD24

AM = Auxotrophic mutant. WT = Wild type

Evaluation of auxotrophic mutants with their parental strains in the large scale of field experiment

Three field experiments were carried out during the growing season of 1998/99 at two different locations. Two of them carried out using a plastic-pots system (Vincent, 1970) containing sterilized sandy and clay soils, which autoclaved at 121°C for one hour among three days, at the first location of Experimental and Agric. Research Unit of Plant Pathology Dept., Fac. of Agric., Mansoura Univ. Sandy soil was washed with a distilled water several times to diminishing chloride ions. However, the third experiment was done under field conditions at the second location of El-Saadia Village, Shirbin Center, Dakhlia Governorate, using natural clay soil (non-sterilized). The experiments in the first location were done using plastic-pots 19 x 22 cm and four replicates were performed for each treatment. However, the experimental design of the third experiment under the field conditions was a randomized complete block with three replicates. Each replicate containing 25 plots, each plot was three meters long and 40 cm between rows, where seeds were sown at one side in one seeded hill, 20 cm apart.

Inoculation

Cultures in mid-log phase growing in nutrient broth of YEM were used for inoculation (Kucey, 1989). Seeds were surface-sterilized with 10% ethanol solution (Dobert and Blevins, 1993) and washed three times with sterilized distilled water. Four-surface-sterilized seeds were planted in each plastic pot. The seeds planted in all experiments were inoculated with approximately 10^8 rhizobia cells from the previous suspension before covered. Seeds were thinned after 3-4 days of germination to three plants per plastic pot and one plant per hill in the field. The plants in the third experiment were watered to field capacity with water as needed until harvest. Cultivated plants in plastic-pots were irrigated using nitrogen free nutrient solution of Bond's modified Crone's stock salts mixture (Allen , 1959). Uninoculated seeds and those inoculated with wild type strains were considered as a negative and positive control, respectively. The plants in all experiments were inoculated after germination with 1.0 ml of rhizobia suspension (10^8 cells/ml) for three times at seven days interval's . The plants in all experiment were fertilized by phosphorus 80 kg/feddan.

Preparation of samples

Six weeks after inoculation, the plants were carefully uprooted and the roots were washed with tap water to remove the adhering clay particles. The nodules were counted and the mean nodule number per plant was calculated. Shoots, roots and nodules were dried at 70°C for 72 hours (Pineda and Nolt , 1990).

Chlorophyll measurment

Chlorophyll a and b were determined after six weeks of planting (WAP) according to Markinney (1941).

Assessment of nodulation and nitrogen fixation

The efficiency of wild type isolates and their mutants was examined for nitrogen fixation by inoculated seeds of faba bean with the cell suspension of each isolate. Some productivity parameter including average weight per nodule (AWN) was calculated according to Pereira *et al.* (1989). An index of nodulation, which removed the effect of plant size, was calculated from the raw data according to Herridge and Betts (1988).

Total nitrogen determination

Samples were digested according to the macro-kjeldahl method and nitrogen concentration was determined by colorimeteric assay (Burris and Wilson, 1957). Nitrogen and protein percentage was calculated on dry weight basis according to Jackson (1973).

Plant yielding

At harvest, four individual plants were taken at random from each experimental plot, on which the mean of the following traits was recorded; number of tillers/plant, number of pods/plant, 100 seed weight (gram), seed yield per plant (gram), and expected seed yield/feddan (kg).

Expected seed yield per feddan of faba bean cultivars

Seeding rates corresponded to approximately 13 emerged plants per m^2 and the expected seed yielding per feddan was calculated to approximately 54600 plant per feddan according to Jensen (1987).

Statistical analysis

Data were subjected to analysis of variance and LSD (P = 0.05 & 0.01), which was used to compare means between the untreated control and individual sample mean, if F-test showed significant treatment effects (Steel and Torrie, 1960).

Results and Discussion

Nodulation and dry matter yield

As shown from the results presented in Table 2, nodules were formed in all treatments, except in control experiment (uninoculated) for both sterilized clay and sandy soils. The obtained numbers and dry weight varied between different isolates. Some auxotrophic mutants of rhizobia had the highest number of nodules, a similar pattern was noticed for the dry weight of nodules.

Some of rhizobial inoculation significantly increased both the number and dry weight of nodules. Some other inoculant of auxotrophic mutants resulted in fewer nodules, but in similar total weight. Similar results were observed for soybean (Chamber and Montes, 1982). The present results are also in agreement with those found by Ramos and Ribeiro (1993), who reported that the treatments with fungicides and seed inoculation resulted in greater nodule weight, seed inoculation produced more nodules than treatments using granular inoculant. Previous studies have shown that seed treatment with fungicides can cause an increase (Lopes and Portugal, 1986), a reduction (Rennie and Dubetz, 1984) or no effect (Kucey and Bonetti, 1988) on nodulation. Ramos and Ribeiro (1993) also found that the fungicide application on seeds increased nodulation, but reduced the occurrence of the inoculated strain in nodules.

TABLE 2. Effect of inoculation with *Rhizobium* fingicide-auxotrophic mutants induced on nodulation of faba bean.

