Ciências Biológicas e da Saúde:

Investigação e Prática

Juan Carlos Cancino-Diaz
(organizador)

VOL II

EDITORA ARTEMIS
2023

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PRÓLOGO

Las ciencias biológicas abarcan diferentes disciplinas, entre ellas la medicina, la epidemiología, la biotecnología y hasta el medio ambiente; que se relacionan con otras ciencias que estudian la salud como la antropología médica. Estas aportan las bases científicas para el mejoramiento de la vida y la salud. En la actualidad, hay un gran interés sobre nuevas investigaciones en ciencias biológicas que ayudan a contestar diferentes inquietudes ocurridas en la vida cotidiana. En este libro, constituido por 16 capítulos, se enfoca en las disciplinas de la salud, la disciplina biotecnológica y la disciplina del medio ambiente.

En la disciplina "Salud y Prácticas", dos artículos están vinculados a desafíos para los profesionales de la salud, uno sobre el manejo de la muerte y otro sobre la maternidad transnacional, en sus aspectos psicosociales y culturales. Estos trabajos son importantes porque demuestran la importancia de actitudes de humanización y empatía por parte de los profesionales de la salud, como parte de sus habilidades y competencias para un abordaje profesional de la muerte y de la maternidad transnacional.

Por otro lado, capítulos que abordan sobre el tópico neurológico están incluidos en esta área: uno de ellos está dirigido a los niños sordos y la aportación del sentido de su vista para el mejoramiento de su salud, y el otro artículo está relacionado con los masajes para el tratamiento de los pacientes con lumbalgia y ciatalgia. Finalizan esta sección trabajos sobre la rehabilitación motora para los pacientes con enfermedad de Huntington, así como un artículo sobre la cadencia musical en la hidrogimnasia y un estudio relacionado con el uso de cannabis para el tratamiento de las enfermedades crónicas. Sin duda, estas aportaciones son de gran interés para el área de la salud.

Un estudio de epidemiología sobre la enfermedad de Chagas en mujeres de edad fértil en el Centro de Atención Primaria de la Salud, en la Cañada (Argentina), demuestra que en algunos lugares la prevalencia de esta enfermedad es alta.

En biotecnología se reportan capítulos sobre el impacto de la malta hacia la actividad de proteasas, la producción de proteína de forraje en *Clitoria* spp, el aislamiento de bacterias celulolíticas y xilanolíticas en Cachiyacu de Lupuna en Perú, y por último una evaluación del efecto gastroprotector de *Anacyclus radiatus*. Estos trabajos aportan investigación nueva sobre aspectos biotecnológicos.

En la parte del medio ambiente, un estudio enfocado sobre la relación del cobre con la fotosíntesis de microalgas, otro capítulo sobre control biológico de *Spodoptera* sp. y dos trabajos sobre el uso de sensores remotos y aplicación en lagos de Chile y la identificación de tóxicos en efluentes urbanos.

El libro está dirigido a la comunidad médica y científica que aporta información relevante en el área de ciencias biológicas; el lector puede tener una visión general de la investigación de estas áreas y comprender la complejidad y diversidad de tópicos relacionados con la biología y la salud.

Juan Carlos Cancino-Diaz

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HORMESIS UNDER OIL-INDUCED STRESS IN *CLITORIA* SPP USED FOR FORAGE PROTEIN PRODUCTION IN SOUTHEASTERN MEXICO[1]

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ABSTRACT: The oil industry is an activity with inherent risks of environmental emergencies due to oil spills and their derivatives. A study was carried out under micro-tunnel conditions for 120 days with the objective of investigating the hormetic effect of oil in 12 variables during the three phenological stages of two species of a legume. A completely randomized experimental design was established with a trifactorial arrangement, two plant species (*Clitoria* sp and *Clitoria ternatea*), five doses of medium fresh oil (0, 3, 10, 20 and 30 g/kg) and three exposure times according to phenological stage (days 30: growth, 75: flowering, and 120: fructification). The results show means with statistical differences in each exposure time in all the variables evaluated. Hormesis occurred in the three phenological stages in the two legumes, it occurred in 17 of the 36 dose-response combinations, both in *Clitoria* sp and *C. ternatea*, which represents 47.2% of the evaluated combinations, it was also found in 11 combinations, 30.5%, the phytotoxic effect of oil represented by the inverse dose-response relationship. The use of both species is recommended because of their adaptability and tolerance to medium crude oil and also because it accumulate crude protein in the foliage and in the pod.
KEYWORDS: Petroleum hydrocarbons. Nodule. Phenological stage. Leguminous.

1 INTRODUCTION

The response of the plant to exposure to chemical substances is manifested in the decrease in growth and production because the metabolic profiles are altered, they also produce secondary metabolites to overcome the stress conditions (Tadeo and Gómez-

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Cárdenas, 2013; Jamil et al., 2018). The adaptation of plants to chemical substances to avoid cellular stress and ensure signaling functions such as the control of cell multiplication and death, has been explained by the control of the levels of reactive oxygen molecules that regulate an antioxidant defense network to avoid cellular toxicity (Mittler, 2002). In addition to these two responses of plants to chemicals, there are hormetic processes at low doses of pollutants (Plice 1949; Eaton and Klaassen, 2005).

Hormesis is a dose-response phenomenon in which both low and high doses stimulate plants (Calabrese, 2012), it is an adaptive response of an organism to a stress factor. The hormetic effect shows an inverted U-shaped dose-response curve, which shows an increase over the control treatment at low doses of oil followed by a decrease at higher doses. In contrast, an oil dose response that forms a J-shaped curve represents a decrease compared to controlling for lower oil doses, followed by an increase at lower doses (Calabrese and Blain, 2009; Calabrese, 2012).

The sensitivity of legumes to exposure to fresh oil has been studied in recent decades by various researchers around the world and they indicate that oil in concentrations equal to and/or greater than 50 g/kg cause damage to cell membranes and death of species such as *Crotalaria* sp, *Crotalaria retusa*, *Leucaena* sp, *Leucanthemum vulgare* and *Mimosa pigra*, also propose that between the ranges of 7.5 to 50 g/kg they are indicators of toxicity to contaminated sites and the indicators are based on growth parameters, biomass production plant and nodules (Rivera-Cruz et al., 2005; Sadat et al., 2014; Walakulu et al., 2020). In other plant species, oil in low and medium doses stimulates the production of root biomass, the number of plants in the tiller and the accumulation of protein in the foliage, and induces a hormesis effect because it increases the carbon content in the soil (Plice 1949; González-Moscoso et al., 2019; Orocio- Carrillo et al., 2019).

