Bioremediation and accelerated phytoremediation of a soil impacted by a hydrocarbons mixture.

Abstract

Waste motor oil (WMO), not having an adequate final confinement, is dumped in agricultural soils, where it easily exceeds 4400 ppm according to the Mexican standard NOM-138-SEMARNAT/SSA1-2012; a concentration that causes loss of fertility. The aims of this research were: i) biostimulation a soil contaminated by 39,000 ppm of WMO with Triton X-100/Tween 80 at 0.5% and mineral solution, ii) phytoremediation by *Phaseolus vulgaris*enhanced with *Methylobacterium symbioticum* and *Xanthobacter autotrophicus* to reduce WMO at lower concentration than the máximum accepted by NOM-138-SEMARNAT/SSA1-2021. The response variables were: a) the initial and final concentration of WMO by Soxhlet, and b) *P. vulgaris*: germination percentage; phenology and biomass at seedling and pre-flowering. The experimental data were analyzed by ANOVA/Tukey 0.05%.

The results showed that biostimulation of soil impacted by 39,000 ppm of WMO with Triton X-100/Tween 80 at 50%, and a mineral solution, decreased to 26,990 ppm in 25 days. Subsequently, phytoremediation by sowing *P. vulgaris* with *M. symbioticum* and *X. autotrophicus* at pre-flowering reduced WMO to 1233 ppm in 50 days, a concentration lower than that accepted by NOM-138-SEMARNAT/SSA1-2012. This supports that accelerated biostimulation and phytoremediation reduced WMO in the soil to recover its productive capacity.

Key words: soil, agriculture, hydrocarbons, emulsification, oxidation, legume, endophytic plant growth promoting bacteria

Introduction

Automotive oil for lubrication and cooling of automobiles is replaced at the end of its useful life. Which generates residual automotive oil (WMO) composed of a mixture of aliphatic and aromatic hydrocarbons (Yang et al., 2016). In Mexico, WMO is classified as toxic waste to avoid damage to the soil. The Mexican regulation known as the general law of ecological balance and environmental protection determines that it be recycled, reused and confined, however, a part is dumped into the soil during its preparation. While another environmental standard called NOM-138-SEMARNAT/SSA1-2012, establishes 4,400 ppm as the maximum concentration of hydrocarbon mixtures such as the WMO allowed in agricultural soil. A higher value of WMO causes a negative effect on physicochemical properties, especially productive capacity (Shukry et al., 2013; Ramadass et al., 2015). An alternative ecological solution is bioremediation via biostimulation initiated with detergents that effectively emulsify the WMO (Lamichhane et al., 2017; Sutthicharoen et., 2023), followed by biostimulation with a mineral solution that balances the concentration of essential minerals in the soil to induce accelerated mineralization of WMO (Anza et al., 2016; Lee et al., 2018), for subsequent sowing a legume tolerant to WMO phytotoxicity such as *Phaseolus vulgaris* whose hydrocarbon phytodegradation capacity is enhanced with *Methylobacterium symbioticum* and *Xanthobacter autotrophicus* genera and species of plant growth-promoting endophytic bacteria that oxidize aromatic hydrocarbons from

Comment [s1]: What is the nature of damage to soil? Mention why this research is undertaken the WMO (Ruikar & Pawar, 2022). Based on the above, the aims of this research were:i) biostimulation a soil contaminated by 39,000 ppm of WMO with Triton X-100/Tween 80 at 0.5% and mineral solution, ii) phytoremediation by *Phaseolus vulgaris* enhanced with *Methylobacterium symbioticum* and *Xanthobacter autotrophicus* to reduce WMO at lower concentration than the máximum accepted by NOM-138-SEMARNAT/SSA1-2021