Inoculum	Nodule number (No plant^{-1})			Nodule dry weight (g plant^{-1})		
	Exp. 1	Exp. 2	Exp. 3	Exp. 1	Exp. 2	Exp. 3
Uninoculated	043	000	000	0.24	0.0000	0.0000
EBW	057	049	029	0.32	0.0463	0.0468
EDSW	083	132	116	0.67	0.0743	0.1529
EDMW	095	094	184	0.37	0.0347	0.1426
ARCW	050	041	011	0.27	0.0223	0.1118
AVRW	091	009	033	0.61	0.0190	0.0830
AMV	095	036	008	0.92	0.0703	0.0183
AMV	065	005	006	0.50	0.0343	0.0034
AMV	089	003	012	0.65	0.0140	0.0053
AMV	096	001	006	0.78	0.0070	0.0041
AMV	098	001	007	0.53	0.0160	0.0318
AMV	078	001	009	0.85	0.0050	0.0170
AMV	077	000	010	0.65	0.0000	0.0030
AMV	068	006	009	0.77	0.0207	0.0050
AMV	139	107	075	0.63	0.0580	0.0855
AMB	069	039	035	0.38	0.0737	0.1235
AMB	041	007	043	0.28	0.0233	0.1271
AMB	079	005	002	0.57	0.0070	0.0150
AMD	075	006	001	0.61	0.0200	0.0002
AMD	116	024	001	0.38	0.0173	0.0706
AMD	065	023	002	0.39	0.0207	0.0014
AMD	084	067	003	0.68	0.0357	0.0084
AMD	076	129	074	0.35	0.0603	0.0573
AMD	193	077	133	0.28	0.0343	0.1018
AMD	109	060	219	0.31	0.0190	0.1030
F-test	**	**	**	**	**	**
LSD 0.05	58.66	034	041	0.30	0.0300	0.0700
LSD 0.01	78.00	045	054	0.40	0.0500	0.0900

* , ** = . Significant at 0.05 and 0.01 probability levels, respectively. Exp. 1 = Field conditions Exp. 2 = Sterilized clay soil. Exp. 3 = Sterilized sandy soil.

Nitrogen fixation was measured by the increase in the dry weight of inoculated plants over that of negative control plants (uninoculated) . Depending upon the inoculum clone used, a large difference in the amount of dry matter yield was observed (Table 3). In this study, the dry weights of plants inoculated with some of fungicide-auxotrophic mutants induced showed significantly increase in dry matter yield over than that of negative control plants. This indicated that the treatment of faba bean seeds with *Rhizobium* prior to sowing has significant effect on the growth of the plant (Lakshmi and Gupta, 1997).

TABLE 3. Effect of inoculation with *Rhizobium* fingicide-auxotrophic mutants induced on shoot and root dry weight of faba bean.

Inoculum	Shoot dry weight (g plant^{-1})			Root dry weight (g plant^{-1})		
	Exp. 1	Exp. 2	Exp. 3	Exp. 1	Exp. 2	Exp. 3
Uninoculated	2.71	1.31	0.54	0.54	0.70	0.71
EBW_1	3.04	1.20	0.53	0.80	0.59	0.52
$EDSW_2$	3.54	1.10	1.10	0.72	0.39	0.50
$EDMW_3$	3.85	1.16	1.71	0.92	0.47	0.91
$ARCW_4$	3.81	0.98	1.25	0.78	0.39	0.78
$AURW_5$	4.87	1.13	0.96	1.00	0.37	0.46
AMV_6	5.63	1.82	0.76	1.27	1.08	1.12
AMV_7	4.25	2.00	0.69	0.95	1.06	1.57
AMV_8	4.48	1.52	0.74	0.92	0.98	0.80
AMV_9	5.78	1.76	0.75	1.38	0.83	1.17
AMV_{10}	5.34	1.38	1.33	0.98	0.81	1.27
AMV_{11}	5.58	1.58	0.85	1.15	0.63	1.82
AMV_{12}	4.90	1.15	0.93	1.02	0.88	0.90
AMV_{13}	4.61	1.21	0.92	0.90	0.56	0.70
AMV_{14}	3.86	1.17	0.85	0.81	0.30	0.50
AMB_{15}	3.29	1.37	0.79	0.66	0.41	0.77
AMB_{16}	2.81	0.86	1.01	0.82	0.32	0.57
AMB_{17}	2.40	1.01	0.91	0.56	0.27	0.74
AMD_{18}	3.36	1.49	0.91	0.90	0.78	1.08
AMD_{19}	3.63	1.65	1.06	0.98	0.46	1.12
AMD_{20}	4.10	1.07	0.78	0.87	0.23	0.82
AMD_{21}	4.40	1.33	0.66	0.86	0.37	0.87
AMD_{22}	3.20	1.77	0.76	0.75	0.42	0.35
AMD_{23}	5.44	1.31	1.02	1.09	0.26	0.73
AMD_{24}	5.61	1.16	1.19	0.98	0.39	0.67
F-test	**	**	**	**	**	**
LSD 0.05	1.04	0.55	0.70	0.28	0.43	0.47
LSD 0.01	1.39	0.73	0.93	0.37	0.57	0.63

** : Significant at 0.01 probability level.

Exp. 1 = Field conditions. Exp. 2 = Sterilized clay soil. Exp. 3 = Sterilized sandy soil.

The stimulation in nodulation and growth of the plant raised from seeds inoculated with *Rhizobium* as compared to uninoculated ones shows the efficiency of the strain of *Rhizobium* in establishing an effective symbiotic relationship. A significant increase in growth characters of plants grown from seed treated with *Rhizobium* - auxotrophic mutants compared to control indicates a synergistic effect. The present results on the interaction of fungicide - auxotrophic mutants induced in *Rhizobium* as used for seed treatment of faba bean are of practical significance because they show not only their compatibility, but also a synergistic effect.