Clitoria is a tropical legume that is widely distributed in nature (Jamil et al., 2018) and in intensive cultivation in pure stands and associated with grasses to be grazed (Correa et al., 2014). The legume *Clitoria* is known as butterfly pea and blue pea in several countries (Mejía, 1984). This plant specie is a tall, slender, herbaceous climbing plant with five leaflets, while the flower color ranges from blue-white with a white or yellow center (Jain et al., 2003). In the humid Mexican tropics, *Clitoria* is found naturally in cattle grazing sites adjacent to oil infrastructure, exposure to fresh oil can occur through the soil and oil breeze, which according to Rivera-Cruz et al. (2016) the petroleum hydrocarbons reach the land with livestock use due to accidental spills of pipelines that carry oil from the wells to the industrial distribution centers.

The sensitivity of plants to exposure to the contaminant can vary according to the dose of oil and the phenological stage. High doses of oil induce in the soil a coating of particles with an oily layer that hinders the absorption of water and exchange of nutrients (Li et al., 1997; Trujillo-Narcía et al., 2012). The development of the root is limited due to the difficulty to assimilate nutrients dissolved in water, as for example in N that its availability is essential for plants, which absorb it, mainly in the form of nitrates and ammonium, for use in the synthesis of protein and other plant organic compounds (Brady and Weil, 2008; Cooper and Scherer, 2012).

Studying the sensitivity of the toxic and hormetic response of the legume *Clitoria* by phenological stages will be a basic knowledge to propose its establishment in oil-contaminated sites in order to produce protein and recover soils degraded by the oil industrial activity. The objectives of this study are as follows, 1) To determine the hormesis effect on growth parameters, production and crude protein content, as well as number, diameter and leghemoglobin of nodules in *Clitoria* sp and *Clitoria ternatea* by phenological stage exposed to soil contaminated with fresh oil, 2) to analyze the type of *Clitoria* that supports stress from total oil hydrocarbons in the soil by phenological stage; finally, 3) recommend the production of integrated protein with native *Clitoria* from the Mexican humid tropics that is used as a forage supplement.

2 MATERIALS AND METODHS

2.1 SOIL AND *CLITORIA* CHARACTERISTICS

Uncontaminated soil (Gleysol) was collected in the Paso y Playa ejido, municipality of Cardenas, Tabasco, Mexico (UTM: 0461719 and 1985998). The soil was collected in March 2019 from a point on the surface horizon (0-30 cm). The soil was dried indoors, sieved through a mesh (2 mm diameter) and the chemical properties of a composite sample were determined before establishing the mixture with different concentrations of fresh oil. The properties of the uncontaminated soil were: clay texture (hydrometer method, Bouyoucos 1962), moderately acidic pH (6.4), extremely rich organic matter content (6.5%) (Walkley and Black, 1934), high inorganic N (55 ppm) (micro-Kjeldahl, vapor entrainment, DOF, 2002) and low cation exchange capacity (9.62 cmol/kg) (1N ammonium acetate method pH 7.0, DOF, 2002).

Clitoria sp (Clisp) and *Clitoria ternatea* L. (Clite) were selected, the first because it is a species that grows wild in the area of influence of particulate and gaseous emissions from the burning pit of the Paredón Battery, Huimanguillo, Tabasco, Mexico, while the Clite was collected in the municipality of Acayucan, Veracruz, Mexico where it is used as a source of protein for cattle.

2.2 EXPERIMENTAL DESIGN AND SOIL PREPARATION

The oil used is the same as that reported by Orocio-Carrillo et al. (2019). It is a medium crude with an API of 10.8°, specific gravity of 0.84 g/cm; the fractions are 56.4, 23.7 and 14% aliphatic, aromatic and polar + resin and asphaltenes, respectively. The experiment was based on a trifactorial arrangement consisting of the plant specie (*Clitoria* sp *and Clitoria ternatea*), the medium oil dose (0, 3, 10, 20 and 30 g/kg of oil) and exposure time associated with three phenological stages (growth, day 30; flowering, day 75 and fruiting, day 120). Each treatment had four repetitions. All treatments were established in a micro-tunnel for 120 days at a variable temperature between 28 to 34 °C. The initial oil concentration (g) in the experimental unit (UE) was sequentially: 0, 6, 20, 40 and 60 g of medium fresh oil in 2000 g of dry soil with a particle diameter of 2 mm. The soil and the oil were manually homogenized with a glass rod. Each UE consisted of a glass beaker (17.5 cm diameter and 22.3 cm high) with 2000 g of dry soil and planted with Clisp or Clite. The 120 UE with plants were irrigated every 24 hours with potable water, maintaining humidity at field capacity (32%). The 12 variables evaluated were plant length (PL), relative growth rate (GR), dry aerial (stem + leaf) biomass (AB), dry root biomass (RB), dry nodule biomass (NB), dry flower biomass (FB), dry pod biomass (PB), number of nodules (NN), nodule diameter (ND), leghemoglobin in nodule (LN), crude leaf protein (LP) and crude pod protein (PP).

2.3 ANALYSIS OF GROWTH, PRODUCTION AND CRUDE PROTEIN

The PL quantification was measured from the base of the stem to the apex using a graduated ruler (cm). The PL was used to determine the GR applying the formula of Hunt et al. (2002): GR = (log PL2-log PL1 / (t2 - t1). Where, long: natural logarithm, PL: plant length, and t: time. The values of AB, RB, NB, FB and PB was quantified by the gravimetric technique after drying the different biomasses at 60 °C for 72 h and weighing the dry materials on a semi-analytical balance (Ohaus, Scout Pro SP 202) with 0.01 g capacity. The NN was evaluated by direct counting, the nodules of each root were separated manually and counted. ND was measured using a Mitutoyo digital vernier with 0.01 mm precision, and leghemoglobin was determined by transverse and longitudinal cuts of the nodule, the presence of red or orange coloration within the nodule was observed. The extraction of N was carried out from 1.0 g of leaves and also of pod by digestion with sulfuric acid and quantified using the micro-Kjeldahl method (Jones et al. 1992). The crude protein content was calculated using the formula: Protein (%) = mL HCl x N x 1.4 PM x 6.25 (AOAC, 1980), where, mL: volume of sulfuric acid, N: exact normality of HCl, and PM is weight of the sample expressed in grams.

