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Original research

Antibiotic-tolerant Rhizobium leguminosarum biovar.vicia in Egyptian Soil

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Abstract:

Extensive use of antibiotics and their mismanagement significantly contribute to the emergence of soil microorganisms that are resistant to antibiotics, especially in the root zone. In this study, twenty-one isolates of *Rhizobium leguminosarum biovar vicia* were isolated from three cultivars of faba- bean that is cultivated in the El Shenab area of Aswan Governorate. For these isolates, morphological, cultural, and biochemical characteristics were examined. 21 isolates of the soil bacterium *Rhizobium leguminosarum* were screened for their response to three widely used antibiotics by using the minimum inhibitory concentration (MIC) test. The isolates were grown in a yeast mannitol ager (YMA) medium, which contains different concentrations (100 – 1000 ppm) of three antibiotics commonly used in Egypt (amoxicillin, cefotaxime, and tetracycline) individually. Six resistant isolates were obtained. The isolates (40A, 40B, and 843B) were the most resistant of the six isolates to antibiotics with high efficiency under the concentration of 1000 ppm for the three antibiotics. The percentage of tolerance and inhibition of the antibiotic for these three isolates was Amoxicillin (72%, 65%, and 45%), Cefotaxime (60%, 55%, and 47%), and Tetracycline (65%, 55%, and 51%) respectively. The three isolates showed high efficiency in breaking down antibiotics. These isolates can be used in agricultural farms that contain antibiotics.

Keywords: antibiotics, *faba bean*, nitrogen fixation, and *Rhizobium leguminosarum.*

1- Introduction

As one of the largest food crops in the world for both human consumption and trade, with the highest nutritional and economic value, faba bean is grown in many temperate and warm countries. Additionally, due to their abundance in protein, phosphorus, iron, and ascorbic acid, faba beans is a favorite winter vegetable of Egyptians and many other people worldwide. Egypt has a total area of about 53874 thousand acres.

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One of the mechanisms that contribute to agricultural production and the food supply is biological nitrogen fixation (Moawad *et al.,* 2004) acheived by a specific group of soil bacteria, which are either free in soil or coexist with leguminous plants . These bacteria typically coexist in large numbers in different soils. (Somasegaran and Hoben, 1994).

The inoculation of legumes with effective rhizobial strains may enhance their ability to fix atmospheric nitrogen. These rhizobia are used in agriculture to boost the productivity of leguminous crops and as a model system to investigate relationships between various spheres of life.

There are a variety of interactions between chemicals poured into soil and the N2-fixing bacteria. The development and spread of N2-fixing bacteria in their environment may be directly hampered by these substances.

Some N_2-fixing bacteria may contribute to the biodegradation of contaminants in the soil and root coat, though this is not certain. It is well known that all nitrogen-fixing bacteria possess an extremely broad enzyme profile and are capable of carrying out extremely complex biotransformation processes (Belay and Assefa 2021, Singh *et al.,* 2013).

Using legumes in general and in particular faba beans as a crop or in pastures has two advantages. The first is the plant's independence from the soil nitrogen, and the second is the possible improve in soil nitrogen levels. As a result, it is possible to use fewer chemical fertilizers and encourage the ability of legume plants to benefit nitrogen fixation and nutrient absorption (Motghare and Gauraha, 2012; Rashid, 2013). However, rhizobia are impacted by various environmental factors, such as antibiotics produced by various organisms in the roots or that are added by humans. As a result, the selection of resistant strains that can compete with antibiotics and increase the crop production (Peix *et al.*, 2001 ; Rashid *et al.*, 2009)

Unfortunately, this issue is getting worse over time, and many of the substances involved are synthetic chemicals that are difficult for the environment to break down. (Moawad *et al.,* 20٠4).

Due to the significance of this sort of biological process, the objectives of this study were as follows:

1) Isolation and identification of rhizobia bacteria isolated from the root nodules of three cultivars of faba bean cultivated in the city of Aswan.

2) Studying the antibiotic resistances of identified isolates toward three efficient antibiotics.

2. Materials and Methods

2.1. First, gather and test bacterial isolates:
In a significant area in the (El Shenab region of Aswan governorate), root nodules from plant samples used of three faba bean Varieties (Giza 40, Giza 843, Giza 716), good to grow at Aswan governorate, to isolate 21 rhizobium strains. The growing regions of Vicia faba in Egypt were gathered for the experiment using nodules. Plants were randomly chosen from farmer's faba beans at the collection sites, which are known to be farmed field faba bean cultivation sites without a history of rhizobium inoculation in nodulating field faba bean. Faba bean nodules were detached from their roots, cleaned in fresh water, and put in a calcium chloride vial for preservation. The wet nodules, which could result in the nodules decomposing, were soaked in calcium chloride. (Yasmin *et al.*, 2020; Zhang *et al.*, 2003).

