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Rhizobium phaseoli tolerant to insecticide on the growth of *Phaseolus vulgaris*

Rhizobium phaseoli tolerante a un insecticida en el crecimiento de *Phaseolus vulgaris*

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Resumen

El género y especie *Rhizobium phaseoli* es usado como inoculante en la producción de *Phaseolus vulgaris* (fríjol), porque en los nódulos de sus raíces, establece una simbiosis para fijar el nitrógeno molecular (FBN) y suplir la demanda de nitrógeno (N) para un crecimiento sano. En el cultivo de *P. vulgaris* se aplican plaguicidas en el control de insectos plaga de raíz, que evitan el efecto benéfico de *R. phaseoli*., por lo que los objetivos de este trabajo fueron aislar y seleccionar *R. phaseoli* tolerante a diazinón. Para ello, *R. phaseoli* se creció en caldo extracto levadura manitol con diazinón y selecciono *R. phaseoli* tolerante al insecticida e inoculó en *P. vulgaris* para determinar la infectividad con base en el número de nódulos, mientras que la efectividad para la FBN en la raíz, de acuerdo al incremento en el peso fresco y seco, en la altura de la planta, y en la capacidad para degradar el diazinón. Se concluye que el *R. phaseoli* tolerante a diazinón fue infectivo y efectivo para el sano crecimiento de *P. vulgaris*.

Abstract

The genus and species of Rhizobium phaseoli are useful as an inoculant for the production of Phaseolus vulgaris (beans) in the root nodules symbiotic stage fixes molecular nitrogen (FN) for supplying nitrogen (N) for healthy growth. In P. vulgaris cropping, pesticides are used to control root insects, which could reduce the beneficial effect of R. phaseoli. The purpose of this work was to isolate and select R. phaseoli diazinon´s tolerance. In that sense, R. phaseoli were cultivated in a medium mixed with diazinon in order to select R. phaseoli tolerant to the pesticide. This mutant of R. phaseoli tolerant to diazinon was inoculated in P. vulgaris the effect was evaluated 45 days later. The response of P. vulgaris was measured by the number of effective nodules at the roots, fresh and dry weight, and the height of the plant. Results showed that R. phaseoli tolerant to diazinon kept its beneficial activity for the healthy growth of P. vulgaris. It was concluded that R. phaseoli tolerant to diazinon were infective and effective for the health growth of P. vulgaris.

Introduction

The symbiosis between legumes and *Rhizobium* is essential in agriculture due to the positive impact it has on the cultivation and consumption of *Phaseolus vulgaris* and other legumes[1]. Inoculating *P. vulgaris* seed with *Rhizobium phaseoli* avoids nitrogen hyperfertilization to the soil, in addition to ensuring the healthy growth and profitable yield of *P. vulgaris*[1] whose grain is considered one of the main in Mexico, in principle due to its high consumption and production, hence the economic importance[2].

One of the problems to ensure a healthy growth of *P. vulgaris*, is the control of insects-pests that damage the root, thus avoiding the beneficial action of *R. phaseoli*[3], first to exert the infectivity or formation of nodules and the effectiveness or the ability of *R. phaseoli* to adequately supply the nitrogen (N) demand for healthy growth. Consequently, pesticides are applied that generally inhibit or kill *R. phaseoli*, however, the information in this regard is contradictory, since it is reported that the growth of *R. phaseoli* is inhibited with minimal concentrations of dichloro diphenyl trichloroethane (DDT) and folidol, being lower than those recommended in the field in the pest control of roots of *P. vulgaris*. While other research reported that *Rhizobium* spp., can be tolerant to pesticides without losing the infective and effective capacity[4,5]. Thus, it has been reported that nodulation in the root system of *P. vulgaris* by *R. phaseoli* is inhibited or reduced due to the toxicity of these pesticides[6,7], which decreases the effectiveness or positive effect for the healthy growth of *P. vulgaris*, basically because they inactivate nitrogenase to fix molecular nitrogen (N_2) or FN_2[8,9].In that sense the objectives of this research was the selection and analysis diazinon-tolerant *R. phaseoli* for the healthy growth of *P. vulgaris*.