2.4 STATISTIC ANALYSIS

The data was analyzed with the SAS version 8.01 program (SAS Inc, 2005). The Shapiro-Wilk test was first used to verify the normal distribution of the data. The equality of variances was tested with the Bartett test. Analysis of mean variance (Tukey, $p \leq 0.05$) of the variables plant length, relative growth rate, dry biomass of the aerial part (stem and leaf), root, nodule, flower was carried out in each plant species and in each phenological stage (exposure time); also in the number of nodules, diameter of the nodule, presence of hemoglobin in nodule, protein in leaves and in the pod. Moreover, the pairwise correlation was evaluated with Pearson's correlation-coefficient, using linear regression, for normally distributed variables with a statistical significance of $p< 0.05$ and 0.01.

3 RESULTS

3.1 EFFECT OF OIL ON PLANT GROWTH

Oil inhibited plant growth in the three stages, the correlation between TPH and PL fluctuated from -0.655** to 0.418* (Table 1). The exposure of the Clisp and Clite plants to five doses of crude oil, for 120 days, originated in the three middle phenological stages with statistically significant differences in PL and GR (Figures 1A and 1B, Table 1). The PL in the growth stage (day 30) was higher in Clisp, it grew 56.5 cm in soil contaminated with 3 g/kg of oil, higher concentrations inhibited the plant growth up to three times, showed U-inver type hormesis in Clisp (Table 2); on the other hand, the growth of Clite was not stimulated by exposure to oil, but not only that, the longest length was 25.1 cm in a plant established in control soil, a value that represents less than half compared to the other species, the hormetic effect was of the reverse type in Clite (Table 2).

This initial noticeable difference suggests that the Clisp species, which grows wild, has a greater natural rooting capacity that promotes initial growth. On the other hand, the responses during flowering (day 75) and fruiting (day 120) show that the oil induced hormesis (Table 2) in the growth of Clite exposed up to 20 g of oil, whereas in Clisp it only occurred during flowering due to the influence of 10 g/kg of oil (Figure 1A). Regarding GR, the trend was similar and complementary to legume stem elongation. The highest daily growth rate occurred in the growth stage of Clisp, the data varied from 0.04 to 1.65 cm/d (Figure 1B), the highest value corresponded to the plant established in soil with 3 g of oil, it was higher 77% compared to the control treatment (1.65 *versus* 0.93 cm). As for the Clite species, the rate registered a negative relationship with the amount of oil, fluctuated from 0.08 cm/d^{-}in the plant that grew in soil with 30 g of oil and increased to 0.41 cm in control soil, the inhibition was 512%. The results in flowering were stimulated by the oil in Clite, it was evident that the greater amount of oil originated the highest daily growth that reached 0.65 cm.

Table 1. Relationships between plant growth, biomass production, nodulation and crude protein content and total petroleum hydrocarbons during the phenological stages of growth (day 30), flowering (day 75) and fruiting (day 120) of *Clitoria* legume.

	PL	GR	AB	RB	NB	FB	PB	NN	LP	PP
Day 30										
TPH	-.655**	- .593**	-.704**	-.630**	NS	-	-	NS	-.710**	-
PL		.950**	.934**	.801**	NS	-	-	NS	NS	-
GR			.902**	.687**	NS	-	-	NS	NS	-
AB				.880**	NS	-	-	NS	NS	-
RB					-.463**	-	-	NS	NS	-
NB						-	-	.648**	NS	-
FB							-	-	-	-
PB								-	-	-
NN									-	-
LP										
Day 75	-.418*	.708**	-.731**	-.645**	-.704**	NS		.682**	NS	-
TPH		NS	.445*	NS	.446*	NS	-	NS	NS	-
PL			-.507**	-.539**	-.382*	NS	-	.419*	NS	-
GR				NS	.653**	NS	-	NS	.559**	-
AB					NS	-.502**	-	-.549**	NS	-
RB						NS	-	-.547**	.519**	-
NB							-	NS	NS	-
FB							-	-	-	-
PB									NS	-
NN										-
LP										-
Day 120										
TPH	-.597**	NS	NS	NS	-.497**	NS	-.396*	.497**	NS	-.923**
PL		NS	NS	NS	NS	NS	NS	NS	NS	NS
GR			.407*	NS	NS	-.390*	NS	NS	NS	NS

TPH: total petroleum hydrocarbons, PL: plant length, GR: relative growth rate, AB: dry aerial (leaf and stem) biomass, RB: dry root biomass, NB: dry nodule biomass, FB: dry flower biomass, PB: dry pod biomass, NN: number of nodules, LP: crude leaf protein, PP: crude protein in pod. *Means with significant differences (Tukey, p = 0.05), **highly significant (Tukey, p = 0.01), and NS: non significant.

Table 2. Hormetic types of dose-response curves of plant growth variables, biomass production, nodulation and protein content of *Clitoria* plants exposed to medium oil during the phenological stages of growth (day 30), flowering (day 75) and fruiting (day 120).

Variable	*Clitoria* sp / day			*Clitoria ternatea* / day		
	30	75	120	30	75	120
Plant length (cm)	U-inv	U-inv	Rever	Rever	Rever	U-inv
Relative growth rate (cm/d)	U-inv	Rever	U-inv	Rever	J	None
Root dry biomass (g)	Rever	Rever	Rever	Rever	Rever	Rever
Aerial dry biomass (g)	U-inv	Rever	U-inv	Rever	Rever	Rever
Nodule dry biomass (g)	U-inv	Rever	Rever	U-inv	U-inv	U-inv
Flower dry biomass (g)	None	U-inv	None	None	U-inv	None
Pod dry biomass (g)	None	None	Rever	None	None	Rever
Number of nodules	U-inv	J	J	U-inv	J	J
Nodule diameter (mm)	J	U-inv	Rever	J	U-inv	Rever
Leghemoglobin in nodule (%)	U-inv	J	Rever	U-inv	J	J
Crude protein in stem-leaf (%)	Rever	U-inv	U-inv	Rever	J	U-inv
Crude protein in pod (%)	None	None	Rever	None	None	Rever
Hormetic curve	7	6	4	5	6	5
None (no curve)	3	2	1	3	2	2
Reverse (inverted curve)	2	4	7	4	4	5

U-inv: Inverted U-shaped dose-response curve, J: J-shaped dose-response curve, Rever: Inversely proportional trend, and None: no trend or no response from the variable.