- Nodule surface sterilization

By submerging the collected nodules in 95% alcohol for 5–10 seconds, the nodules were surface sterilized. The nodules were then submerged for 3–4 minutes in 0.1% Clorox. Then, six rounds of sterile water were used to cleanse these nodules. After being broken up, the nodules were streaked on the YMA agar medium. The remaining collected nodules were kept until subsequent bacterial isolation in a falcon tube filled with silica gel.

- Rhizobia were isolated from faba bean root nodules.

The following ingredients were present in the YMA agar medium: The following ingredients were used in 1 liter of deionized water: K_2HPO_4 (0.5 g), $MgSO_4$.7HO (0.2 g), NaCl (0.2 g), $CaCO_3$ (0.2 g), Mannitol (10 g), Yeast Extract (0.4 g), and Agar (20 g). An HCl solution was used to bring the medium's pH to 7.0. The Rhizobium strains were added to the medium and allowed to grow for a week.

On the medium, colonies of the strains were grown, and their morphology and appearance were scrutinized. The strains were identified using the YMA agar medium with bromothymol blue as an indicator. (Zhang *et al.*, 2003).

The plates were incubated for around 7 days at 28 °C. By repeatedly stumbling on YMA plates, single colonies were purified. Single colonies underwent individual Gram staining, morph culture, and biochemical analyses following a 2-day purification process. Every week, we observed how the rhizobial strains responded to this medium. Rhizobial strains that grow quickly change the medium yellow as a result of the production of acid, while rhizobial strains that grow slowly turn the medium blue as a result of alkali production (Somasegaran; Hoben, 1994).

2.2. Antibiotics Sensitivity Assay:

The sensitivity of rhizobial isolates to three antibiotics—amoxicillin (Amox), cefotaxime (Cefo), and tetracycline (Tetra) The preparation of concentrations starting from 100 ppm to 1000 ppm was investigated by measuring the O.D. and inhibition zone at high doses of each antibiotic. 21 isolates in primitive concentrations from 100 to 500 grew for the three antibiotics, but from the beginning of the concentration of 600, resistance to some isolates appeared.

2.3.- Evaluation the impact of antibiotics on slowing of rhizobial growth at high antibiotics concentrations

Fresh rhizobial isolate grown on YMA slants was transferred to sterile test tubes containing 10 ml of distilled sterile water, and the suspension was homogenized by vortex with each antibiotic to allow the final concentration of the antibiotic in the media in 5 concentrations (600, 700, 800, 900, and 1000 ppm). To each flask containing medium that has been appropriately dosed with the antibiotic, one milliliter of the rhizobial suspension was added. At a temperature of 28 °C, the flasks were incubated on a shacking incubator (150 rpm) for 2, 4, 6, 8, and 10-day intervals were used to collect samples from the flasks.

To compare the growth of rhizobia in the presence of the antibiotic with that grown normally on the particular growth medium for rhizobia, control flasks without any of the antibiotics were included (Tena *et al.*, 2016; and Tsegaye *et al.*, 2015).

3.Results and Discussion

3.1. Isolation and description of rhizobia from faba bean nodules

Twinty one rhizobial isolates were extracted from faba bean nodules that were collected from various locations within the Aswan governorate. The features of the isolate are shown in Table (1).

Litmus milk's created zone produced an alkaline reaction. These isolates grew uniformly turbidly in yeast extract mannitol broth medium. According to Somasegaran and Hoben's (1994) definition of the Rhizobium type, certain morphological and colony characteristics have been confirmed.