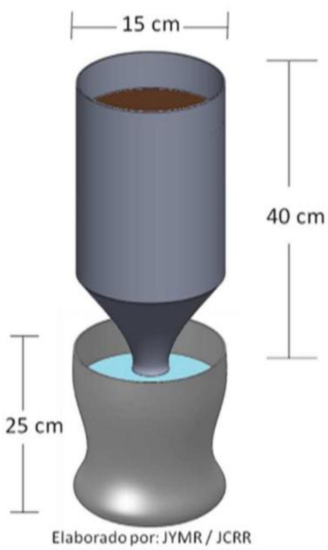
Materials and methods

Origin of the *R. phaseoli* isolated. *R. phaseoli* were isolated from the roots of *Phaseolus* var Bayo, in congo red yeast extract mannitol agar (CRYEMA) with the following composition (g/L): K_2HPO_4 0.5, $MgSO_4$ 0.2, NaCl 0.1, mannitol 1.0, yeast extract 1.0, congo red 10.0 mL/L (from a 1:500 solution), agar 20.0 g, 1000.0 mL distilled water, pH 7.0 according to Vincent[10], collected from the experimental agricultural field of the National Institute of Forestry, Agricultural and Livestock Research (INIFAP), Aldama, Tamaulipas, Mexico, were designated with the codes: ReD, ReDa and ReD1, they are part of the collection of the Environmental Microbiology laboratory of the Institute of Biological Chemical Research (IIQB) -Universidad Michoacana de San Nicolás de Hidalgo (UMSNH)[1,10].

Selection of *R. phaseoli* tolerant to diazinon. In this trial, pesticides frequently applied in Aldama Tamaulipas, Mexico, were tested, such as: diazinon (O, O-diethyl O-2-isopropyl-6-methylpyridin-4-yl phosphorothioate) - Servin (1-naphthyl N- methyl carbamate), c/pesticide was added to the CRYEMA in concentrations of: 250, 300, 500 and 100 ppm, in a nephelometric flask, 1.0 mL equivalent to 1 x 10^6 CFU/mL of *R. phaseoli* was inoculated per 100 mL of yeast mannitol extract broth (YMEB) plus diazinon or Servin in concentrations of: 200, 300, 400 and 1000 ppm, these flasks were incubated for 24 to 36 h in shaking/200 rpm, for adaptation and selection of *R. phaseoli* to the increasing concentration of diazinon and Servin. The adaptation of *R. phaseoli* to the increase in the concentration of each pesticide was achieved by detecting the growth expected with that increase, compared to the normal growth of *R. phaseoli* in the nephelometric flask in YMEB without diazinon or Servin under the same conditions. incubation time, temperature and time[4].

Test of infectivity and effectiveness of *R. phaseoli* resistant to diazinon in *P. vulgaris*. The diazinon-tolerant strains of *R. phaseoli* were selected (since Servin inhibited the growth of *R. phaseoli* from the first concentration so it was no longer used).

