



Bioremediation and Accelerated Phytoremediation of a Soil Impacted by a Hydrocarbons Mixture

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Waste motor oil (WMO), without adequate final confinement, is spill in agricultural soil, there, 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 of agricultural soil impacted 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 maximum accepted by NOM-138-SEMARNAT/SSA1-2021. The response variables were: a) the initial and

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final concentration of WMO by Soxhlet, and b) *P. vulgaris*: germination percentage; phenology and biomass at seedling and pre-flowering stages. The experimental data were analyzed by ANOVA/Tukey 0.05%, with the program Statgraphics Centurion XVI. II.

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 the maximum, accepted by NOM-138-SEMARNAT/SSA1-2012. This supports that is possible to accelerate biostimulation and phytoremediation reduced WMO in the agriculture soil to recover its productive capacity.

Keywords: Soil, hydrocarbons; emulsification; oxidation; legume; endophytic plant growth promoting bacteria.

1. INTRODUCTION

Automotive oil for lubrication and cooling of automobiles, is replaced at the end of its useful life. That, generates waste motor oil (WMO): a mixture of aliphatic and aromatic hydrocarbons [1]. In Mexico, WMO is classified as toxic waste, to avoid environmental damage. 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 spill into the agriculture soil during its preparation. Besides that, 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 [2,3], since WMO is toxic for all living forms related with organic matter mineralization related to soil's fertility [4,5]. An alternative ecological solution is bioremediation via biostimulation initiated with detergents that effectively emulsify the WMO [6,7,8], followed by biostimulation with a mineral solution, that balances the concentration of essential minerals, in the agriculture soil, to induce accelerated mineralization of WMO [4], for subsequent sowing a legume tolerant to WMO phytotoxicity such as *Phaseolus vulgaris* [9,10,11,12,13], inoculated with hydrocarbon phytodegradation capacity, is enhanced with *Methylobacterium symbioticum* and *Xanthobacter autotrophicus*, genera and species, of plant growth-promoting endophytic bacteria, that are able to oxidize aromatic hydrocarbons, from the WMO [11,5]. Based on the above, the aims of this research were: i) biostimulation of 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 maximum accepted by NOM-138-SEMARNAT/SSA1-2021.

2. 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 19° 39' 27", north latitude 100° 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, according is showing in Table 1, at the end of this period of time, the concentration of WMO, was determined by the Soxhlet method, as a single response variable. In the second phase, that is showing in Table 2, for agriculture soil phytoremediation impacted by WMO remaining, from biostimulation was performed. To do this, *P. vulgaris* seed, were disinfected with sodium hypochlorite (Clorox ^{MR}) 0.6%/2.5 min, rinsed 6 times with sterile water, disinfected 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.*

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 (+); not added (-)

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

<i>Phaseolus vulgaris</i> in agriculture soil*	<i>Methylobacterium symbioticum</i>	<i>Xanthobacter autotrophicus</i>	Water	Mineral solution (%)
Relative control	-	-	-	100
Negative control	-	-	+	-
Treatment 1	+	-	-	50
Treatment 2	-	+	-	50
Treatment 3	+	+	-	50

*(n)= number of repetitions = 6; added(+); not added (-)

autotrophicus and mixture in relation 1:1 (v/v) with a cell concentration equivalent to 1.5×10^8 CFU/mL by viable plate count (VPC) in nutrient agar, and 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.

3. RESULTS AND DISCUSSION

In Table 3, it is shown that, the initial biostimulation by: 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 a negative control (NC). These results support that 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 [6], then for biostimulation by mineral solution, induced the mineralization of the WMO by native aerobic heterotrophic microorganisms of this environment [9,5].

In Table 4, the germination percentage of *P. vulgaris* enhanced with *M. symbioticum* and *X. autotrophicus* fed with 50% mineral solution in soil polluted by 26,990 ppm of WMO remaining from biostimulation, with 83.33% germination, statistically different numerical value compared to 56.67% of *P. vulgaris* sown in the same soil not polluted by WMO, fed with 100% mineral solution or relative control (RC); and with 50% germination of *P. vulgaris* in the same soil impacted by 38,204 ppm of WMO used as a 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 phytohormones that induced a rapid and better of germination [14,11,15]. When both *M. symbioticum* and *X. autotrophicus*, were able to use, as a source of carbon and hydrocarbons energy of WMO, thereby reducing the phytotoxicity of WMO [2,10,16], that inhibit the germination of seeds, as showed in fig. 1 [17].