Table (1): Characteristics of rhizobial isolates from faba bean nodules

Isolates code	Gram Stain	Motility	Growth rate	Catalase	Oxidase	pH change
716 A	-	+	F	+	+	A
716 B	-	+	F	+	+	A
716 C	-	+	F	+	-	A
716 D	-	+	F	+	-	A
716 E	-	+	F	+	+	A
716 F	-	+	F	+	+	A
716 G	-	+	F	+	-	A
40 A	-	+	F	+	+	A
40 B	-	+	F	+	+	A
40 C	-	+	F	+	-	A
40 D	-	+	F	+	+	A
40 E	-	+	F	+	+	A
40 F	-	+	F	+	-	A
40 G	-	+	F	+	-	A
843 A	-	+	F	+	+	A
843 B	-	+	F	+	+	A
843 C	-	+	F	+	+	A
843 D	-	+	F	+	+	A
843 E	-	+	F	+	-	A
843 F	-	+	F	+	-	A
843 G	-	+	F	+	-	A

F: fast growth. A: acid PH

3.2. The impact of three antibiotics on the development of twenty-one faba bean rhizobial isolates:

The rhizobium isolates respond differently to the tested antibiotics based on their inherent antibiotic resistance. Table (2) revealed numerous variations among the nodulating Rhizobium isolated against the major 5 concentrations. The isolates (716 A, 716 B, 40 A, 40 B, 843 A, and 843 B) were the most tolerant. Furthermore, isolates 40A and 40B were more responsive to the agonist at five concentrations and resistant to it (Adnan, 2010 and Kenasa *et al.,* 2014).

Tetracycline concentrations of 400 and 500 were effective against the three isolates 40D, 716C, 716D, 843C and 843D, whereas other concentrations did not show the same effect .

Also, Amoxicillin (Amox) was showed the same activity at concentration 400 and 500ppm against the isolates 40C, 40F, 40G, and 716G However,cefotaxime (Cefo), inhibited the growth of the isolates 40D, 716C, and 716D at doses of 400 and 500 ppm. These findings revealed various trends in relation to the tested antibiotics. The rhizobial isolates were distinguished using this method by (Gasim *et al.* 2015; Melak *et al.* 2018).

Table (2): Effect of three antibiotic concentrations on the development of isolates of Rhizobium leguminosrum biover viciae.

Isolate code	Amoxicillin (Amox)					Cefotaxime (Cefo)					Tetracycline (Tetra)				
	100	200	300	400	500	100	200	300	400	500	100	200	300	400	500
40 A	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++
40 B	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++
40 C	++	++	+	+	-	++	++	++	+	+	++	++	++	+	+
40 D	+++	++	++	+	+	++	++	+	-	-	++	++	+	-	-
40 E	++	++	++	+	+	+++	+++	+++	++	+	++	++	++	+	+
40 F	++	++	+	-	-	+++	++	++	++	++	+++	++	++	+	+
40 G	+++	++	+	-	-	+++	++	++	++	++	+++	++	++	+	+
716 A	++++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++++	++++	+++	+++	+++
716 B	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++++	+++	+++	+++	+++
716 C	+++	++	++	+	-	+++	++	+	-	-	+++	++	+	-	-
716 D	++	++	++	+	+	+++	++	+	-	-	++	+	+	-	-
716 E	+++	++	++	+	+	+++	++	++	+	+	+++	++	++	+	+
716 F	+++	++	++	+	+	+++	++	+	+	+	+++	++	+	+	+
716 G	+++	++	+	-	-	+++	++	++	+	+	+++	++	++	+	+
843 A	++++	++++	+++	+++	++	++++	++++	++++	+++	+++	++++	++++	+++	+++	+++
843 B	++++	+++	+++	+++	+++	++++	++++	+++	+++	+++	++++	++++	+++	+++	+++
843 C	+++	++	+	+	-	++	++	+	-	-	++	++	+	-	-
843 D	+++	++	++	+	+	+++	++	+	-	-	+++	++	+	-	-
843 E	+++	++	+	+	+	+++	++	++	+	-	+++	++	++	+	-
843 F	+++	++	++	+	+	+++	++	++	+	+	+++	++	++	+	+
843 G	+++	++	++	+	+	+++	++	++	+	+	+++	++	++	+	+

Table (3) Summarize the results showed a discrepancy in the multiple resistance to antibiotics, it was found that 14 isolates (66%) could resist antibiotic A1 at the concentration of 400, while at the concentration of 500 for the same antibiotic, almost half of the isolates (52%) were 11 isolates could grow. Whereas 13 isolates were found that recorded equivalent percent among rhizobial

isolates which resistant to another two antibiotics, at concentrations 400 and 500 (Haciseferogullari *et al.*, 2003).