Photosynthetic responses to nitrogen fixation

It is evident from Table 4 that there was a significant increase in formation of chlorophyll (a), (b) and total chlorophyll by many of auxotrophic mutants, as well as, their parental strains at both, field conditions and sterilized sandy soil.

This is in agreement with those reported by Hunt *et al.* (1985), who found that the photosynthetic capacities of leaves can change dramatically in response to photon flux density (Björkman, 1981), nitrogen supply or atmospheric CO_2 concentration (Griffin and Seemann, 1996). The results obtained here are in accordance with those of Sharma *et al.* (1982), who found that inoculation of lentil seed with 8 R. *leguminosarum* strains increased the leaf chlorophyll and nodule leghaemoglobin contents. These contents were positively correlated with nodule dry weight, dry matter and seed yields and total N uptake/plant.

TABLE 4. Effect of inoculation with fungicide-auxotrophic mutants induced *Rhizobium* on chlorophyll formation (mg/g fresh weight) of faba bean.

Inoculum	Chlorophyll (a) formation**			Chlorophyll (b) formation**			Total chlorophyll contents		
	Exp. 1	Exp. 2	Exp. 3	Exp. 1	Exp. 2	Exp. 3	Exp. 1	Exp. 2	Exp. 3
Unin*	0.26	0.97	0.30	0.82	3.52	1.03	1.09	4.49	1.33
EBW	0.35	0.69	0.41	1.92	4.30	1.66	2.27	4.99	2.10
EDSW	0.27	0.79	0.54	1.35	3.14	2.28	1.62	3.94	2.82
EDMW	0.25	0.94	0.62	0.98	4.16	2.54	1.23	5.10	3.17
$ARCW_4$	0.21	0.85	0.49	1.80	3.71	2.02	2.01	4.56	2.51
$AURW_2$	0.49	0.77	0.53	0.64	2.88	2.22	1.13	3.65	2.76
AMV	0.37	0.97	0.20	1.00	2.45	1.58	1.38	3.10	1.78
AMV	0.36	0.85	0.27	1.36	3.46	1.81	1.73	4.31	2.08
AMV_8	0.43	0.96	0.42	3.03	3.81	2.24	3.46	4.78	2.66
AMV	0.55	0.90	0.33	2.36	4.66	1.81	2.91	5.56	2.15
AMV	0.58	0.64	0.30	2.65	3.88	1.69	3.20	4.53	1.99
AMV	0.50	0.63	0.33	2.52	2.47	1.81	3.02	3.11	2.14
AMV_{12}	0.47	0.83	0.48	2.34	2.97	2.75	2.81	3.81	3.23
AMV	0.42	0.85	0.55	2.17	2.92	2.95	2.60	3.77	3.50
AMV	0.34	0.77	0.47	1.91	3.62	2.37	2.26	4.39	2.85
AMB	0.32	1.04	0.55	2.10	3.85	2.63	2.42	4.93	3.18
AMB	0.34	0.95	0.35	2.30	3.83	2.75	2.64	4.78	3.10
AMB_{17}	0.30	0.95	0.58	2.30	2.76	2.51	2.60	3.71	3.09
AMD	0.23	0.79	0.65	1.82	4.00	3.06	2.05	4.79	3.71
AMD	0.24	0.95	0.49	2.82	3.71	2.26	3.06	4.66	2.75
AMD	0.28	1.04	0.42	1.86	3.93	1.52	2.14	4.97	1.94
AMD_{21}	0.32	0.98	0.55	1.71	3.80	1.99	2.03	4.78	2.54
AMD_{22}	0.24	0.90	0.71	1.40	3.49	3.13	1.64	4.40	3.84
AMD	0.39	1.16	0.58	1.95	3.75	2.34	2.34	4.90	2.92
AMD_{24}	0.18	0.72	0.57	1.29	3.90	2.56	1.47	4.62	3.12
F-test	*	NS	**	**	NS	**	**	NS	**
LSD 0.05	0.21		0.19	0.80		0.93	0.86		1.07
LSD 0.01	0.28		0.25	1.08		1.24	1.15		1.43

* = Uninoculated. ** = Data was taken at seven weeks from sowing.
NS = Not significant. *, ** = Significant at 0.05 and 0.01 probability levels, respectively

The present results suggest that many of auxotrophic mutants and their parental strains who showed significantly increase in chlorophyll formation under the field conditions, also showed similar nature under sterilized sandy soil conditions. This stimulation in chlorophyll formation of the plant raised from seeds inoculated with *Rhizobium*-auxotrophic mutants as compared to uninoculated ones shows the efficiency of the strain of *Rhizobium* in establishing an effective symbiotic relationship. This indicates a synergistic effect.

Grain yield

As shown from the results presented in Table 5 the treatments of seeds with some of *Rhizobium*-auxotrophic mutants induced by fungicides had a negative effect on yield, although the results were not significant between treatments in sterilized clay soil for both seed yield per plant, 100 seeds weight and expected grain yield per feddan. On the other hand, significant differences were obtained between treatments under the field conditions, for both seed yield per plant and expected grain yield per feddan. Seeds treated with some of *Rhizobium* -auxotrophic mutants appeared to be a better effect on seed yield per plant and expected grain yield per feddan.

TABLE 5. Yield components in faba bean (*Vicia faba*) as influenced by inoculation with fungicide-auxotrophic mutants induced of *Rhizobium leguminosarum* biovar *viciae* under field conditions (Exp. 1) and sterilized clay soil (Exp. 2).