Figure 1 Leonard' jar



Therefore, the seeds of *P. vulgaris* var Bayo (donated by the Secretary of Agriculture, Livestock, Fisheries, Rural Development and Food of the Mexican government) were inoculated with *R. phaseoli* resistant to diazinon in Leonard's jars (figure 1) there the soil was placed in the upper part of Leonard's Jar and the mineral solution or water in the lower part of the Jar, both parts were connected with a 20 cm long cotton strip, for movement of the liquid by capillary action[5], The soil where *P. vulgaris* was sowing had the follo-wing physicochemical properties: it was a lateritic with pH 6.64, clay texture 40.56 %, sand 0.76 %, silt 37.8 %, field capacity 30.08 %, organic matter humidity 4.57 %, poor in total nitrogen 0.1 %, field capacity 30.08 %, and cation exchange capacity of 4.61 mg/100 g. This soil was sieved with a mesh of No. 20, it was solarized 48 h at 70 °C, to avoid pests and diseases, Leonard's Jars were placed in the greenhouse under the following average micro-climatic conditions were: the temperature of 23.2 °C, with a brightness of 450 $\mu mol \bullet m^{-2} \bullet s^{-1}$ and a relative humidity of 67 %. *P. vulgare* with *R. phaseoli* was fed with a mineral solution with the following chemical composition: 1 molar of K_2HPO_4, KH_2PO_4, $CaCl_2$, $MgSO_4$, traces of $FeSO_4$, 1.0 mL of minor elements solution in 1000 mL of distilled water adjusted to pH 7 which was pasteurized (90 °C/10 min). Later 45 days later, the effect of diazinon-tolerant *R. phaseoli* strains in *P. vulgaris* was evaluated by means of the following response variables, plant height (PH), leaf diameter (LD) (cm), as well as the dry weight of the foliage (DWF), number of effective nodules in the root its dry weight and the dry weight of the root[10]. Evidence that *R. phaseoli* degraded (coometabolism) diazinon in culture medium.

The diazinon-tolerant *R. phaseoli* strains were inoculated in a nephelometric flask, with congo red mannitol yeast extract broth (CRMYEB) with diazinon, the flasks were shaken at 300 rpm until the stationary phase, by means of a viable count that was reached at 30 h of incubation according to the growth curve of *R. phaseoli*, in comparison with the same *R. pha-seoli* in CRMYEB without diazinon. This test sho-wed the coometabolism of *R. phaseoli* to degrade diazinon in combination with mannitol used as a carbon source[4,11-13].

Results

Table 1 Effect of diazinon-tolerant *Rhizobium phaseoli* on the growth of *Phaseolus vulgaris* var Bayo

Strain	ReD	ReDa	ReD1
No. of effective nodules (red)[1]	19[c*]	20[c]	39[a]
Leaves color	Intense green	Green	Intense green

[1]n=20*equal letters without statistical differences according to Tukey (p<0.05)

Table 1 shows the effect of diazinon-tolerant *R. phaseoli* strains on the growth of *P. vulgaris* as a function of the number of effective nodules that determine a plant health condition with 19, 20, 39 generated respectively by the strains of *R. phaseoli* resistant to diazinon called ReD, ReDa and ReD1.

Regarding the color of *P. vulgaris* leaves, they were indicative that the isolated infected the roots of *P. vulgaris* formed red nodules (effective), which indicates the presence of leghemoglobin associated with its capacity to fix N_2 and the color intense green on the leaves[1].

Table 2 Comparison of the infectivity and effectiveness of wild *Rhizobium phaseoli* and diazinon-tolerant *Rhizobium phaseoli* on the growth of *Phaseolus vulgaris* var Bayo

[1]Infectivity stability and effectiveness parameters	***R. phaseoli* silvestre**			***R. phaseoli* tolerante a diazinón**		
	ReD	**ReD1**	**ReDa**	**ReD**	**ReD1**	**ReDa**
Leaf diameter (cm)	3.7[a]*	3.9[a]	3.5[a]	3.2[a]	3.6[a]	3.1[a]
Plant height (cm)	23[b]	29[a]	22[b]	19.1[c]	24.9[b]	18.0[c]
Foliage dry weight (g)	5.0[a]	3.9[b]	4.0[b]	4.9[a]	3.6[b]	3.9[b]
Dry weight of effective nodules (mg)	490[b]	498[a]	130[c]	488[b]	509[a]	100[c]
Number of effective nodules (red)/plant	25[b]	29[a]	22[b]	21.0[b]	27.0[a]	19.0[b]
Root dry weight with nodules (g)	3.2[a]	3.8[b]	4.9[a]	4.0[b]	3.5[b]	4.2[a]

[1]n=20, [*]Equal letters without statistical differences according to Tukey (p<0.05).