Table (3): Numbers and percentages of microbial isolates resistant to high concentrations of antibiotics

Antibiotic and concentrations	A1		A2		A3	
	400	500	400	500	400	500
total isolates	21	21	21	21	21	21
number of resistant isolates	14	11	13	13	13	13
Percent %	66	52	62	62	62	62

Where: A1 Amoxicillin **A2** Cefotaxime **A3** Tetracycline

3.3.Column diagrams illustrating the rhizobial isolates that are the most tolerant

Six isolates were found to be rhizobia tolerant to three antibiotics as a result of research done to assess how the application of antibiotics affected the growth inhibition of rhizobial isolates. There are six faba bean nodule-derived isolates in this group. With the help of gradient antibiotic concentrations, these isolates were further investigated to determine the impact of three antibiotics on the column graph of each isolate. The previously specified procedures for rhizobial suspension preparation, inoculation, growth conduction, and sampling were followed. (Moawad *et al.*, 2005).

In the growth medium, the antibiotic concentrations were 600, 700, 800, 900, and 1000 ppm. Before rhizobial inoculation, the media was treated with antibiotics as previously described. Column graphs of each isolate in media with and without control antibiotics were blotted to assess how each isolate's growth was affected by the concentration of each antibiotic.

3.4. Effect of elevated antibiotics concentrations on the potential inoculant faba bean rhizobial isolates

According to (Crepon et al., 2010; Datta et al., 2015; Hajjamet al., 2016), the results were in agreement with this study.

Table (4) showed that the faba bean rhizobial isolates differed in their tolerance to the various concentrations of the antibiotics in the media. None of the concentrations of the three antibiotics was capable to eliminate the isolates 40A, 40B, and 843B growth.

At the concentration of 600 ppm the most tolerant isolate to A1 was isolates 40A and 40B, and to A2 isolate all isolates. The increase of antibiotics concentration to 900 and 1000 ppm retarded the growth of the majority of the isolates. The retardation was higher at higher antibiotic concentrations. Some isolates showed distinct tolerance to the antibiotics. Isolates 40A, 40B, and 843B markedly highly tolerated 600 and 700 ppm three antibiotics concentrations, and lowly tolerated 800, 900, and 1000 ppm three antibiotics concentrations whereas isolates 716A tolerated all concentrations except 1000 ppm A3.

Isolate 716A is more resistant isolate to antibiotics than 716B, especially with A1 and A3. Isolate 716B has the lowest retardation with 900 and 1000 ppm A1 and also at concentrations 700, 800, 900, and 1000 ppm A3.

These two isolates had the same trend with A2, where 843A recorded intermediate resistance with 600 ppm A1, A2, and A3, and low resistance with an A2 and A3 concentrations 800, 900, and 1000 ppm, finally the presence of no retardation with A1 concentrations 900 and 1000ppm (Temesgenet *et al.,* 2015).

Table (4): Effect of high concentrations of three tested antibiotics on the Six *Rhizobium leguminosrum biover viciae* isolates in the growth medium.

isolates code	Amoxicillin(ppm)					Cefotaxime (ppm)					Tetracycline(ppm)				
	600	700	800	900	1000	600	700	800	900	1000	600	700	800	900	1000
40 A	+++	++	++	++	++	++	++	++	+	+	+++	+++	++	++	+
40 B	+++	++	++	++	+	++	++	+	+	+	++	++	+	+	+
716 A	++	++	+	+	+	++	++	+	+	+	++	+	+	+	-
716 B	+	+	+	-	-	++	++	++	+	+	+	-	-	-	-
843 A	++	+	+	-	-	++	++	+	+	+	++	+	+	+	+
843 B	++	++	+	+	+	++	++	+	+	+	+	+	+	+	+

Abbreviations: + = normal growth; ++ = moderate growth; +++ = high growth; - =no growth

3.5. Resistance and inhibition (%) on the growth of six *Vicia faba* rhizobial isolates affected by five high concentrations of three antibiotics.

The growth was measured as optical density at 660 nm and transferred to inhibition percentage as in materials and methods.

The faba bean rhizobial isolate 40A was very resistant to all antibiotics compared with the isolate 716A faba bean. The inhibition percentages of such resistant isolates were significant at antibiotics concentration of 1000 ppm (Graham, 2008;Mehta and Nautiyal 2001) .

The effects of five concentrations of three antibiotics (Amoxicillin, Cefotaxime, and Tetracycline) on 6 faba bean rhizobia isolates were investigated. Data are presented in Figures (1-3). Shows that the antibiotic A1 concentration of 900 and 1000 ppm had toxic effects on 716B and 843A isolates of *Rhizobium leguminosrum* biover viciae, while isolate 716B cannot resist antibiotic A3 concentrations of 700, 800, 900, and 1000 ppm, and also the same toxic effect of antibiotic A3 concentration 1000 with isolate 716A. However, a lower inhibition effect was observed at lower concentrations (600 ppm) of antibiotics, (Berrada *et al.,* 2012; Mutch and Young 2004).