Inoculum	Seed yield / plant		100 seeds weight (g)		Expected grain yield (kg feddan^{-1})	
	Exp. 1	Exp. 2	Exp. 1	Exp. 2	Exp. 1	Exp. 2
Uninoculated	26.62	0.78	51.23	31.33	1453.33	042.58
EBW$_1$	15.64	1.49	50.17	45.67	0853.33	081.66
EDSW$_2$	21.02	0.92	58.80	65.33	1147.33	050.22
EDMW$_3$	23.07	1.78	48.33	59.67	1259.33	097.19
ARCW$_4$	18.91	1.31	60.24	47.33	1032.33	071.34
AURW$_5$	15.72	1.33	47.62	52.73	0858.00	072.44
AMV$_6$	13.54	2.07	47.90	43.53	0739.00	113.20
AMV$_7$	22.05	1.44	59.23	55.00	1203.33	078.81
AMV$_8$	14.16	1.71	54.22	51.90	0773.00	093.55
AMV$_9$	22.66	1.85	54.20	55.57	1237.33	100.83
AMV$_{10}$	34.17	2.26	56.00	50.67	1865.67	123.40
AMV$_{11}$	27.38	2.37	59.77	47.50	1494.33	129.40
AMV$_{12}$	15.46	1.22	49.90	52.33	0844.00	066.43
AMV$_{13}$	20.89	1.53	56.07	55.50	1141.00	083.54
AMV$_{14}$	09.33	2.55	59.61	61.00	0509.33	139.06
AMB$_{15}$	18.13	1.46	51.99	69.83	0989.67	079.90
AMB$_{16}$	17.61	2.20	58.86	53.13	0961.33	119.94
AMB$_{17}$	18.25	1.28	46.77	55.77	0996.33	069.89
AMD$_{18}$	16.18	1.81	44.11	54.70	0883.33	098.83
AMD$_{19}$	13.09	0.72	52.55	40.17	0714.67	039.31
AMD$_{20}$	21.37	1.24	55.98	48.00	1166.33	067.52
AMD$_{21}$	15.98	1.59	55.15	59.47	0872.33	086.63
AMD$_{22}$	21.33	1.65	58.23	54.07	1164.33	089.91
AMD$_{23}$	16.41	1.63	57.08	63.00	0895.67	089.00
AMD$_{24}$	14.93	2.28	54.33	58.03	0815.00	124.49
F-test	*	NS	NS	NS	*	NS
LSD 0.05	10.68				583.34	
LSD 0.01	14.28				779.79	

* = Significant at 0.05 probability level. NS = Not significant.

Uninot. = Uninoculated

The results also showed that some of *Rhizobium*-auxotrophic mutants and their wild type isolates appeared to have a better effect (under sterilized clay soil) on seed yield per plant, 100 seeds weight and grain yield per feddan, than uninoculated control plants. The present results is in agreement with those of Ram *et al.* (1984), who reported that treatment of seeds with *Rhizobium* followed by fungicide proved best in terms of plant height, nodule number and oven-dry weight of nodules. The same authors also found that this treatment had a positive effect on yield, as well as, treatment of seed with fungicide before inoculation with *Rhizobium* appeared to have a better effect on plant growth, but treatment with *Rhizobium* alone or fungicide alone seemed to be better for nodulation. Treatment of seeds with *Rhizobium* (under field conditions), resulting in low estimate of seed production than uninoculated control plants. In contrast, some isolates of auxotrophic mutants appeared to have a better effect on seed production than their parental wild type isolates. This are in accordance with Ram *et al.* (1984), who found that, with *Rhizobium* treatment, the seed yield was greater between 4.5 to 6.6%, lending support to the findings of Rai *et al.* (1977)

TABLE 6. Effect of seed treatment with rhizobia auxotrophic mutants induced on nodulation parameters of faba bean plants grown through a symbiotic relationship with *Rhizobium*.

Inoculum		Average weight/nodule			Nodulation index (%)		
		Exp. 1	Exp. 2	Exp. 3	Exp. 1	Exp. 2	Exp. 3
Uninoculated		0.0060	0.0	0.0	9.00	0.0	0.0
EBW_1		0.0057	0.00085	0.00180	10.58	3.21	9.16
$LDSW_2$		0.0080	0.00059	0.00140	18.62	6.72	14.08
[illegible]		0.0040	0.00039	0.00079	9.63	3.00	8.32
$ARCW_4$		0.0059	0.00054	0.01382	7.42	2.29	8.40
$AURW_5$		0.0073	0.00330	0.00161	12.67	1.66	7.80
AMV_6		0.0096	0.00190	0.00240	16.65	3.76	1.72
AMV_7		0.0104	0.00600	0.00046	11.96	1.88	0.48
AMV_8		0.0074	0.00390	0.00037	15.34	1.10	0.70
AMV_9		0.0079	0.00390	0.00048	13.74	0.36	0.49
AMV_{10}		0.0062	0.01600	0.00684	9.89	1.10	4.80
AMV_{11}		0.0109	0.00500	0.00212	15.33	0.29	2.64
AMV_{12}		0.0089	0.00170	0.00030	13.33	0.18	0.34
AMV_{13}		0.0114	0.00260	0.00036	17.11	1.85	0.54
AMV_{14}		0.0045	0.00053	0.00112	17.53	4.92	9.71
AMB_{15}		0.0058	0.00210	0.00597	12.16	2.88	18.15
AMB_{16}		0.0073	0.00330	0.00425	9.99	2.63	11.96
AMB_{17}		0.0074	0.00190	0.00251	25.01	0.66	1.51
AMD_{18}		0.0092	0.00340	0.00005	18.12	1.36	0.02
AMD_{19}		0.0038	0.00110	0.03533	11.26	1.03	7.49
AMD_{20}		0.0055	0.00100	0.00028	10.83	2.03	0.15
AMD_{21}		0.0083	0.00050	0.00260	15.83	2.86	1.28
AMD_{22}		0.0057	0.00045	0.00077	11.34	3.30	7.70
AMD_{23}		0.0015	0.00045	0.00080	5.27	2.62	9.78
AMD_{24}		0.0028	0.00031	0.00060	5.64	1.64	11.15
F-test		**	**	**	*	**	**
LSD	0.05	0.0036	0.00578	0.01122	8.99	2.45	7.37
	0.01	0.0048	0.00772	0.01500	12.03	3.27	9.86

