



Research Article

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Coexistence of *Agrobacterium tumefaciens* and *Rhizobium etli* in Soil and Inside the Roots of *Phaseolus vulgaris*

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Abstract

In the nodules induced by *Rhizobium* in legumes there is evidence that it is not the only genus that invades the roots of these plants as a consequence of the absence of mineral nitrogen and water stress, which forces the legume to establish symbiosis with *Rhizobium*. However, from the infection to the formation of the nodule, other genera are involved, although it is not clear what role they play in the symbiosis. The objectives of this work were:

a) to demonstrate that the existence in the soil, of beneficial endophytes, such as *R. etli* and phytopathogens such as *A. tumefaciens*, are able to compete for the formation of nodules or galls.

b) That *R. etli* and *A. tumefaciens* by competition will determine that *P. vulgaris* will have N₂ fixation nodules, with healthy growth or *P. vulgaris* shown chlorotic plant due to damage galls. For this, soil was inoculated in *P. vulgaris*, the effect of the formation of nodules or galls, was measured by phenology, the nodule or gall formers were isolated and inoculated in *P. vulgaris*, including total nitrogen and phenology: leaf color, plant height, number and color of nodules were measured.

All isolates were identified by biochemical traits, and some molecular test as well presence of DNA plasmid.

The results demonstrated that the beneficial *R. etli* strains contained the *Inf+* and *Nif+* plasmids, but *A. tumefaciens* a *Ti* plasmid, since both are genetically close relatives. These results show the promiscuity of *A. tumefaciens* with *R. etli* upon isolation, that reduces the possibility of isolating, *R. etli* and facilitates the recovery of *A. tumefaciens*. It is concluded that in the same nodule, there is also competition, and the dominant one will determine what relationship it will have with *P. vulgaris* as a symbiotic or parasitic relationship according to environmental conditions.

Keywords: Soil; Nitrogen; Competition; Endophytes; Symbiosis; Parasitism; Positive Ecological Interactions

Introduction

Phaseolus vulgaris or common bean is a legume, that depends on the availability of inorganic nitrogen compounds as enough water in the soil solution to establish a symbiotic relationship with *Rhizobium etli* [1-6] or be invaded by *Agrobacterium tumefaciens*, that makes it sick with tumors or galls [7,8]. Both genus and bacterial species, share a common genetic origin, with plasmids related to biological nitrogen fixation (*Nif+*) by *R. etli* [9] and/or galls that, with some damage mechanisms compromise plant health [10] can live in the soil and even within the nodules or galls [11-14]. *R. etli*, by invading and penetrating the root hairs, to induce the formation of a root hypertrophy or nodule that allows to *R. etli* to fix N_2 [15,16]. This ability depends on plasmids for nodulation (*Nod+*), as well as the biological nitrogen fixation (*Nif+*) [2,4]. Both infection and nodulation, allow the same nodule to be invaded, by more than one endophytic genus and species [5,6].

Thus, it is possible that *A. tumefaciens* a plant pathogen coexists with *R. etli* [7,8]. The ability to compete of *R. etli* against other endophytic bacteria, as well as *A. tumefaciens* will depend on that, will make the nodule beneficial for *P. vulgaris*, or whether it will become a gall and cause plant disease [8,11,12]. Today is well known that in all type's soils both *A. tumefaciens* and *R. etli* as like other microbial types as actinomycetes as *Micromonospora* sp [15,17-19] in that sense, according its genetic ability to compete for invading any root system of the specific legume could be a positive or negative ecological interaction among endophytic microorganisms will be success [1,3,5-9]. The objectives of this work were:

- To demonstrate that the existence in the soil of beneficial endophytes such as *R. etli* and phytopathogens such as *A. tumefaciens* are able to compete for the formation of nodules or galls,
- That *R. etli* and *A. tumefaciens* by competition will determine that *P. vulgaris* will have N_2 fixation nodules with healthy growth or *P. vulgaris* shown chlorotic plant due to damage galls.

Material and Methods

Agricultural soil samples: the soil sample comes from the "Ejido el Refugio" Municipio. from Cadereyta de Jiménez, N.L, México and stored in polyethylene bags at a temperature of 28 30°C Analysis of the physicochemical properties of the soil. The pH, organic matter content, total nitrogen, electrical conductivity, and texture were measured according to established methods [13,14].