Materials and methods

This research was carried out in stages in the greenhouse of the Environmental Microbiology Laboratory of the Chemical-Biological Research Institute of the Universidad Michoacana de San Nicolás de Hidalgo (UMSNH), Morelia, Mich., Mexico. In the first phase, agricultural soil used for growing *Zea mays* (maize) was collected located at 19º 39' 27" north latitude 100º 19' 59" west longitude, with an altitude of 1820 meters above sea level with a temperate climate; on an agricultural land called “La Cajita" of the Zapata Tenancy in the municipality of Morelia, Mich., Mexico at km 5 of the Morelia-Pátzcuaro highway, Mich., Mexico. The soil was sieved with No. 20 mesh and solarized at 70°C/48 h and minimized the problem of pests and diseases, then for every 1.0 kg it was artificially contaminated by 39,000 ppm of WMO (from a local mechanical workshop), then as a first biostimulation was emulsified with a mixture of detergents: Triton For 20 days, as shown in Table 1, at the end of this period of time, the concentration of WMO was determined by the Soxhlet method, with a single response variable.In the second phase, soil phytoremediation impacted by WMO remaining from biostimulation wasperformed. To do this, *P. vulgaris*seed were desinfectad with sodium hypochlorite (Clorox^{MR}) 0.6%/2.5 min, rinsed 6 times with sterile water, desinfectad with alcohol 70%/5 min, rinsed 5 times with sterile water. Then for every 10 seeds were inoculated with 1.0 mL of a suspension of *M. symbioticum* and individual *X. autotrophicus* and mixture in relation 1:1 (v/v) with a cell concentration equivalent to 1.5x108 CFU/mL by viable plate count (VPC) in nutrient agar with pH adjusted to 7. Soil moisture remained at 80% of field capacity. The response variables of measures were: emergency days and germination percentage; phenology: seedling height (PH) and radical length (RL); and biomass: fresh and dry weight, aerial and radical (FAW/FRW) and (DAW/RDW) at seedling and prefloration level,as well as the final WMO concentration by Soxhlet. The experimental data were subjected to a variance analysis (ANOVA) by the comparative mean test of Tukey HSD P-0.05% with the program Statgraphics Centurion XVI. II.

Comment [s2]: Provide chemical composition of experimental soil

Comment [s3]: How this value was arrived at

Comment [s4]: How was this experiment carried out ? is it pot experiment

Table 1. Experimental design for the biostimulation of a soil impacted by 39,000 ppm of waste motor oil (WMO)for 25 days

Agriculture soil*	39,000 ppm of WMO	Triton X-100 and Tween 80 at 0.5%	Mineral solution (%)
Relative control	-	-	100%
Negative control	+	-	-
Treatment	+	+	+
*=number of repetitions or (n) = 6; added (+); no added (-)			

Table 2. Experimental design for impacted soil phytoremediation with a remaining concentration of waste motor oil from biostimulation

Phaseolus vulgaris**in agriculture soil***	***Methylobacterium symbioticum***	***Xanthobacter autotrophicus***	**Water**	**Mineral solution (%)**
Relative control	-	-	-	100
Negative control	-	-	+	-
Treatment 1	+	-	-	50
Treatment 2	-	+	-	50
Treatment 3	+	+	-	50
*(n)= number of repetitions = 6; added(+); no added (-)				

Results and discussions

Table 3. Concentrationof waste motor oil inagriculture soilremaining, after 20 days of biostimulation.

Agriculture soil impacted by 39,000 ppm of waste motor oil (WMO)*	Final concentration
Soil polluted by WMO irrigatedonly with water or negative control	38,204 ppm**
Soil biostimulated by WMO biostimulated byTriton X-100 and Tween 80 at0.5% plus mineral solution at 50%	26,990^{b}
*n=6 **differentlettersshowedstatistically different according to ANOVA/Tukey $p<0.05$%.	

In Table 3, it is shown that the initial biostimulation with a mixture of: Triton X-100 and Tween 80 to 0.5% and with a mineral solution reduced the WMO in agricultural soil from 39,000 to 26,990 ppm after 20 days biostimulation period time, there was statistically different numerical value compared to 38,204 ppm of WMO in soil no biostimulated used as negative control (NC). This results support that the mixture of Triton X-100 and Tween 80 to 0.5% were able to emulsify the WMO due to desorption of this mixture of hydrocarbons of the soil surface (Cheng et al., 2017), then for biostimulation with mineral solution, induced the mineralization of the WMO by native aerobic heterotrophic microorganisms of this environment (Nasr, 2019).