Figure (1) indicate that antibiotic A1 tested toxic and had a remarkable effect on faba bean isolates 40A, 40B, 716A, and 843B with all concentrations. While the antibiotic caused complete inhibition of 716B and 843A isolates in a medium supplemented with 900 and 1000 ppm. Whereas; in the medium supplemented with antibiotic A1 concentration of 800 ppm, the percentages of inhibition for the same isolates reached 40(%) and 40(%), respectively, (Alghamdi, *et al.,* 2012; Allito *et al.,* 2015).

On the other hand, isolates 40A and 40B respectively recorded the highest resistance 72% and 65% with an A1 concentration of 1000 ppm.

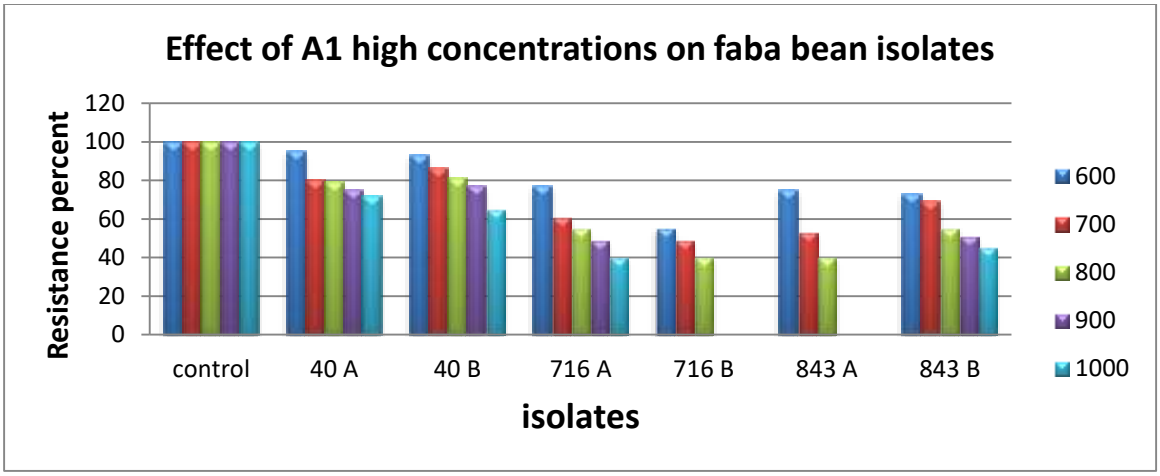

Figure (1): Resistance (%) of six faba bean rhizobial isolates affected by high five concentrations of antibiotic amoxicillin.

Data in Figure (2) show that the six isolates varied in their resistance to antibiotic A2. Isolate 843A and 843B were the least resistant, where the percentages of retardation at 1000 ppm of antibiotic A2 were 40% and 47%, respectively. Isolates 40A and 40B from faba bean were more resistant and recorded 60% and 55% respectively with the antibiotic A2 at a concentration of 1000 ppm (Belay and Assefa, 2011; Othman and Tamimi, 2016).

But also all of the isolates had strong resistance to A2 concentrations 600 and 700 ppm, where isolate 843B recorded 21(%) and 25(%) retardation on 600 and 700 ppm concentrations respectively. The Isolate 716A was moderate resistant by growth retarding 17(%) with 1000 ppm concentration and 55(%) with 600 ppm concentration. The same isolate 716B was moderate resistance by growth retarding 52(%) with 1000 ppm concentration and 21(%) with 600 ppm concentration (Ahmed and Abdelmageed, 2015; Tena et al., 2016).