In table 2, where according to the literature it was observed that the diazinon-tolerant *R. phaseoli* coding as ReD, ReDa and ReD1 had a positive effect on the LD, and PH of *P. vulgaris* with 19.1, 24.9, 18 cm, as well as in the DWF, these numerical values were statistically different in comparison with the wild *R. phaseoli* isolates unable to grow in the presence of diazinon. Like the diazinon-tolerant *R. phaseoli* isolates in the number of effective red nodules, the dry weight that included that of the complete root, not statistical difference compared to the analogous numerical values registered in *P. vulgaris* with wild *R. phaseoli* non-diazinon tolerant.

Table 3 shows that *R. phaseoli* strains grown in CRYEMA with diazinon generated a number of colony forming units (CFU) with a statistically different numerical value compared to wild *R. phaseoli* isolated (wild) in CRYEMA without diazinon, which indicates that the isolates subjected to the maximum concentration of diazinon were physiologically adap-ted to the insecticide, although there was a reduction in the UFC *R. phaseoli*, since it is a natural selection which caused the death of the cells of *R. phaseoli* that are susceptible to diazinon toxicity[4].

Table 4 shows the growth as CFU, registered in diazinon-tolerant *R. phaseoli* isolates, the literature indicates that it may be due to an action of coometabolism when they use mannitol for the degradation of diazinon, in this table it was evident that when *R. phaseoli* grew in mannitol and diazinon, a decrease in the number of colonies was registered as CFU, because although *R. phaseoli* degraded it, it was toxic for a part of the total population of *R. phaseoli*, compared to when it grew in the CRYE-MA without diazinon, which represents the amount of the expected CFU growth, while by suppressing mannitol and diazinon, the absence of both caused a drastic decrease in the *R. phaseoli* population, because the other components of CRYEMA: like yeast extract and Congo red were insufficient for *R. phaseoli* to reproduce at the level registered when the culture medium contained mannitol as sole source of carbon and energy[1] .

Table 3 Comparison of the stability of resistence of *Rhizobium phaseoli* to diazinon compared to wild *Rhizobium phaseoli*

Isolated	*CRYEMA with diazinon[1] CFUx10^2	CRYEMA without diazinon[1] CFUx10^2
ReD	26[c]**	44[b]
ReD1	30[c]	49[b]
ReDa	40[b]	61[a]

[1*] average of 4 repetitions in CFU = Colony Forming Units x10^2/mL [*]CRYEMA = congo red yeast extract mannitol agar.
[*]Equal letters without statistical differences according to Tukey ($p<0.05$).

Table 4 Diazinon coometabolism by insecticide tolerant *Rhizobium phaseoli* isolates comparad to the growth without insecticideon the wild *Rhizobium phaseoli*

Strain	[2]Congo red yeast extract mannitol agar without diazinon [1]CFUx10^2	Congo red yeast extract mannitol agar with diazinon[1] CFUx10^2	Congo red yeast extract agar without mannitol or diazinon[1] CFUx10^2
ReD	39[c]*	15.[d]	0.1[e]
ReD1	50[b]	23[c]	0.9[e]
ReDa	60[a]	31[c]	0.7[e]

[1]CFU = Colony Forming Units/mL; *Equal letters without statistical differences according to Tukey ($p<0.05$).

Discussion

Table 1 shows that a positive response of *P. vulgaris* was observed based on the number of effective nodu-les, which supports that diazinon-tolerant *R. phaseoli* conserved plasmids that contain infectivity and effectiveness in the roots of *P. vulgaris*, for healthy growth, because there was insufficient mineral nitrogen in the soil for the growth of *P. vulgaris*[1,5,6,12].

Table 2 shows that the diazinon-tolerant *R. phaseoli* strains conserved the plasmids responsible for nodulation or infectivity, which were red in color, indicating the presence of leghemoglobin, a protein related to the ability to fix N_2 (effectiveness), consequently *P. vulgaris* synthesized enough chlorophyll, for the intense green color of the leaves, a phenological characteristic of a healthy growth when *P. vulgaris* is symbiosis with strains of *R. phaseoli* that are infective and effective [7,10,13].