Comment [s5]: What was triggering action of these chemical on reduction of WMO? What was percent reduction of WMO

In Table 4, the germination percentage of *P. vulgaris*enhanced with *M. symbioticum and X. autotrophicus* fed with 50% mineral solution in contaminated soil by 26,990 ppm of WMO remaining from biostimulation, with r 83.33% germination, statistically different numerical value compared to 56.67% of *P. vulgaris* sown in a soil without WMO, fed with 100% mineral solution or relative control (RC); and with 50% germination of P. vulgaris in the soil impacted by 38,204 ppm of WMO used as negative control (NC). The germination of the seeds of *P. vulgaris*enhanced with *M. symbioticum and X. autotrophicus*, supports the transformation of the metabolites of the germination of the seeds into phytohormons that induced a rapid and better of germination (Chan-Quijano et al., 2020). While both *M. symbioticum* and *X. autotrophicus* used as a source of carbon and hydrocarbons energy of WMO, thereby reducing the phytotoxicity of WMO (Kochhar et al., 2022), that inhibit the germination of seeds, as showed in figure 1.

Table 4. Percentage of germinationof*Phaseolusvulgaris* with*Methylobacterium symbioticum* and *Xanthobacter autotrophicus*in impactedsoil by 26,990 ppm of WMO remaining from biostimulation.

*Phaseolus vulgaris**	Germination percentage (%)
Uninoculated, in unpolluted soil fed with 100% mineral solution or relative control (RC)	56.67^{d**}
Uninoculatedin soil impactedby38,204 ppm of WMO irrigated only with wateror negative control (NC)	50.00^{e}
Enhanced with *M. symbioticum*in soil polluted by WMOremained from biostimulation	63.33^{c}
Enhanced with *X. autotrophicus* in soil by WMO remaining from biostimulation	66.67^{b}
Enhanced with*M. symbioticum* and *X. autotrophicus*in soil polluted by WMO remaining from biostimulation	83.33^{a}

*n=6 **different letters showed statistically different according to ANOVA/Tukey p<0.05%.

Figure 1. Effect of *Methylobacterium symbioticum* and *Xanthobacter autotrophicus* on germination of*Phaseolus vulgaris* after 9 days of sowing in soil polluted by 26,990 ppm of waste motor

oil.

RC (relative control): *P. vulgaris* uninoculated with *M. symbioticum and X. autotrophicu*s fed with 100% mineral solution in soil no polluted WMO. NC (negative control): *P. vulgaris* uninoculated with *M. symbioticum* and *X. autotrophicus* uninoculated, irrigated with water in soil polluted by 38,204 ppm WMO (negative control), T1: *P. vulgaris* with *X. autotrophicus* fed with 50% mineral solution in soil polluted by WMO remaining from biostimulation (Treatment 1), T2: *P. vulgaris* with *M. symbioticum* fed with 50% mineral solution in soil polluted by WMO remaining from biostimulation (Treatment 2), T3: *P. vulgaris* with *M. symbioticum* and *X. autotrophicus* fed with 50% mineral solution in soil polluted by WMO remaining from biostimulation (Treatment 3).

Table 5. Phenology and seedling biomass of *Phaseolus vulgaris* enhanced with *Methylobacterium symbioticum* and *X. autotrophicus* during phytoremediation of soil contaminated by 26,990 ppm of from biostimulation.

Comment [s6]: Mention when this data was recorded?