*, ** = Significant at 0.05 and 0.01 probability levels, respectively.

Exp. 1 = Field conditions. Exp. 2 = Sterilized clay soil. Exp. 3 = Sterilized sandy soil

It is evident from Table 6 that there was a significant increase in average weight/nodule due to inoculation, by some of auxotrophic mutants at three different experiments. Moreover, there was a significant increase in nodulation index following inoculation treatments with some auxotrophic mutants, as well as, their parental isolates, as compared to untreated control. These results revealed that the treatment of faba bean seed with *Rhizobium*- auxotrophic mutants prior to sowing may has significant effect on nodulation parameters nitrogen fixation and growth characters of the plants. A significant increase in these parameters from seed treated with *Rhizobium* indicates a synergistic effect.

The results demonstrated the variability in nodulation parameters in faba bean as response to inoculation with *Rhizobium*-auxotrophic mutants and their parental wild type isolates. The low values in nodulation index obtained in this study by many of auxotrophic mutants induced were not entirely unexpected in light of the problems encountered in soils not previously cropped with faba bean and featuring elevated levels of soil NO^{-3}. Results of previous research had shown that the normal rate of inoculation was insufficient to optimize nodulation of Bragg soybean sown into a rhizobia-low soil (Herridge *et al.,* 1987) because of poor survival of the inoculant rhizobia and that nodulation levels could be increased with increased levels of applied inoculant (Herridge *et al.,* 1984). In both of the Australian studies, levels of nodulation were dependent upon the sizes of the populations of *B. japonicum* established in the seedling rhizospheres (Herridge *et al.,* 1987). As shown from the present study, nodules were formed in all treatments, except for in both sterilized clay and sandy soils, but average weight/nodule and nodulation index varied between inoculants, according to strains activity in their symbiotic relationship.

Traditionally, it has long been recognized that nodule number is generally not correlated with the effectiveness of a strain since ineffective strains often form numerous small nodules (Kuykendall *et al.,* (1996).

Nodulation index indicated that nodule mass increased concurrent with nodule number, and specific activity in fixing nitrogen was constant (Hunter and Kuykendall, 1990). After six weeks of growth, even in soil already populated with faba bean-nodulating *Rhizobium,* plants inoculated with some of auxotrophic mutants were significantly better nodulated than those inoculated with others of them. The present results agreed well with Kuykendall *et al.*

(1996), who found fourty-four percent more nodules were produced by the mutant strain of Bradyrhizobium japonicum compared to its progenitor. This is in accordance with their earlier findings in greenhouse studies under single-strain conditions (Hunter and Kuykendall, 1990).

Total nitrogen accumulation

As shown from the results presented in Table 7, plants inoculated with *Rhizobium* auxotrophic mutants and their wild type isolates accumulated significant values of nitrogen, most of them effectively fixed N_2 under three different experiments. Uninoculated plants accumulated between 3.53 and 77.82 mg N plant^{-1}. Inoculated plants accumulated N_2 with average ranged between 55.84 and 216.31 mg plant^{-1} under the field condition. However, the inoculated plants under sterilized clay soil accumulated N_2 15.85 and up to 67.35 mg plant^{-1}. Under sterilized sandy soil , the inoculated plants accumulated between 3.87 and 29.75 mg N plant^{-1}. This is due to the growth of plants in each experiment and also depended on the native rhizobia population in the field in the first experiment. Most of auxotrophic mutants, as well as, some of their parental strains revealed a significant increase in the shoot protein percentage than uninoculated ones. The advantages of more protein percentage in the shoots was potentially more useful than others for animal feeding. A significant increase in nitrogen fixation and also in protein percentages of plants grown from seed treated with fungicide-auxotrophic mutants of *Rhizobium* compared to control indicates a synergistic effect. As shown from the results, most of auxotrophic mutants gave good average yields of N in all treatments, for their high efficiency in accumulating N. Several good N_2 fixers of isolates were listed among the present study for shoot data. The results obtained in this study are in agreement with those found by Gil *et al*. (1997), who demonstrated that the N content in the shoot biomass was always higher in the legumes than in grass. However, *B. humidicola* in monoculture showed the highest total N accumulation (18.1g m^{-2}) due to its higher growth rate. Legumes in monocrop had a very significant proportion of N derived from biological N_2- fixation (BNF) (37-69%), with C. pubescens as the better N_2 -fixing legume (51-69%). The present results are also in agreement with those found by Kucey and Bonetti (1988), who reported that strains which showed high levels of nitrogen fixation also resulted in higher seed yields than strains less effective for the symbiosis.