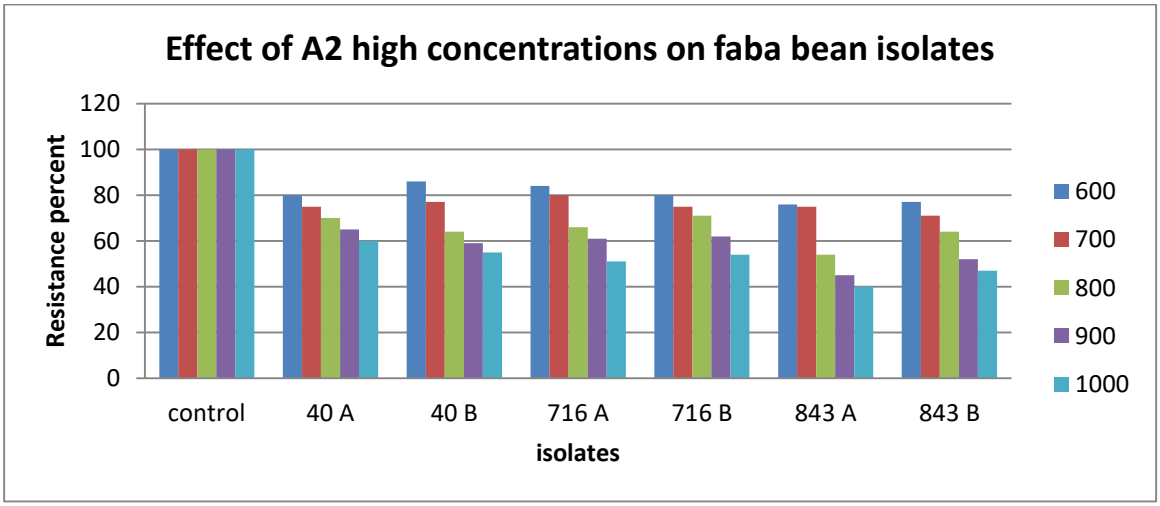

Figure (2). Resistance (%) of six faba bean rhizobial isolates affected by high five concentrations of antibiotic cefotaxime.

The reaction of the six isolates obtained from field nodules to antibiotic A3 shows remarkably significant inhibition (40 - 95 (%)) as a compound with normal growth at medium without antibiotic, this is in agreement with (Adnan, 2010 and ; Sheikhet *et al.*, 2020).

The highest retardation was recorded with isolate 40A on medium supplemented with 1000 ppm A3, whereas the lowest was observed with isolate 843A treated with 1000 ppm A3. That isolate 716B cannot grow with A3 concentrations (700, 800, 900, and 1000). The isolates showed different sensitivity to the three concentrations of the antibiotic (Figure. 3), Chen, *et al.*, (2018) agrees with these results.

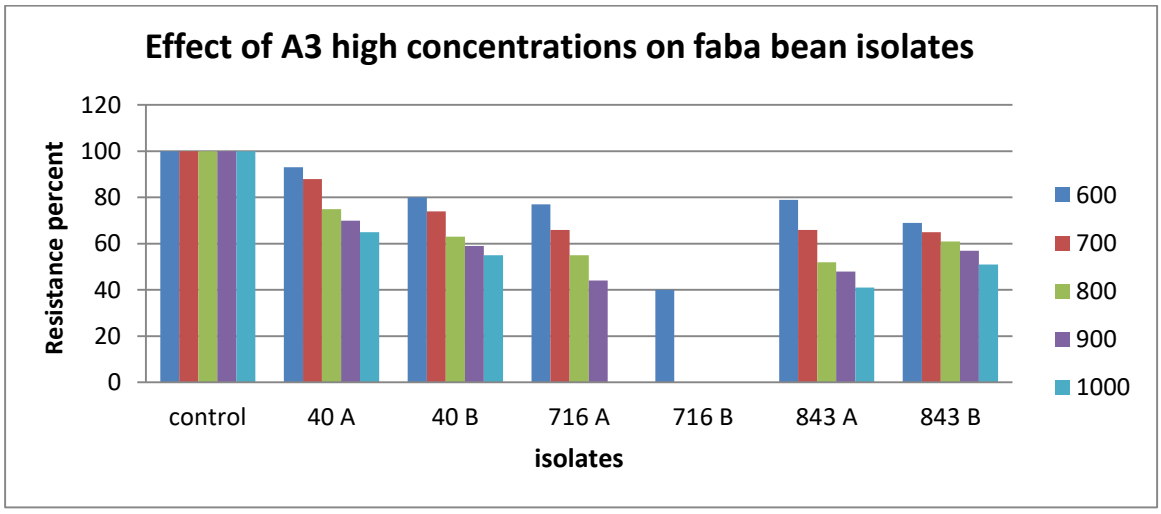

Figure (3): Resistance (%) of six faba bean rhizobial isolates affected by high five Concentrations of the antibiotic tetracycline.

Conclusion

The results of this study demonstrated the ability of isolates 40A, 40B, and 843B to flourish in antibiotic-toxic environments. The ability of their confined isolates to break down the antibiotics is demonstrated by this study. The rhizobial isolates were acquired using enrichment culture for 21 rhizobial isolates from 3 different faba bean species nodules that were sown in Shenab region soil in the Aswan governorate.

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