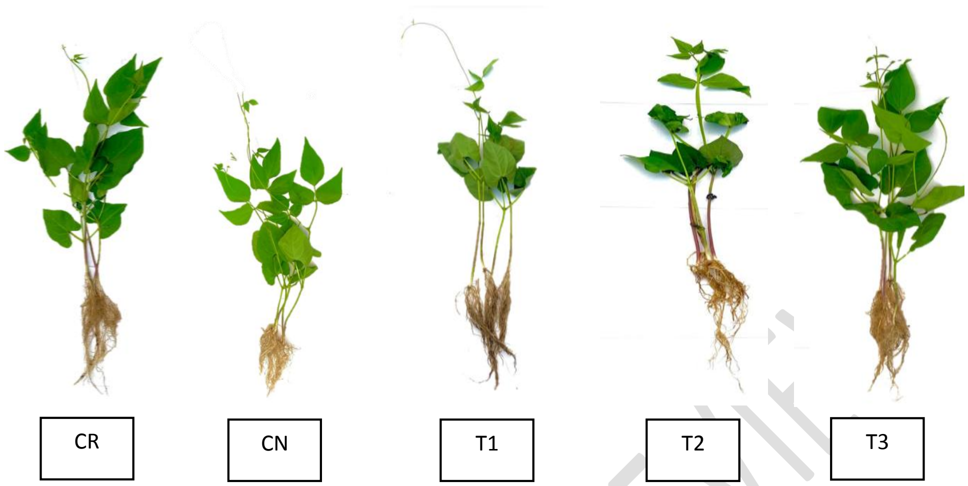
*Phaseolus vulgaris**:	Plant height (cm)	Radical length (cm)	Fresh weight (g)		Dry weight (g)	
			Aerial	Radical	Aerial	Radical
Uninoculated, in soil unpolluted by waste motor oil fedwith mineral solution at 100%	37.68[b**]	17.30[b]	3.11[b]	1.53[a]	1.61[c]	0.22[bc]
Uninoculated, in soil polluted by 38,204 ppm of WMO irrigatedonly water	23.65[d]	11.15[c*]	1.63[c*]	0.61[c*]	0.23[d]	0.06[c]
Enhanced with *M. symbioticum*in soil polluted by WMOremainingfrom biostimulation	37.50[b]	21.80[ab]	3.70[ab]	1.13[b]	1.93[b]	0.31[b]
Enhanced with*X. autotrophicus*in soil polluted byWMO remaining from biostimulation	31.38[c]	21.65[ab]	3.18[b]	1.35[ab]	1.12[c]	0.14[bc]
Enhanced with*M. symbioticum* y *X. autotrophicus* in soilpolluted by WMO remaining from biostimulation	49.58[a]	24.53[a]	4.12[a]	1.58[a]	2.37[a]	1.17[a]

*n=6 ** **different letters showed statistically different according to ANOVA/Tukey p<0.05%.

Table 5 shows the phenology and biomass at seedling level of P. vulgaris improved with M. symbioticum and X. autotrophicus, in the phytoremediation of the soil impacted by 26,990 ppm of ARA remaining from biostimulation, that registered 49.58 cm PH and 24.53 RL both numerical values were statistically different compared to 37.68 cm PH and 17.30 cm RL of *P. vulgaris* in non WMO soil fed with 100% mineral solution or relative control (RC). In the biomass of *P. vulgaris* enhanced with *M. symbioticum* and *X. autotrophicus*, 4.12 g of AFW and RFW 1.58 g was registered, as well as 2.37 g of ADW and 1.17 g of RDW statistically different numerical values compared to 3.11 g of AFW; 1.53 g RFW 161 g ADW and 0.22 g RDW of *P. vulgaris* uninoculated with *M. symbioticum* and *X. autotrophicus* sown in soil not contaminated by WMO used as relative control (RC). Increasing phenology and biomass of *P. vulgaris* enhanced with *M. symbioticum* and *X. autotrophicus*, supports that a reduction in WMO concentration was due to the radical activity of *P. vulgaris* and the transforming capacity of metabolites inside its root by *M. symbioticum* and *X. autotrophicus* (Madariaga-Navarrete et al., 2017; Gouthami et al., 2023), as shown in the enhanced*P. vulgaris* response with *M. symbioticum* and *X. autotrophicus* in figure 2.