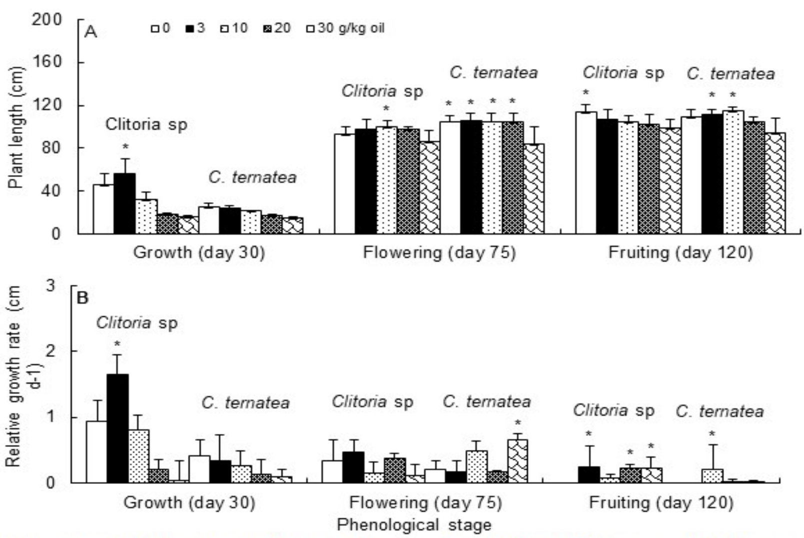

Figure 1 Effect of oil on length (A) and relative growth rate (B) of *Clitoria* sp and *Clitoria ternatea* exposed to crude oil during the three phenological stages. Values represent mean ± SD, n = 4. Asterisk within each phase indicate significant difference at the 5% level according to the LSD test.

The exposure of the plant to oil caused hormesis (Table 2) during fruiting (day 120), it was more accentuated in Clisp, where doses 3, 20 and 30 g of oil promoted 0.21 to 0.24 cm of daily growth. On the other hand, in control soil it did not register growth. Oil inhibited the growth of the plant on day 30 (0.593**), promoted (0.708**) during flowering (day 75) and in the fruiting stage (day 120) it was not significant (Table 1).

3.2 EFFECT OF OIL ON THE PRODUCTION OF PLANT BIOMASS

Figure 2 shows that oil affected the formation of leaf, stem, root and nodule biomass throughout the vegetative cycle of the two legumes. Statistical differences occurred between the means of the evaluated treatments. The type of hormesis in the four types of plant biomass was varied. In the case of the root that interacts directly with oil, the two evaluated species were affected by oil, for this reason it was classified as reverse hormesis, otherwise it was in the NB because in four of the six cases it was of the hormetic type U-inver (Table 2). Comparison of the two species shows that Clisp response on day 30 (Figure 2A) was positive because in control soil it produced 0.37 g of RB, whereas in Clite it was 0.12 g, this difference represents 308%, these data suggest a better adaptation of the Clisp species to oil. The greater amount of roots in the flowering and fruiting stages was also noticeably higher in this species, in addition the oil caused phytotoxicity in the plants exposed to 3, 10, 20 and 30 g of oil. On day 75 the data of both legumes not exposed to oil was 1.8 and 0.65 g of RB, it was higher by 277% and decreased to 174% on day 120. Oil and root biomass registered negative correlation in the growth stage (-0.630**) and in flowering (-0.645**), there was no effect on fruiting (PB) (Table 1). The aerial biomass production also registered the same trend as the root biomass, although the adaptation process did occur due to the stress derived from the 3 g dose of medium oil (Figure 2B). Amounts less than 1.0 g of dry foliage and stems produced the plants of both legumes harvested on day 30 and increased significantly in the following two phenological stages. On day 75 Clite formed the highest amount of foliage and stems (AB), statistically it was similar in the plants exposed to 0, 3 and 10 g/kg of oil, the values varied from 24 to 25 g of foliage and stem; the phytotoxic effect of oil originated 44% lower foliage biomass. The data from the collection on day 120, in full fruiting period, show that example of U-inv hormesis, the value was 37 g, was 3 g (8.1%) higher than the biomass of the plant established in soil without oil.

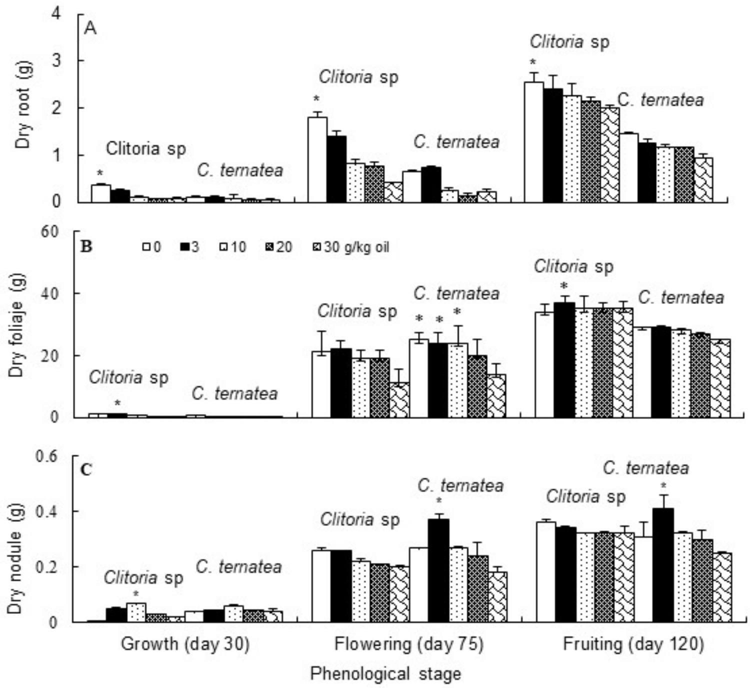

Figure 2 Effect of oil on dry biomass of root (A), foliage (stem and leaf) (B) and nodule (C) according to phenological stage of *Clitoria* sp and *Clitoria ternatea* exposed to crude oil for 120 days. Values represent mean ± SD, n = 4. Asterisk within each phase indicate significant difference at the 5% level according to the LSD test.