Isolation of *A. tumefaciens* and *R. etli*

From agricultural soil, decimal dilutions were made in 0.85% (NaCl) saline solution, of the soil that were used to inoculate the *P. vulgaris* var *Bayo* seedling in Leonard Jars that was sterilized at 121°C, *P. vulgaris* used as absolute control (AC), was only irrigated with water current, *P. vulgaris*, used as relative control (RC), and fed White's nutrient solution for plants with the following composition is: 3 molar solutions of NH_4NO_3 , and 1 molar of K_2HPO_4 , KH_2PO_4 ,

$CaCl_2$, $MgSO_4 \cdot 7H_2O$, $FeSO_4$ solutions were added 1ml/1000 of distilled water, the pH was adjusted to 7.0, which was boiled for 20 min. The *P. vulgaris* var *Bayo* seed in the jar was disinfected with 0.2% $HgCl_2$ and rinsed 5-8 times with sterile distilled water. When *P. vulgaris* developed the first leaves, it was inoculated with the dilution of soil. 45 days later, when the galls or nodules appeared, they were selected according to the position, shape and color [14,17].

Isolations of *Agrobacterium tumefaciens* and *Rhizobium etli* from Galls and Nodules

The galls and/or nodules were disinfected with ethanol and then washed with sterile distilled water 8 times with 30% H_2O_2 . Galls and nodules crushed in a sterile mortar with 0.85% saline solution, streaked on Congo red yeast extract agar with the following composition (g/L) K_2HPO_4 0.5, $MgSO_4 \cdot 7H_2O$ 0.2, NaCl 0.1, yeast extract 1.0, mannitol, Congo Red 10ml, 10.0, agar 18.0 (YEMCR) distilled water 1000 ml, adjusted pH of 6. The Petri dishes were incubated at 30 °C/ 48 h and reseeded in YEMCRA. Isolates were carried out by monthly periodic reseeded in inclined YEMCRA, at a temperature of 15 °C [14,19].

Identification of Isolated Galls and Nodules

Infectivity and effectiveness micro and macroscopic morphology of the morphological and microscopic characteristics considered, there were: Gram and macroscopic size, shape, size, elevation and color of the colony and biochemical profile [1,3,5]. For ineffectiveness, *P. vulgaris* was inoculated and the following were evaluated: the number, shape, color and position of the nodule or nodules [6]. The effectiveness depends on foliage color, plant height and total nitrogen content based on the Kjeldahl method.

Biochemical Tests

Use of citrates as the sole carbon source, growth on glucose peptone agar, reaction in litmus milk or whey formation, Congo red absorption, resistance to 2% NaCl, hydrolysis of gelatin and casein, production of H_2S in sulphite agar bismuth, and production of 3 Ketolactose [6,10].

Detection of plasmids of *A. tumefaciens* and *R. etli*

In *Agrobacterium* and *Rhizobium* isolates from the soil of Cadereyta, Jiménez, NL, México, specific plasmids for the formation of Ti tumors (galls) or root nodules were searched to confirm the negative (parasitism) or positive (symbiosis) action of infection (*Inf+*) and (*Nif+*) of biological fixation of molecular nitrogen (N_2) in the roots of *P. vulgaris* var. *Bayo* according to what has been described in the literature [2,4,15,16].

Results and Discussion

Obtaining isolates of *A. tumefaciens* and *R. etli* of *P. vulgaris* nodulated "Leonard Jar" inoculated with the soil sample from the "Ejido el Refugio" Municipality of Cadereyta Jiménez, NL, México 4 isolates were obtained, which were assigned the following codes: *A. tumefaciens*: At4, *R. etli* 8, **Table No. 1** shows physicochemical

properties of the agricultural soil used as a source of isolation of both: *A. tumefaciens* and *R. etli*, where the survival capacity of both pathogenic and beneficial endophytic bacteria was easy to isolated them, observed despite the properties of the soil: crumbly sandy texture, low content of organic matter, and mineral nitrogen, slightly alkaline pH, and saline [1,5,12].