Comment [s7]: The data shown in table 5 is solely due to microorganisms? what about the carry over effect of chemicals used in bio stimulation on the crop
What is the disposal of the crop after phytoremediation?
Why this crop was selected ? is it better scavenger of hydrocarbons

Figure 2. *Phaseolus vulgaris* phenology enhanced with *Methylobacterium symbioticum* and *Xanthobacter autotrophicus* to seedling during phytoremediation of soil polluted by26,990 ppm of waste motor oilafter 50 days.



RC (relative control): *P. vulgaris* uninoculated with *M. symbioticum and X. autotrophicu*s fed with 100% mineral solution in soil no polluted WMO. NC (negative control): P. vulgaris uninoculated with *M. symbioticum and X. autotrophicus* uninoculated, irrigated with water in soil polluted by 38,204 ppm WMO (negative control), T1: *P. vulgaris* with *X. autotrophicus* fed with 50% mineral solution in soil polluted by WMO remaining from biostimulation (Treatment 1), T2: *P. vulgaris* with *M. symbioticum f*ed with 50% mineral solution in soil polluted by WMO remaining from biostimulation (Treatment 2), T3: *P. vulgaris* with *M. symbioticum* and *X. autotrophicus* fed with 50% mineral solution in soil polluted by WMO remaining from biostimulation (Treatment 3).

Table 6. Phenology and prefloration biomass of *Phaseolus vulgaris* enhanced with *Methylobacterium symbioticum* and *Xanthobacter autotrophicus* in phytoremediation of contaminated soil by 26,990 ppm of waste motor oil (WMO) after 50 days

Phaseolus vulgaris:*	Plant height (cm)	Radical length (cm)	Fresh weight (g)		Dry weight (g)	
			Aerial	Radical	Aerial	Radical
Uninoculated in soilunpolluted fed	58.46^{c**}	33.44^{b}	5.34^{b}	2.55^{b}	2.76^{b}	1.18^{b}

with mineral solution at 100%						
Uninoculated in soilpolluted by 38,204 ppm of WMO irrigated only water	44.54^{cd}	21.52^{c}	3.98^{c}	1.92^{c}	1.46^{b}	1.07^{b}
Enhanced with*M. symbioticum*in soil polluted by WMO remaining from biostimulation	96.3^{a}	38.5^{a}	6.70^{a}	4.61^{a}	2.97^{a}	2.19^{a}
Enhancedwith *X. autotrophicus*in soil polluted byWMOremaining from biostimulation	85.14^{b}	39.42^{a}	6.33^{a}	4.65^{a}	2.89^{a}	2.27^{a}
Enhanced with *M. symbioticum* and *X. autotrophicus* remaining from biostimulation	91.1^{a}	44.46^{a}	5.10^{b}	4.38^{a}	2.82^{a}	2.25^{a}
*n=6 **different letters showed statistically different according to ANOVA/Tukey $p<0.05\%$.						

Table 6 shows the phenology and pre-flowering biomass of *P. vulgaris enhanced with M. symbioticum* and statistically different numerical values compared to the 58.46 cm PH and 33.44 cm RL of *P. vulgaris*uninoculated in the soil unpolluted by WMO. In the biomass, *P. vulgaris* improved with *M. symbioticum* and*P. vulgaris*uninoculated sown in the soil not contaminated by WMO used as RC, as shown in figure 2. In the phenology and biomass of *P. vulgaris*enhanced with *M. symbioticum* and *X. autotrophicus,* its supports that when both of them colonizing the interior of the roots used radical metabolism compounds to transform them into phytohormones that induced a dense root system by biostimulation with the mineral solution for maximum uptake and at the same time to mineralize aromatic hydrocarbons remaining from the WMO, that allowed a similar root growth of *P. vulgaris*that was observed on unimpacted soil by WMO (Kafle et al., 2022; Meištininkas et al., 2023.