Correlation between oil an AB (leaf and stem) was negative during qrowth (-0.704**), fruting (-0.731**) and there was no effect on fruiting (Table 1). Regarding the biomass of the nodule, oil induced a U-inver type hormetic effect (Figure 2C, Table 2). On day 30, the highest amount of biomass was 0.07 g in Clisp under the effect of 10 g of oil and decreased to 0.02 g in the plant exposed to 30 g. The biomass increased in the samplings carried out during flowering and fruiting, in both phenological stages the biomass of the nodules was higher in Clite exposed to 3 g of oil. The dry weight in the flowering stage was 0.37 g and in control it decreased to 0.27 g, equivalent to 37% less; the same information but corresponding to fruiting increased to 0.41 in soil with 3 g of oil and 0.31 in the control, again it was lower 32% (Figure 2C). Oil affected the biomass of the nodule during flowering (-0.704**) and also during fruiting (-0.497**) (Table 1). The FB collected on day 75 was also inhibited by oil in Clisp but hormesis occurred in Clite in soil with 3 g of oil; As for the PB of the pod on day 120, it was also inhibited by oil, the reduction was 43% in Clisp and increased to 53% in the other species (Table 3). Crude oil registered a negative correlation (-0.396*) with the PB on day 120 (Table 1).

Table 3 Effect of oil on the production of dry flower and pod biomass of *Clitoria* sp and *Clitoria ternatea* plants exposed to crude oil for 120 days.

Dry biomass (g)

Flower (day 75)		Pod (day 120)	
Clitoria sp	*C. ternatea*	*Clitoria* sp	*C. ternatea*
5.0 ± 0.08e*	5.72 ± 0.08b	11.54 ± 0.34a	9.59 ± 0.32ab
5.74 ± 0.01b	5.87 ± 0.04a	8.47 ± 0.55bc	7.60 ± 0.22bc
5.62 ± 0.03bcd	5.53 ± 0.05cd	7.83 ± 0.47bc	6.25 ± 0.05c
5.65 ± 0.07bc	5.68 ± 0.03b	8.37 ± 0.14bc	8.81 ± 0.33bc
5.51 ± 0.16d	5.62 ± 0.06bcd	8.08 ± 0.21bc	6.25 ± 0.27c

*Different letters in columns of each plant organ indicate statistical differences (Tukey, p = 0.05, a> b, n = 4).

3.3 EFFECT OF OIL ON THE NUMBER, SIZE AND PRESENCE OF LEGHEMOGLOBIN OF NODULES

The exposure of legumes for 120 days to oil caused statistical differences in the quantity, size and presence of leghemoglobin in nodules in the three phenological stages, it caused type J hormesis in the three evaluated stages (Figure 3A, Table 2). The results of day 30 show that the amount of nodules was stimulated in Clite but mainly in Clisp that formed 84 nodules in plant exposed to 10 g of oil and increased to 125 nodules during flowering (day 75), these values represent increases of 551 and 227% with respect to the number of nodules of the control treatment. This trend was maintained in the fruiting stage in Clisp although with less difference compared to the control, the plants exposed to 30 g of oil formed 144 nodules and in the control plant it was 75 nodules, it decreased 48% (Figure 3A). The presence of oil in the soil induced increases in the number of nodules during flowering (0.682**) and also in the fruiting stage (0.497**). The nodule size was sensitive to oil, it only increased in size in both legumes exposed to 3 g of oil in the growth stage, it also occurred in Clisp at the same dose but in the flowering stage (Figure 3B). The largest size of the Clisp nodule on day 30 was 2.32 mm and the smallest size 1.21 mm in the control plant, the difference was 92%; the same trend but of a lesser magnitude was identified in Clite, it was 33%. The results obtained in flowering showed greater statistical differences in Clisp. The largest average nodule was also found in Clisp with 3.88 mm and in the control plant it was 3.49 mm, it decreased to 2.76 mm in the plant exposed to 30 g of oil, the size decreased to 41% due to the effect of exposure to oil.

The nodulation during fruiting did not register hormesis, on the contrary, only in the control plant the nodules with larger diameters (4.64 mm) were formed and it decreased in both legumes due to the increase in the amounts of oil added in the soil (Figure 3B). The viability of the N-fixing bacteria within the nodules, evaluated through the presence of reddish coloration derived from leghemoglobin, was positive in both plant species in the growth and flowering stages, and partially in fruiting (Figure 3C). Here inverted U-type hormesis occurred during the growth stage and J-type in flowering. Leghemoglobin in nodule reached 90% of the Clisp nodules exposed to 10 g of oil, whereas in the control soil was 66.7% and decreased to 36.7% in soil with 30 g of oil.

The response of the legume Clite showed the same trend but with less damaging effect, it varied from 36% in soil with 30 g of oil and increased to 76% when the plant was exposed to 10 g of oil. The largest percentage value was 86.7% in nodules during flowering in Clite and it was statistically similar in soils with 20 and 30 g of oil (Figure 3C). On day 120, Clisp showed that oil inhibited the leghemoglobin in nodules, in control it was 98% and decreased to 66.7% in the most contaminated soil; On the other hand, the Clite data show a positive trend, the oil promoted the accumulation in nodules: in the control plant, leghemoglobin was found in 56.7% of the nodules and increased to 83.3% in the plant exposed to 30 g of oil for 120 days.