TABLE 7. Effect of inoculation with *Rhizobium* auxotrophic mutants induced and their parental wild type isolates on N accumulated in the biomass of faba bean.

Inoculum		Total N (g / shoot)			Protein (%) in the shoots		
		Exp. 1	Exp. 2	Exp. 3	Exp. 1	Exp. 2	Exp. 3
Uninoculated		0.078	0.019	0.0035	17.94	09.14	04.06
EBW_1		0.101	0.024	0.0039	20.85	12.69	04.58
$EDSW_2$		0.142	0.044	0.0121	25.08	25.39	06.87
$EDMW_3$		0.065	0.026	0.0297	10.50	14.35	10.92
$ARCW_4$		0.106	0.024	0.0187	17.48	15.06	09.33
$AURW_5$		0.150	0.016	0.0091	19.25	08.56	05.94
AMV_6		0.157	0.028	0.0067	17.41	09.52	05.56
AMV_7		0.164	0.045	0.0094	24.19	14.27	08.62
AMV_8		0.195	0.051	0.0166	27.25	21.10	13.96
AMV_9		0.200	0.067	0.0086	21.67	23.90	07.14
AMV_{10}		0.216	0.049	0.0096	25.27	22.23	07.58
AMV_{11}		0.145	0.060	0.0117	16.83	23.62	08.71
AMV_{12}		0.164	0.047	0.0170	21.12	25.39	11.46
AMV_{13}		0.158	0.039	0.0171	21.50	20.62	11.79
AMV_{14}		0.132	0.036	0.0172	21.31	19.50	12.75
AMB_{15}		0.112	0.035	0.0100	21.21	15.89	07.89
AMB_{16}		0.111	0.023	0.0123	24.69	16.66	07.62
AMB_{17}		0.056	0.030	0.0111	14.50	18.96	07.62
AMD_{18}		0.131	0.031	0.0156	24.56	13.00	10.81
AMD_{19}		0.149	0.045	0.0163	25.83	17.12	09.69
AMD_{20}		0.106	0.034	0.0094	16.17	19.89	07.39
AMD_{21}		0.078	0.044	0.0071	11.15	20.75	06.71
AMD_{22}		0.106	0.053	0.0059	20.69	18.77	04.87
AMD_{23}		0.193	0.041	0.0217	22.15	19.77	13.23
AMD_{24}		0.176	0.039	0.0141	19.56	21.00	07.39
F-test		**	**	**	**	**	**
LSD	0.05	0.032	0.015	0.0053	1.83	2.08	1.49
	0.01	0.043	0.020	0.0071	2.44	2.79	1.99

** = Significant at 0.01 probability level.

Exp. 1 = Field conditions. Exp. 2 = Sterilized clay soil. Exp. 3 = Sterilized sandy soil

References

Allen, O.N. (1959) *Experimental in Soil Bacteriology.* Burgess Pub. Co. Minn, Minnesota, USA.

Björkman, O. (1981) Responses to different quantum flux densities. *Encyclopedia of Plant Physiology* (NS), Vol. 12 A Physiological Plant Ecology I (eds O.L. Lange, P.S. Nobel, C.B. Osmond and H. Ziegler), pp. 57-107. Springer-Verlag, Berlin.

Burris, R.H. and Wilson, P.W. (1957) Methods for measurement of nitrogen fixation. In *Methods in Enzymology.* Vol. III. Eds. S.P. Colwick and N.O. Kaplan, PP. 355-366. Academic Press, N.Y.

Chamber, M.A. and Montes , F. J. (1982) Effect of some seed disinfectants and methods of rhizobial inoculation on soybean (Glycine max L. Merrill). *Plant and Soil,* **66,** 353.

Dahiya, J.S. (1991) Cajaflavanone and cajanone released from *Cajanus cajan* (L. Millsp) roots induce nod genes of *Brady Rhizobium* sp. *Plant and Soil,* **134,** 297.

Dobert, R.C. and Blevins, D.G. (1993) Effect of seed size and plant growth on nodulation and nodule development in lima bean (*Phaseolus lunatus* L.). *Plant and Soil,* **148,** 11.

Gil, J.L., Guenni, O. and Espinoza, Y. (1997) Biological N_2-fixation by three tropical forage legumes and its transfer to *Brachiaria humidicola* in mixed swards. *Soil Biol. Biochem.,* **29**(5/6): 999.

Griffin, K.L. and Seemann, J.R. (1996) Plants, CO_2 and photosynthesis in the 21^{st} century. *Chemistry and Biology,* **3,** 245.

Harborne, J.B. (1971) Distrivution of flavonoids in the leguminosae. In *Chemotaxonomy of the leguminosae.* Eds. J. B. Harborne, D. Boulter and B.L. Turner, PP. 31-67. Academic Press, London.

Harborne, J.B. (1984) Phenolic compounds. In *Phytochemical Methods.* A guide to modern tevhniques of plant analysis. 2^{nd} Edition Ed. J.B. Harborne. PP. 45-85. Chapman and Hall, London.

Herridge, D.F. and Betts , J.H. (1988) Field evaluation of soybean genotypes selected for enhanced capacity to nodulate and fix nitrogen in presence of nitrate. *Plant and Soil,* **110** : 129.