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 agriculture soil, impacted by 26,990 ppm of WMO, 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*, that registered: 4.12 g of AFW and RFW 1.58 g, 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 polluted by WMO used as relative control (RC). An increasing in 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* [18,19,20], as shown in the enhanced *P. vulgaris* response with *M. symbioticum* and *X. autotrophicus* in (Fig. 2).

Table 3. Concentration of waste motor oil in agriculture soil remaining, after 20 days of biostimulation

Agriculture soil impacted by 39,000 ppm of waste motor oil (WMO)*	Final concentration
Soil polluted by WMO irrigated only with water or negative control	38,204 ppm**
Soil biostimulated by WMO biostimulated by Triton X-100 and Tween 80 at 0.5% plus mineral solution at 50%	26,990 ^b

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

Table 4. Percentage of germination of *Phaseolus vulgaris* with *Methylobacterium symbioticum* and *Xanthobacter autotrophicus* in impacted soil by 26,990 ppm of WMO remaining from biostimulation

<i>Phaseolus vulgaris</i> *	Germination percentage (%)
Uninoculated, in unpolluted soil fed with 100% mineral solution or relative control (RC)	56.67 ^{d**}
Uninoculated in soil impacted by 38,204 ppm of WMO irrigated only with water or negative control (NC)	50.00 ^e
Enhanced with <i>M. symbioticum</i> in soil polluted by WMO remained from biostimulation ((Treatment 1)	63.33 ^c
Enhanced with <i>X. autotrophicus</i> in soil by WMO remaining from biostimulation (Treatment 2)	66.67 ^b
Enhanced with <i>M. symbioticum</i> and <i>X. autotrophicus</i> in soil polluted by WMO remaining from biostimulation (Treatment 3)	83.33 ^a

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



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

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

<i>Phaseolus vulgaris</i> †:	Plant height (cm)	Radical length (cm)	Fresh weight (g)		Dry weight (g)	
			Aerial	Radical	Aerial	Radical
Uninoculated, in soil unpolluted by waste motor oil fed with 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 irrigated only water	23.65 ^d	11.15 ^{c*}	1.63 ^{c*}	0.61 ^{c*}	0.23 ^d	0.06 ^c
Enhanced with <i>M. symbioticum</i> in soil polluted by WMO remaining from biostimulation	37.50 ^b	21.80 ^{ab}	3.70 ^{ab}	1.13 ^b	1.93 ^b	0.31 ^b
Enhanced with <i>X. autotrophicus</i> in soil polluted by WMO remaining from biostimulation	31.38 ^c	21.65 ^{ab}	3.18 ^b	1.35 ^{ab}	1.12 ^c	0.14 ^{bc}
Enhanced with <i>M. symbioticum</i> and <i>X. autotrophicus</i> in soil polluted 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%.

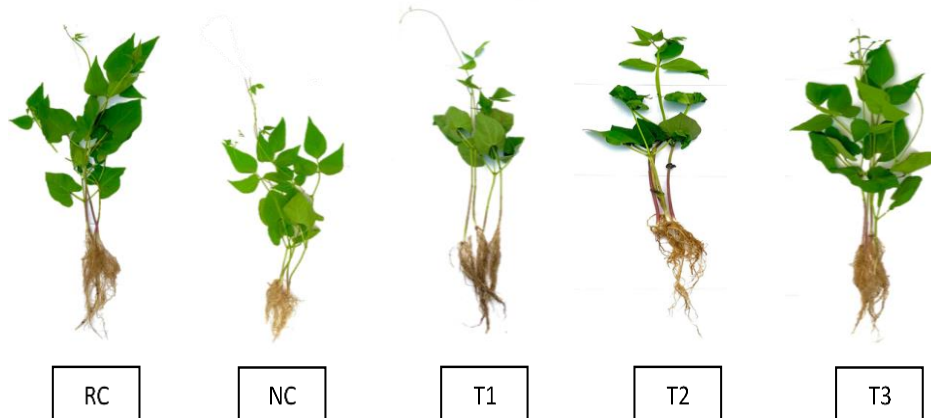


Fig. 2. *Phaseolus vulgaris* phenology enhanced with *Methylobacterium symbioticum* and *Xanthobacter autotrophicus* to seedling during phytoremediation of soil polluted by 26,990 ppm of waste motor oil after 50 days