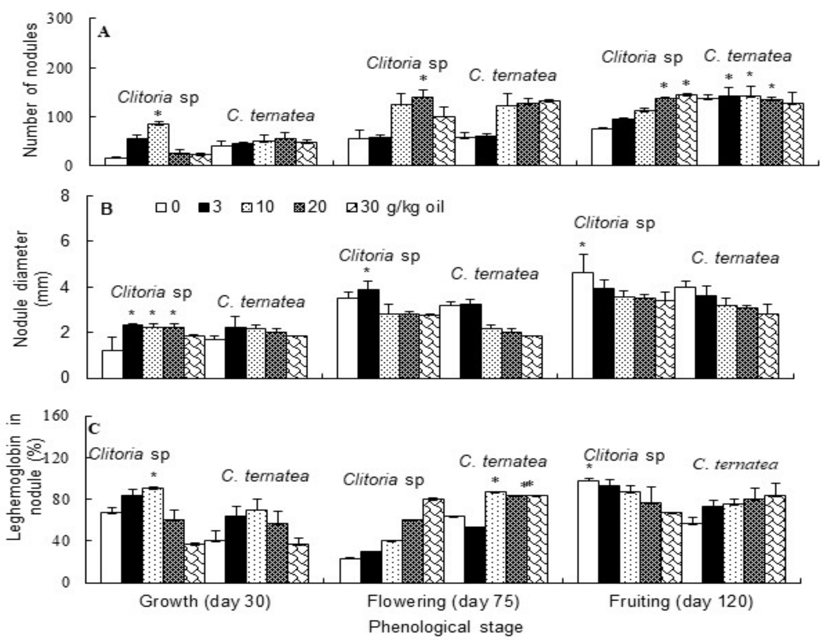

Figure 3 Effect of oil on number of nodule (A), nodule diameter (B) and presence of leghemoglobin in nodule (C) according to phenological stage of plants of *Clitoria* sp and *Clitoria ternatea* exposed to oil for 120 days. Values represent mean ± SD, n = 4. Asterisk within each phase indicate significant difference at the 5% level according to the LSD test.

3.4 EFFECT OF OIL ON THE ACCUMULATION OF CRUDE PROTEIN IN FOLIAGE AND POD

The crude protein contents in the foliar biomass and in the pod of both plant species registered statistical differences (Figure 4). The amounts of protein in leaves were higher in Clite in the three stages evaluated (Figure 4A). On day 30 the values fluctuated from 9.08 to 15.7% in Clisp and in Clite from 12.35 to 18.43%, in the flowering stage it varied from 15.92 to 19.14% in Clisp and changed to 14.51 to 21.22% in Clite, finally, in the fruiting protein amounts in Clisp decreased in the range 13.63 to 14.87% and increased to 18.03 to 20.69% in Clite. The oil effect caused inverted U-type hormesis in both species during flowering and fruiting (Table 2) but hormesis did not occur in the initial growth stage. The crude protein accumulated in the pod of both legumes shows a constant decrease as the amount of oil in the soil increases (Figure 4B). The highest amount of protein in Clisp was 20.67% in the pod of the plant established in the soil without oil and decreased to 13.87% in the soil with 30 g of oil, the reduction was 49% compared to the control plant. The information corresponding to Clite registered a similar trend, the lowest was 13.85% in the most contaminated soil and increased to 21.17% in control soil, this represents 53% less protein (Figure 4B). The relationship between oil and crude protein in foliage biomass was affected (-0.710**) in the growth stage but not in flowering or fruiting. The correlation between the protein content in the pod and the oil dose was also negative (-0.923**) in the fruiting stage.

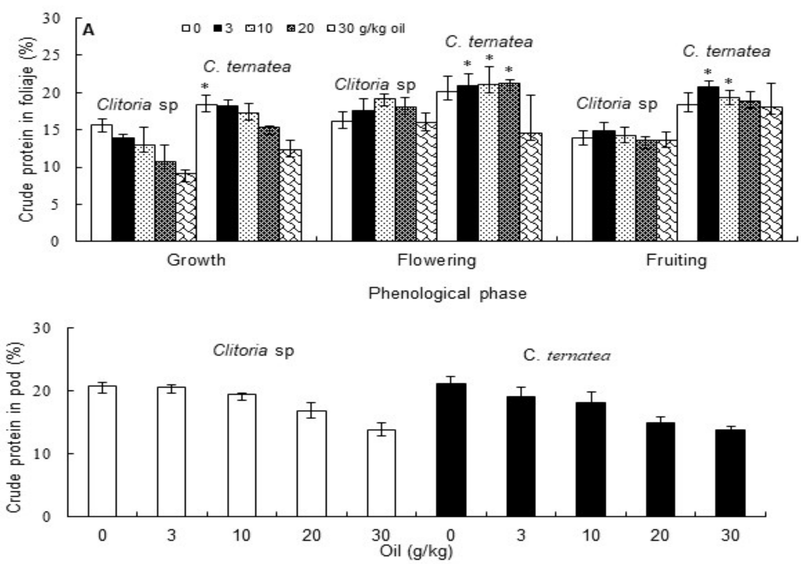

Figure 4 Accumulation of crude protein in aerial biomass (A) and in pod (B) of plants of *Clitoria* sp ar *Clitoria ternatea* exposed for 120 days to different doses of crude oil. Values represent mean ± SD, n 4. Asterisk within each phase indicate significant difference at the 5% level according to the LSD test.

4 DISCUSSION

4.1 EFFECT OF OIL ON PLANT GROWTH

Oil inhibited plant growth in all three phenological stages. The higher PL and GR of Clisp with respect to Clite was different because it registered, in less time, adaptation to oil during the first 30 days in the vegetative growth stage. The best response of the growth of Clisp is associated with the greater amount of the root system, it suggests greater absorption of nutrients, therefore this explains the greater daily growth rate.The best adaptation and tolerance of Clisp to oil occurred, probably, because it is a wild plant sensitive to environmental stimuli, but not Clite, which is a forage species introduced from Australia to Mexico in 1968 (Córdoba and Ramírez 1993), it has been improved in Mexico for half a century using conventional genetic procedures (Bravo, 1971; Villanueva et al., 2004; Medel et al., 2012; García-Ferrer et al., 2015). It is known that the stem and the root are structures capable of maintaining indefinite growth due to the permanent activity of vegetative meristems, which respond to adaptation to environmental changes and activate new development programs (Segura, 2013).

Our results show that the PL and GR of both species of *Clitoria*, exposed to 0, 3, 20 and 30 g of oil, show similar trends in the three phenological stages. Although the literature consulted only reports global data, that is, it does not separate the effect according to the phenological stage, different species of legumes exposed to TPH, or its derivatives, originate the decrease in PL (Adieze et al., 2012; Bento et al., 2012; Rivera-Cruz et al., 2012; Kreslavski et al., 2014) but in other cases hormetic effects occur because the pollutant promotes plant growth (Radwan et al., 2005; Bento et al., 2012; Kummerová et al., 2013; Agoun-Bahar et al., 2019; Walakulu et al., 2020).