Table 3 shows the evidence that the growth of *R. phaseoli* in yeast mannitol extract agar (YMEA) with diazinon based on CFU, was the result of a natural selection of the physiological adaptation to diazinon, without the risk of losing the capability to grow in the presence of diazinon, the numerical value of the CFU, was reduced in relation to the same variable of CFU, of the wild strains of *R. phaseoli*, because during the selection a part of this population susceptible to diazinon died, therefore, the number of CFU of *R. phaseoli* tolerant to diazinon was lower[14-16]. As has been reported for other genera, species of the *Rhizobiaceae* family such as

Bradyrhizobium spp., which also co-metabolize and/or mineralize other applied pesticides, in conventional agriculture in the control of insects-pests that attacking the roots of legu-mes[15,16]. The capability to degrade insecticides similar to diazinon, is a common biochemical property in nature according to those reported for other bacterial groups other than *R. phaseoli* such as: *Pseudomonas,* as well as actinomycetes: *Streptomyces*, *Micromonospora*, *Thermoactinomyces*, *Nocardia* and *Mycobacterium*[4,12,13] and other genera and species of soil fungi, and/or those endophytic living in association with plant roots of the type: *Aspergillus fumigatus*, *Cunninghamella elegans*[17], *Penicillium citreoni-grum*[18]. *Rhizopus nodosus*, includes the human pathogenic enterobacteria: *Escherichia coli*, *Salmonella bongori* that are capable of mineralizing diazinon, the above supports this is because both eukaryotes and prokaryotes have this genetic property of resistance to diazinon, because they are related to human activities of plant and water management that are part of agriculture[19-21].

Table 4 shows the tolerance of *R. phaseoli* to diazinon was the results of the physiological adaptation capacity of this genus and species, which also has the codified genome using as only source carbon some compounds similar to diazinon, so if there is another simple carbon source that, when is oxidized, made easy the degradation of the pesticide, a biochemical action known as coometabolism. What was showed that when *R. phaseoli* tolerant to diazinon grew in CRYEMA and diazinon, compared to the inhibition of growth of *R. phaseoli* in CRYEMA without mannitol or diazinon[4,8,11,14]. The selection of strains of *R. phaseoli* tolerant to pesticides is useful in conventional agriculture, its has been reported on *Bradyrhizobium japonicum* strains for producing *Glycine max* to avoid attacking of pest insects [15,22,23]. For this reason *R. phaseoli* is recommended in agricultural issues where *P. vulgaris* has a problem with insect pest destroying roots and limiting its healthy growth, underline due diazinon is applied for producing *P. vulgaris* in doses similar to those evaluated in this research[12], since diazinon is applied at the begining growth steps of *P. vulgaris* without evidence that its persistence in *P. vulgaris* tissues and/or seeds[14]. Based on the above, its concluded that it is possible to isolate diazinon-tolerant strains of *R. phaseoli* that are highly infective and effective for a healthy growth of *P. vulgaris* without risk of lost caused by pest insects destroying its root system.

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Conflicts of interest

The authors of this article declare that there is no conflict of interest in the planning, execution and writing of the research carried out, nor with those people and institutions that financed it.

Ethical considerations

The approval of the research by the Ethics Committee of the Universidad Michoacana de San Nicolás de Hidalgo, Mexico followed the guidelines established by that committee.

Authors' contribution to the article

Santoyo Pizano Gustavo, planning of the experiment and statistical analysis of all results. *Hernán-*

dez Mendoza José Luis, update of the literature and partial review of the literature for the experimental results. *Marquez-Benavides Liliana*, analysis of results and criticism of the discussion of results. *De Luna-Esquivel Gustavo*, support for the experimental design and execution of experiments with *Phaseolus vulgaris* and *Rhizobium phaseoli*. *Sánchez-Yáñez Juan Manuel*, Planning, direction of the experiments, comprehensive analysis of results, writing of the article, responsible for the financial support of the research.

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