Table 2 shows the phenology and nodulation of *P. vulgaris* var Bayo inoculated with soil from Cadereyta, Jimenez, N. L. México, to demonstrate the existence of native *R. etli*, a genus and species common in soils of México as described in Table 1. In which the healthy growth of *P. vulgaris* fed with the mineral solution with

100% ammonium nitrate (NH_4NO_3) was evident with a plant height (PH) of 35 cm, leaves of 8 cm in diameter, green without nodules, compared to *P. vulgaris* with 30 cm of PH, green leaves with 7cm in diameter and with 25 red nodules, indicating the active presence of *R. etli* infective and effective and *P. vulgaris* with 26 cm of PH, with leaves of 6 dm in diameter with 25 red nodules indicating the presence of *R. etli* infective and effective [20] and 10 white ones that suppose the existence of *A. tumefaciens* [17-19]. That shows the survival and competition for the radical system of *A. tumefaciens* and *R. etli* in the soil [21,22].

Table 1: Physico and chemical soil* proprieties of agricultural soil Cadereyta de Jiménez, Nuevo León, México.

Texture	Sandy crumbly
Organic matter	1.83% (poor)
pH	7.4 (light alkaline)
Total, nitrogen	0.95% (poor)
Moisture percent	12.8% (poor)
Electric conductivity	Light saline
*Soil sample deep: 25 cm	

Table 2: Phenology of *Phaseolus vulgaris* var Bayo inoculated with agricultural soil from Cadereyta, Jiménez, N. L, México.

* <i>P. vulgaris</i>	Phenology			
	Plant height (cm)	Leaf size (cm)	Leaf color	Number of nodules and color/plant
Relative control 100% NH_4NO_3	35.0 ^{a**}	8 ^a	Green	0 ^d
Absolute control Irrigated water	23.0 ^d	3.5 ^d	Yellow	0 ^d
Inoculated with agricultural soil	30.0 ^b	7 ^b	Green	25 ^c pink
	26.0 ^c	6 ^c	Green	35 ^a pink
26.0 ^c		6 ^c	Green	30 ^b pink
	20 ^d	3 ^d	Yellow	10 ^d white
*n=12 **different letters were statistically different according to ANOVA-Tukey at 0.05.				

When Table 3 shows the percentage of protein in *P. vulgaris* inoculated with *A. tumefaciens*, with *R. etli*, with the 50% NH_4NO_3 dose in the mineral solution compared to uninoculated *P. vulgaris* fed with the 100% NH_4NO_3 dose and uninoculated *P. vulgaris* irrigated only with water in soil poor in mineral nitrogen. Where *P. vulgaris* with *R. etli* 8 registered 17.50% protein as a consequence of the formation of nodules that *R. etli* formed to fix N_2 that favored the healthy growth of the plant compared to *P. vulgaris* fed with 100% NH_4NO_3 without inoculating that registered 19.95% protein, an amount equivalent to the need of the legume to be healthy, in evident difference with *P. vulgaris* with *A. tumefaciens*-9 with 14.70 % protein that shows a problem of nutritional deficiency: this

experiment showed what the literature reports that both genera coexist in the soil, which can benefit or sicken the plant depending on the ability to survive in the soil and then compete for the radical infection of the legume to induce a positive or negative effect [22,23].

Table No. 4 show effect of *R. etli* of the *P. vulgaris* inoculated with the strains in relation to *P. vulgaris* used as a control with and without mineral nitrogen fertilizer as NH_4NO_3 . A difference is observed between them because the one inoculated as the *R. etli* 8 strain achieved good size, green color of the foliage, and normal wet weight, although the nodules were few, they were large and pink,

while in those inoculated with *R. etli* 9 and *R. etli* 10, the color of the foliage was yellowish and the nodules were abundant but small and white. When plasmids were extracted from the *Agrobacterium* isolate, the existence of the *Ti* plasmid that causes root galls in *P. vulgaris* was confirmed. Plasmids associated with *Inf+* for root infection and *Nif+* for biological N_2 fixation were also confirmed (data not shown). Confirming that both genera *A. tumefaciens* and *R. etli* exist in the soil for a diseased or healthy *P. vulgaris*

according to phenology, biomass and protein content [22-24]. These characteristics are of infectivity but ineffectiveness in fixing N_2 , since the presence of nodules is not a guarantee that plants can benefit from N_2 gas provided by bacteria. Because the *R. etli* 8 strain was considered infective and effective, however, this strain can be effective in one host and achieve parasitism in others [21,25,26]. Therefore, it is not possible to conclude that a strain is effective or ineffective in absolute terms [27,28].