In the first case, there are reports indicating that the height of *Centrosema* sp and *Pueraria* sp legumes is inhibited by exposure to soil contaminated with 10% oil, but 1% stimulated 6.4% the height of *Centrosema* sp (Adieze et al., 2012), our results also coincide in the decrease in the height of the legume *Leucaena leucocephala* exposed to 5 and 7.8% of fresh crude oil and also weathered (Rivera-Cruz et al., 2012). Other results (Bento et al., 2012) show that the plant response to exposure varies according to the species and type of oil. *Acacia angustissima* and *Mimosa caesalpinnifolia* plants grew without limitations despite the presence of high levels of oil, on the other hand, *Mimosa artemisiana* significantly decreased its growth (Bento et al., 2012). Regarding polyaromatic hydrocarbons, the effect of doses lower than 4 mg of naphthalene induces the growth of *Pisum sativum* and also the plant conserved the chlorophyll and carotenoid contents (Agoun-Bahar et al. 2019) but 7 mg/kg of fluoranthene caused toxic effects and inhibited the growth of the root of *Pisum sativum* (Kummerová et al., 2013). Our results highlight that the PL of Clisp was greater in the growth stage (day 30) than that of Clite, but over time in the flowering stages (day 75) and fruiting (day 120) the length registered the same trend and scale in both species, it was only different in the Clisp flowering. The GR was higher in the growth, in the following two stages it decreased and it was similar, possibly caused by processes associated with the adaptation of the plant.

Oil causes alterations in the soil, in plant tissues and cells, also in the photosynthetic process. The hydrophobic effect causes a severe decrease in humidity in the soil, the loss of humidity reaches up to 58% of the field capacity, so that the nutrient transport decreases (Trujillo-Narcía et al., 2012), this causes nutritional disorders causing lower plant growth, incidence of chlorosis and foliar necrosis (Römheld, 2012). The repercussions on the tissue and the plant cell are manifested in the reduction of root hair growth, deformation of the primordia of the lateral roots, reduction of the cells of the rhizodermis and of the outer layers of the primary cortex, as well as the formation of white spots that later turn into necrotic lesions (Alkio et al., 2005; Kummerová et al., 2013). Other phytotoxic effects are revealed in the deformation of the chloroplast and the mitochondria, causing the collapse of these cellular structures (Liu et al., 2009). Although oil causes toxic effects in all plant organs, the ability to adapt to oil suggests new physiological processes associated with the radical exudates that induce hormesis. In this regard, it has been reported that as the time of exposure of the root to oil increases, it manages to break the surface tension in the soil through radical exudates such as suberin, which favors the exchange of nutrients (Newman and Reynolds, 2004), which suggests that it is an adaptation process represented by the expansion of the root system that improves the absorption and transport of nutrients.

4.2 EFFECT OF OIL ON THE PRODUCTION OF PLANT BIOMASS

Oil caused phytotoxic effects in the biomass production of the legumes studied. The decrease in the amounts of leaf-stem, root and nodule biomass throughout the vegetative cycle was inversely related to the dose of oil incorporated into the soil. Unlike the ability of *Leersia hexandra* grass, which has the hormetic capacity to inhibit the decrease in root and leaf biomass (Orocio-Carrillo et al., 2019), various species of legumes are affected when exposed to oil. The legumes *Centrosema brasilianum* and *Calopogonium mucunoides* decreased biomass up to 15% due to the effect of 5% crude oil (Merkl et al., 2004), the same effect was caused by the polyaromatic phenanthrene, anthracene and fluorene in *Phaseolus vulgaris* (Paškova et al., 2006), the legumes *Pueraria* sp and *Centrosema* sp were also affected by exposure to 5 and 10% crude oil.

The root and fruit biomass in Clisp sp and Clite are sensitive to exposure to day 30, 75 and 120 of oil. The root is the first organ to be fully exposed to contaminants in the soil and therefore the effect on the roots is important in evaluating the susceptibility of plants to contaminants (Rivera-Cruz et al., 2012; Walakula et al. 2020). Root growth involves various biochemical and physiological processes (Masakorala et al., 2013a). Our findings of the negative effect of root oil are in agreement with previous studies on *Medicago sativa, Vigna radiata*, *Phaseolus vulgaris* and *Vigna unguiculata* grown in soil with hydrocarbons (Schwab et al., 2006; Masakorala et al., 2013b; Arias-Trinidad et al., 2017; Akhter et al., 2018). The inhibition recorded in the production of root biomass in Clisp and Clite at days 30, 75 and 120 of exposure to oil suggests that the composition of medium fresh oil that are hydrophobic compounds (e.g., benzene, toluene, ethylbenzene and xylene and short-chain aliphatic compounds) that can bind to the root surface, creating a hydrophobic environment that leads to limited water and nutrient uptake by the plant (Li et al., 1997; Issoufi et al., 2006; Trujillo-Narcía et al., 2012). The lack of exchange of solutes from the outside to the inside of the root cell alters the activity of the apical meristem in the formation of young cells and secondary roots at the tip of the root (Salisbury and Ross, 2000).

Another organ that decreases due to the oil effect is the fruit biomass in Clisp and Clite, it suggests that the contaminant was a stress factor that altered the route of transmission of information and gene regulation (Tadeo-Gómez and Cárdenas, 2013) altering the pollen-stigma protein recognition that inhibits the development of the pollen tube in the flower, which is basic for the formation of the seed (Agustí, 2013). However, in general Clisp produced higher pod biomass (8.86 g) compared to Clite with 7.72 g (Table 3).

The foliar biomass of Clisp to contaminated sites is stimulated by low doses of oil at days 31, 75 and 120 of oil exposure, suggesting that the processes of cell division, growth and elongation in aerial apical meristems develop in a more intense compared to Clite to form stem and leaf (Salisbuy and Ross, 2000) as a response to stress induced by the contaminant. Flower formation in Clisp was stimulated by low and medium doses of oil, while Clite by low doses, an inverted U-shaped hormetic response (Table 2).

The increase in foliar biomass and flowers due to oil stress in the soil suggests the overcompensation and alteration of homeostasis in these legumes, which has been described as a response to organic and inorganic chemical substances from plants (Stebbing, 1982; Calabrese and Baldwin, 1997). It has also been suggested that this growth stimulus at low concentrations of a compound may be due to a feedback mechanism; and physiological control mechanisms in organisms are believed to have evolved to overreact to a small deviation from the physiological norm (Stebbing, 1982; Swart et al., 1995). Some authors have even suggested that petroleum hydrocarbons may be promoters of plant growth and reproduction (Maliszewska-Kordybach and Smreczak, 2000).