Herridge, D.F., Roughly, R.J. and Brockwell J. (1984) Effect of rhizobia and soil nitrate on the establishment and functioning of the soybean symbiosis in the field. Aust. *J. Agric. Res.,* **35**: 149.

Herridge, D.F., Roughly, R.J. and Brockwell , J. (1987) Low survival of *Rhizobium japonicum* inoculate leads to reduced nodulation, nitrogen fixation and yield of soybean in the current crop but not in the subsequent crop. Aust. *J. Agric. Res.,* **38**: 75.

Hombrecker, G., Brewin N.J. and Johnston, A.W.B. (1981) Linkage of genes of nitrogenase and nodulation ability on plasmids of *Rhizobium leguminosarum* and *Rhizobium phaseoli.* Mol. Gen. Genet., **182** : 133.

Hooykaas, P.J.J.,Van Brussel, A.A.N., den Dulk-Ras, H., Van Slogteren, G.M.S.and Schilperoort, R.A. (1982) *Sym* plasmid of *R. trifolii* expressed in different Rhizobial species and *Agrobacterium tumefaciens.* Nature (London), **291**: 351.

Hunt, E.R.; Weber, J.A. and Gates, D.M. (1985) Effects of nitrate application on *Amaranthus powellii* Wates. I. Changes in photosynthesis, growth rates and leaf area. *Plant Physiology,* **79**: 609.

Hunter, W.J. and Kuykendall, L.D. (1990) Enhanced nodulation and nitrogen fixation by a revertant of a nodulation defective *Brady Rhizobium japonicum* tryptophan auxotroph. *Appl. Environ. Microbiol.,* **56**: 2399.

Hunter, W.J. and KuykendallL, D. (1995) Symbiotic properties of 5- methyltryptophan - resistant mutants of *BradyRhizobium japonicum. Plant and Soil,* **173**: 293.

Jackson, M.L. (1973) *Soil Chemical Analysis.* Prentic-Hall of India. Private Limited, New Delhi, pp. 38-251.

Jensen, E.S. (1987) Inoculation of pea by application of *Rhizobium* in the planting furrow. *Plant and Soil,* **97**: 63.

Kape, R., Parniske, M. and Werner , D. (1991) Chemotaxis and nod gene activity of *Brady Rhizobium japonicum* in response to hydrocinnamic acids and isoflavonoids. *Appl. Environ. Microbiol.*, **57**: 316.

Karanja, N.K. and Wood, M. (1988) Selecting *Rhizobium phaseoli* strains for use with beans (*Phaseolus vulgaris* L.) in Kenya: Tolerance of high temperature and antibiotic resistance. *Plant and Soil,* **112** : 15.

Kucey, R.M.N. (1989) .Response of field bean (*Phaseolus vulgaris* L.) to levels of *Rhizobium leguminosarum* bv. *phaseoli* inoculation in soils containing effective indigenous *R. leguminosarum* bv. *phaseoli* populations. *Can. J. Plant Sci.,* **69**: 419.

Kucey, R.M.N. and Bonetti, R. (1988) Effect of resicular arbuscular mycorrhizal fungi and captan on growth and N_2 fixation by *Rhizobium* - inoculated field beans. *Can. Soil Sci.,* **68**: 143

Kuykendall, L.D., Hashem, F.M. and Hunter, W.J. (1996) Enhanced competitivenes of a *BradyRhizobium japonicum* mutant strain improved for nodulation and nitrogen fixation. *Plant and Soil,* **186**: 121.

Lakshmi, B.S. and Gupta, J. P. (1997) Effect of thiophanate-methyl and *Rhizobium* seed treatment on nodulation and growth of soybean. *Indian Phytopath.*, **50** (2) : 294.

Lopes, E.S. and Portugal, E.P. (1986) Compatibilidade enter o trataento de sementes d amendoium comfungicidas, sobrevivencia de *Rhizobium* e nodulacao. *Bragantia,* **45**:293.

Markinney, G. (1941) Absorption of light by chlorophyll solution. *J. Biol. Chem.,* **140**: 315.

Pereira, P.A.A., Burris R.H. and Bliss F.A. (1989) ^{15}N-dinitrogen fixation potential of genetically diverse bean lines (*Phaseolus vulgaris* L.). *Plant and Soil,* **120**:171.

Pineda, P. and Nolt, J.K. (1990) Response of bean varieties to inoculation with selected *Rhizobium* strains in El-Salvador. *Turrialba,* **3**: 410.

Rai, R., Singh, S.N. and Murtnza , M.D. (1977) Differential response of *Rhizobium* strains of bengal-gram (*Cicer arietinum* L.). *Curr. Sci.*, **46** (6) : 572.

Ram, G., Chandrakar, B.S. Misra , M.K. and Katre, R.K. (1984) Effect of seed treatment with *Rhizobium* and dithane M-45 on plant height, nodulation and yield of chickpea. *Indian J. Agric. Sci.,* **54**(3): 214.

Ramos, M.L.G. and Ribeiro , W.Q. (1993) Effect of fungicides on survival of *Rhizobium* on seeds and the nodulation of bean (*Phaseolus vulgaris* L.). *Plant and Soil,* **152**:145.

Rennie, R.J. and Dubetz, S. (1984) Effect of fungicides and herbicides on nodulation and N_2 fixation in soybean fields lacking indigenous *Rhizobium japonicum*. *Agron. J.*, **76**(3): 451.

Rolfe, B.G. (1988) Flavones and isoflavones as inducing substances of legume nodulation. *Biofactors,* **1**: 3 .