Table 3: Protein, percent in *Phaseolus vulgaris* var *Bayo* sown, in sterile soil inoculated with *Rhizobium etli* 8, and *Agrobacterium tumefaciens*-9 native strain from soil, Cadereyta, Jiménez, N, México.

<i>Phaseolus vulgaris</i> */**	Protein per cent (%)
Relative control fed 100% NH_4NO_3	19.95 ^{a***}
Absolute control uninoculated irrigated with water	13.47 ^c
<i>Rhizobium etli</i> -8	17.5 ^b
<i>Agrobacterium tumefaciens</i> -9	14.7 ^c
*n=12 **Kjeldahl method, ***different letters were statistically different ANOVA-Tukey at 0.05.	

Table 4: Phenology and fresh biomass of *Phaseolus vulgaris* var *Bayo* with native strains of *Agrobacterium tumefaciens*-9 and *Rhizobium etli*-8 from soil of Cadereyta, Jiménez, Nuevo León, México.

Phenology and Fresh Aerial and Radical Biomass						
		Plant height (cm)	Leaf size (cm)	Leaf color	Number and color of nodules	Fresh aerial weight (g)
* <i>Phaseolus vulgaris</i>	Relative control fed 100% NH_4NO_3	75 ^{a**}	5.5 ^a	Green	-	12.0 ^a stem 8.5 ^a root
	Absolute control irrigated water	50 ^c	1.3 ^d	Yellow green	-	5.1 ^d 4 ^c
<i>P. vulgaris</i> native strains	<i>R. etli</i> 8	70 ^a	4.0 ^b	Green	19 large pink	9.0 ^b 6.8 ^b
<i>A. tumefaciens</i> 9		63 ^b	2.0 ^c	Yellow green	15 White small	6.7 ^c 4.5 ^c
*n=12 **different letters were statistically different according to ANOVA-Tukey at 0.05 (-) = non-nodulated roots.						

Table 5 shows the effect of *A. tumefaciens* 9 and *R. etli* 8 on the protein percentage of *P. vulgaris* fed with a mineral solution and 50% NH_4NO_3 of the recommended dose for the *Bayo* variety. It was observed that *R. etli* infected and formed nodules with the pigment leghemoglobin to protect the nitrogenase, that allowed it to fix N_2 for healthy growth of *P. vulgaris*, with 17.0% protein, a percentage value with no statistical difference from the protein percentage in *P. vulgaris* not inoculated with *R. etli*, fed with the mineral solution and 100% NH_4NO_3 recommended for this variety of *P. vulgaris* 17.5%. In contrast to the protein percentage of *P. vulgaris* with *A. tumefaciens*, that by infecting and parasitizing the roots of *P. vulgaris* caused the lowest percentage of protein with 4.3%, even compared to *P. vulgaris* not inoculated with *A. tumefaciens* and *R. etli* sown

in soil poor in mineral nitrogen, fed without any mineral solution only with water [27,28]. This result demonstrates the coexistence in the soil of both genera and endophytic species, that compete for the colonization of the *P. vulgaris* roots [1,2,29-31], to colonize and establish a symbiotic or parasitic relationship depending on that has the best response to the physicochemical conditions of the soil, especially the insufficient concentration of mineral nitrogen and soil moisture [5,6,11,13], that in the end will determine which will predominate in the *P. vulgaris* roots, justifying the selection of native *Rhizobium* isolates from the soil or red nodules with high leghemoglobin content [14,17,19,23,25].

Table 6 shows the biochemical profile of *R. etli* isolated from the soil of Cadereyta, Jiménez, N. L, México; poor in organic matter,

mineral nitrogen, and restricted humidity that was isolated in *P. vulgaris* var *Bayo* which had a saccharolytic activity and not proteolytic activity in accordance with some biochemical tests such as the reaction in Litmus mil: acid, negative to hydrolyze casein and gelatin, inability to grow on glucose peptone agar, not producing H_2S , and not absorbing Congo Red compared to a reference strain of *R. etli* that confirms that in that soil of Cadereyta, Jiménez, N. L. México this *R. etli* is native as indicated by the literature either to

lactose production is a primary test to separate *R. etli* strains from *A. tumefaciens* since it is only positive for this species and for *A. tumefaciens*, placing the isolated strains within the first species as no galls were found or tumor in the plant since it is only a common inhabitant of the *rhizosphere*. Protein determination was used to evaluate the nitrogen contributed by *R. etli* and the infection by *A. tumefaciens* to the plant, because it is considered the best way to assess the effectiveness of a strain [8-10].