4.3 EFFECT OF OIL ON THE NUMBER, SIZE AND PRESENCE OF LEGHEMOGLOBIN OF NODULES

The exposure of both species of *Clitoria* to the five doses of oil for 120 days, caused different effects in the three phenological stages evaluated. According to our results, the number of nodules registered a hormetic response (Table 2) due to the influence of oil, promoted the formation of this important plant organ in the biological fixation of N. The distribution of the number of nodules per plant was variable but always greater in soils contaminated with 20 to 30 g of petroleum. Different studies coincide with our results because the amount of nodules increases, for example in *Vicia faba* exposed to 1% oil (Radwan et al., 2005) but other scientific reports identified that both oil in doses of 3 and 5% and with 75 mg of polycyclic aromatic hydrocarbons affected the formation and growth of nodules in *Medicago sativa* plants (Wetzel and Werner, 1995), *Centrosema brasilianum* and *Calopogonium mucunoides* (Merkl et al., 2004), and by effect of 60 to mg 80 mg of anthracene and pyrene over *Pachyrhizus erosus* and *Clitoria ternatea* (Somtrakoon et al., 2018).

The stimulation of NB in Clisp and Clte stands out with a J-shaped hormetic dose-response curve at day 30, and an inverted-U-shaped dose-response curve of Clite at days 75 and 120 induced by exposure to low dose (3 and 10 g of oil). This information suggests that Clite in the three times exposed to fresh oil regulates environmental stress by increasing plant cell division in the root, rhizobium site of interaction for the formation of nodule cell mass which is the site where rhizobium fixes N_2 and reduces to ammonia (Madigan et al., 2015). Another factor that influences the behavior of the NB is the relationship with the NN; at day 30 highly significant and positive relationship NB*NN (0.648**), at day 75 highly significant negative (-0.547**) and at day 120 without any relationship between these variables.

An explanation of these relationships may be that the NN in Clisp and Clite at days 30. 75 and 120 present dose-response curves in the form of J (Figure 3A) while the NB in Clisp and Clite presents three different dose response curves (Figure 2C). The increase in NN suggests that *Clitoria* in oiled soil stimulates the release of flavonoids and the release of nod-factors by rhizobium multiplying in the rhizosphere (Lambers et al., 1998), rhizobium adhere to the root hairs of *Clitoria*, forms the infection filament, and the plant cell divides forming the nodule (Madigan et al., 2015). Similar results have been reported in soil contaminated with chromium, where *Trifolium* is capable of nodulation, although chromium concentrations are lethal for a free-living rhizobium. The fact shows how symbiosis can offer bacteria a permissive niche in a toxic environment (Suartini, 2001).

4.4 EFFECT OF OIL ON THE ACCUMULATION OF CRUDE PROTEIN IN FOLIAGE AND PODS

The crude protein content registered statistical differences in the three phenological stages in the aerial biomass (leaf) and also in the sheath of Clisp and Clite. The results show that the effect of doses of 3, 10 and 20 mg/kg of HTP caused hormesis in Clisp and Clite during flowering and fruiting (days 75 and 120) but not in the initial growth stage (day 30). On the other hand, the effect of oil, on the leaf and on the pod, was reverse hormesis since the plant reduced protein production in both species. This positive plant response is associated with adaptation mechanisms probably related to light growth factors, water, CO_2 and mineral nutrients (Gárate and Bonilla, 2013) which are the most important growth factors. The accumulation of protein in the plant is the result of physiological processes in the plant that interact with various environmental components. The factors light, temperature, air humidity, water availability, mineral nutrient content in the soil and carbon dioxide influence photosynthesis, in turn growth and the differentiation of plant organs (Azcon-Bieto et al., 2013). The content of essential nutrients in plant tissues is explained by the interaction of the genetic endowment, the availability of nutrients in the soil and by the phenological moment or age of the plant according to the organ or plant tissue considered (Gárate and Bonilla 2013). Unlike Orocio-Carrillo et al. (2019) that identified a strong hormetic effect in the exposure of *Leersia hexandra* grass up to 238 g/kg of TPH, our results show that Clisp and Clite can adapt to low doses of less than 30 g of TPH. Other studies have also shown that *Leucaena leucocephala* was also damaged and that the amount of N in plant tissue decreased sharply (Rivera-Cruz et al., 2012). The decrease in protein content may be related to the damage that oil causes in root hairs, cells, chloroplasts and mitochondria (Alkio et al., 2005; Liu et al., 2009; Kummerová et al., 2013), in the same way to the decrease in available water in the soil induced by the hydrophobic effect of oil (Li et al.,1997; Trujillo-Narcía et al., 2012).

5 CONCLUSION

The effect of the combination species-dose-time of exposure to oil induced a better response in growth, biomass, number and diameter of nodules, as well as in the accumulation of crude foliar and pod protein in *Clitoria ternat*ea.

Clitoria sp is a wild legume with a greater tendency to form hormesis in response to variables of growth, production, nodulation and raw protein accumulation. *Clitoria* sp showed 31 dose-response curves in the form of hormesis, while *C. ternatea* 26, in addition *Clitoria* sp formed higher root and aerial dry biomass, and also accumulated 17 and 18% aerial and pod protein. These responses suggest recommending the wild species *Clitoria* sp for establishment in environments stressed by oil contamination.

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SOBRE O ORGANIZADOR

Dr. Juan Carlos Cancino Diaz - Egresado de la Escuela Nacional de Ciencias Biológicas (ENCB) del Instituto Politécnico Nacional (IPN), México, con la licenciatura en Ingeniero Bioquímico. Estudios de posgrado en la misma institución con la especialidad de maestría en Bioquímica y doctorado en Inmunología. Actualmente es profesor e investigador de la ENCB-IPN impartiendo la catedra de Microbiología veterinaria para los Químicos Bacteriólogos Parasitólogos. El área de investigación es sobre el estudio de la biología de *Staphylococcus epidermidis*, con una alta producción de artículos científicos en revistas científicas de prestigio. Ha desempeñado como director de tesis de licenciatura, maestría y doctorado. Tiene una patente otorgada por el instituto mexicano de la propiedad intelectual y cuatro en curso de aprobación. Es miembro del sistema nacional de investigadores de México nivel II. Es editor de un libro sobre *Staphylococcus epidermidis* que está en curso de publicación y cinco capítulos de libro sobre su área de investigación.

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