Sharma, P.K., Chahal, V.P.S. and Rewari , R.B. (1982) Studies on relationship between chlorophyll content and nitrogen fixation in lentil (*Lens esculenta* L.) nodulated by different strains of *Rhizobium leguminosarum*. *Ind. J. Microbiol.,* **22** (4), 291.

Somasegaran, P. and Hoben, H.J. (1985) To collect nodules and isolate Rhizobium. In: *Methods in legume - Rhizobium Technology.* PP. 4-21. United States Agency for International Development.

Steel, R.G.D. and Torrie , J.H. (1960) *Principles and Procedures of Statistics.* McGraw-Hill, Toronto, Ont., 481 p.

Sundaresh, H.N. and Hiremath , P.C. (1982) Pesticides. 16:22 (Cited from Lakshmi and Gupta, 1997, *Indian Phytopath.,* **50** (2) : 294.

Vincent, J.M. (1970) A Manual for the Practical Study of the Root Nodule Bacteria. IBP15. *Blackwell Scientific Publications.* Oxford.

Wildin, K.D. and Kennedy, B.W. (1983) Phytopathology, **73** : 429-434. (Cited from Lakshmi and Gupta, 1997, *Indian Phytopath.,* **50** (2):294.

(*Received* 3 /4 2001;
accepted 28 /7 /2002)

إستجابة الفول البلدي للتلقيح بطافرات الرايزوبيم المستحدثة بواسطة المبيدات الفطرية

خليفه عبد المقصود زايد ، على ماهر محمد العدل ، محمود عبد المقصود ناصف و عبد الغنى عبد الغنى زهرى*

قسم الوراثة - كلية الزراعة - جامعة المنصورة -المنصورة
و* معهد بحوث الأراضى والمياه والبيئة ، مركز البحوث الزراعية القاهرة - مصر .

إن إستعمال المبيدات الفطرية لحماية البذور والجذور من الأمراض المحمولة فى التربة يعتبر ضرورياً فى إنتاج الفول البلدى تحت ظروف الرى فى الأراضى المصرية. ولسوء الحظ فإن إستعمال هذه المبيدات ربما يكون غير متوافق مع تلقيح الفول البلدى بالرايزوبيوم لما تسببه هذه المبيدات من أضرار وراثية لسلالات الرايزوبيوم التى تقيم علاقة تكافلية مع هذا العائل البقولى. وتعتبر عملية تكوين العقد الجذرية والتى فيها يتم تثبيت الآزوت الجوى عملية معقدة يدخل فيها تغيير الجينات التى لها علاقة بالمعيشة التكافلية بين الميكروب والعائل البقولى. وفى هذا البحث تمت دراسة العلاقة التكافلية بين الطافرات الكيموحيوية والمستحدثة بواسطة المبيدات الفطريه فى الرايزوبيوم والعائل البقولى لها. وقد أوضحت النتائج مدى كفاءة بعض الطافرات الكيموحيوية فى إحداث زيادة معنوية لمعدل تكوين العقد الجذريه والوزن الجاف لكل من النبات وللعقد الجذريه ، على النقيض من ذلك كانت كفاءة البعض الآخر من الطافرات محدودة فى معدل تكوين العقد الجذريه. وقد نتج عن العديد من الطافرات الكيموحيويه زيادة معنويه فى معدل تكوين الكلورفيل وهذا يعكس مدى كفاءة تلك السلالات فى تكوين علاقة تكافليه ذات كفاءة مع العائل البقولى لها. وقد أوضحت النتائج أن معاملة البذور ببعض طافرات الرايزوبيا ربما يكون لها تأثير إيجابى يفوق السلالات الأبوية لها فى معدل إنتاج النباتات من البذور. وفى هذا الصدد فقد حقق التلقيح بطفرة العوز الغذائى AMV_{10} زيادة نسبيه فى معدل إنتاج النباتات من البذور قدرها ٤٨، ١١٨ ٪ وذلك فى التجربة التى أجريت فى التربة

الطينيه المعقمة. ولقد تراوحت نسبة النيتروجين المتراكم بالمجموع الخضرى للنباتات غير الملقحة في التجربة التى أجريت تحت الظروف الحقليه مابين ٣,٥٢ إلى ٧٧,٨٢ مللى جرام للنبات ، بينما فى النباتات الملقحة تحت نفس الظروف الحقلية تراوحت القيم مابين ٥٥,٨٤ إلى٢١٦,٣١ مللى جرام للنبات. وعلى العموم أظهرت النباتات التى قد تم تلقيحها بالرايزوبيا تحت الظروف الحقلية أقصى معدل من قيم النيتروجين المتراكم بالمجموع الخضرى لها مقارنه بتلك الموجودة في النباتات التي قد نمت فى كل من التربة الطينيه والرمليه المعقمة. ولذلك فإن النتائج المتحصل عليها من هذه الدراسة تعكس مدى أهمية تلقيح النباتات بسلالات الرايزوبيا المتخصصه والفعاله من الناحيه الوظيفيه وعلاقة ذلك بمدى كفاءة تكوين العقد الجذريه وتثبيت الآزوت الجوى ومدى إرتباط هذه الصفات بمعدل النمو فى المجموع الخضرى وأثره بالتالى على زيادة محصول النباتات من البذور ومحتوى المجموع الخضرى من البروتين هذا بدوره يعتبر هاماً فى زيادة القيمة الغذائية للنباتات التى تستخدم فى تغذية حيوانات المزرعة.