Table 5: Protein per cent in *Phaseolus vulgaris* var *Bayo* with *Agrobacterium tumefaciens* 9 and *Rhizobium etli* 8.

<i>P. vulgaris</i> +	Protein per cent*
Relative control NH_4NO_3	17.5 ^{a*}
Absolute control without NH_4NO_3	12.2 ^c
<i>R. etli</i> 8	17.0 ^a
<i>A. tumefaciens</i> 9	4.3 ^e
+n= 12 *Kjeldahl, **Different letters were statistically ANOVA-Tukey at 0.05	

Table 6: Biochemical profile of *Rhizobium etli* 8 from nodules of *Phaseolus vulgaris* var *Bayo*.

	<i>R. etli</i> Reference strain	<i>R. etli</i> 8
Citrates	-	-
Litmus milk	a	a
Growth on peptone glucose agar	-	-
Resistance to NaCl 2%	-	-
Casein hydrolysis	-	-
H_2S produccion	-	-
3 Ketolactose synthesis	-	-
Gelatin hydrolysis	-	-
Red Congo absorption	-	-
(+) = positive reaction; (-) = negative reaction		

Table 7: Biochemical profile of *Agrobacterium tumefaciens* from galls of *Phaseolus vulgaris*.

	<i>A. tumefaciens</i> reference strain	<i>A. tumefaciens</i> 9
Citrates	-	+
Peptone glucose agar	-	+
Litmus milk	b	b
Resistance to NaCl 2%	-	+
Casein hydrolysis	+	+
H_2S production	+	+
3 Ketolactose synthesis	+	+
Gelatin hydrolysis	+	+
(+) = positive reaction, (-) =not growth, (b) =alkaline reaction		

Table No. 7 shows the behavior of the strains isolated from the nodules in relation to a reference standard of *R. etli*. It is observed that strains At1, At2, At and At4 differ notably from the reference strain, while *Retli* 8, *R.etli*.9 and *R. elti* 10 coincide with the same, the above was corroborated by the Biuret reaction which is positive for *R. etli* and negative for *A. tumefaciens* inability to hydrolyze casein, gelatin, resistance to 2% NaCl and the non-production of H₂S, in Bismuth sulfite agar, which proves that the strains *R. etli* 8, *R. etli* 9 and *R. etli* are just one *R. etli* strain. These complementary data can be used to classify them in the genus because, as mentioned, some bacteria are incapable of infecting the roots of legumes, so this data was also used. Indicates the biochemical tests between the strains At1, At2, At4 and the reference strain *A. tumefaciens* where essentially to classify them in a genus the production of 3 Ketolactose, resistance to 2% NaCl, growth in glucose peptone agar and use of citrates as the only carbon source [1,10].

Conclusion

It was evident that a wide diversity of genera and species like *A. tumefaciens* and *R. etli* that are microorganisms that coexists in the soil, each with the genetic capacity to survive the physicochemical conditions of the soil. They can then detect the presence of substances derived from germination and roots and then establish positive or negative interactions with legumes such as *P. vulgaris*. This is the case with *A. tumefaciens* and *R. etli*, which share genetic characteristics that cause *A. tumefaciens* to parasitize *P. vulgaris*, although this does not cause its death. This is in contrast to *R. etli*, which, by responding to the lack of mineral nitrogen and low humidity, helps the plant cope with this type of stress and subsequently grow healthily. This supports the need for microbial selection through the use of plants, which allows for the differentiation between positive and negative interactions. As well as pink-red nodules were observed with *R. etli* and white galls of *A. tumefaciens* on the same plant coming from the same agricultural soil.

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Conflicts of Interest

The authors declare that there is no type of conflict of interest in its planning, execution and writing with the institutions involved, as well as those that financially supported this research.

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