Figure 3. Phenology of *Phaseolus vulgaris*enhanced with Methylobacterium symbioticum and *Xanthobacter autotrophicus* at pre-flowering in the phytoremediation of soil contaminated by 3000 ppm of waste motor oil (WMO).

RC (relative control): *P. vulgaris* uninoculated with *M. symbioticum and X. autotrophicus* fed with 100% mineral solution in soil no polluted WMO. NC (negative control): P. vulgaris uninoculated with *M. symbioticum and X. autotrophicus* uninoculated, irrigated with water in soil polluted by 38,204 ppm WMO (negative control), T1: *P. vulgaris* with *X. autotrophicus* fed with 50% mineral solution in soil polluted by WMO remaining from biostimulation (Treatment 1), T2: *P. vulgaris* with *M. symbioticum f*ed with 50% mineral solution in soil polluted by WMO remaining from biostimulation (Treatment 2), T3: *P. vulgaris* with *M. symbioticum* and *X. autotrophicus* fed with 50% mineral solution in soil polluted by WMO remaining from biostimulation (Treatment 3).

Table 7. Concentration of waste motor oil in agricultural soil remaining from biostimulation and phytoremediation at 25 and 50 days

Agriculture soil pollutedby 25,990ppm of waste motor oil (WMO) remaining from biostimulation	Final concentration (ppm) at	
	seedlingat 25 days	Pre-flowering at 50 days
Unpolluted soil by WMO fed with mineral solution at 100% or relative control (RC)	0.0	0.0
Soil polluted by WMOnon biostimulated or phytoremediated irrigated only water or negative control (NC)	38,204^{b}**	34,949^{c}
Soil polluted by WMO biostimulatedand phytoremediatedwith*Phaseolus vulgaris*enhanced with*Methylobacterium symbioticum*	17, 470^{a}	1,735^{b}
Soil polluted by WMO biostimulated and phytoremediated with*P. vulgaris*enhanced with*Xanthobacter autotrophicus*	16, 956^{a}	1,640^{b}
Soil polluted by WMO biostimulated and	17,035^{a}	1, 233^{a}

phytoremediatedwith*P. vulgaris*enhanced with*M. symbioticum* y *X. autotrophicus*		
*n=6 **different letters showed statistically different according to ANOVA/Tukey p<0.05%.		

Table 7 shows the concentration of WMO in the soil at the end of phytoremediation sowing*P. vulgaris* enhanced with *M. symbioticum* and up to 1233 ppm of WMO in 50 days, a concentration that was lower than the maximum limit accepted by NOM-138-SEMARNAT/SSA1-2012.

This numerical value was statistically different compared to 34,949 ppm in soil impacted by WMO non biostimulated or phytoremediated used as negative control (NC). This results supports that in the soil impacted by WMO, biostimulation with Triton X-100 and Tween 80, and then subsequent biostimulation by applying the mineral solution, the concentration of WMO was reduced (Lee et al., 2018), to allow seed sowing and growth of *P. vulgaris*enhanced with *M. symbioticum* and *X. autotrophicus* that since The interior of the roots of *P. vulgaris* converted organic compounds from root metabolism into phytohormones that increased the mineral uptake capacity and the mineralization of aromatic hydrocarbons from the WMO (Ruikar & Pawar, 2022; Tonelli et al., 2022), to reduce the WMO concentration to a value below the maximum established by NOM-138-SEMARNAT/SSA1-2012.

Conclusion

The results of this research support that an agricultural soil contaminated by 39,000 ppm of WMO can be bioremediated in less than period of 60 days by biostimulation with Triton X-100 and Tween 80 to reduce WMO concentration and to allow phytoremediation with *P. vulgaris*enhancedwith *M. symbioticum* and *X. autotrophicus* for relative rapidsoil fertility recovering.

Institutionalreviewboardstatement

Comment [s8]: No such sweeping statement unless backed up by relevant data

This study was conducted in accordance with the guidelines of the Declaration of Helsinki and was approved by the ethics committee of the university.

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