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*, irrigated with water sown, in soil polluted by 38,204 ppm WMO or (negative control), T1 (treatment 1): *P. vulgaris*, with *X. autotrophicus* fed, with 50% mineral solution, sown in soil polluted by WMO, remaining from biostimulation. T2 (treatment 2): *P. vulgaris* with *M. symbioticum*, fed with 50% mineral solution, sown in soil polluted, by WMO remaining from biostimulation. T3 (treatment 3): *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 shows the phenology and pre-flowering biomass, of *P. vulgaris* enhanced, with *M. symbioticum* with statistically, different numerical values, compared to 58.46 cm PH and 33.44 cm

RL of *P. vulgaris* uninoculated in agriculture soil, unpolluted by WMO. In the biomass, *P. vulgaris* improved with *M. symbioticum*. Compared to *P. vulgaris* uninoculated fed with mineral solution

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

<i>Phaseolus vulgaris</i> *:	Plant height (cm)	Radical length (cm)	Fresh weight (g)		Dry weight (g)	
			Aerial	Radical	Aerial	Radical
Uninoculated in soil unpolluted fed with mineral solution at 100%	58.46 ^{c**}	33.44 ^b	5.34 ^b	2.55 ^b	2.76 ^b	1.18 ^b
Uninoculated in soil polluted 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 <i>M. symbioticum</i> 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
Enhanced with <i>X. autotrophicus</i> in soil polluted by WMO remaining from biostimulation	85.14 ^b	39.42 ^a	6.33 ^a	4.65 ^a	2.89 ^a	2.27 ^a
Enhanced with <i>M. symbioticum</i> and <i>X. autotrophicus</i> 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%.

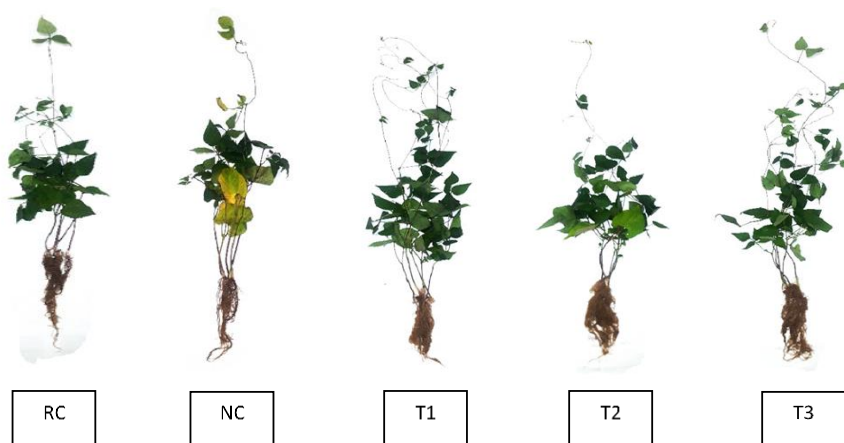


Fig. 3. Phenology of *Phaseolus vulgaris* enhanced with *Methylobacterium symbioticum* and *Xanthobacter autotrophicus* at pre-flowering in the phytoremediation of soil polluted by 3000 ppm of waste motor oil (WMO)

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 (treatment 1): *P. vulgaris* with *X. autotrophicus* fed with 50% mineral solution in soil polluted by WMO remaining from biostimulation. T2(treatment 2): *P. vulgaris* with *M. symbioticum* fed with 50% mineral solution in soil polluted by WMO remaining from biostimulation. T3(treatment 3): *P. vulgaris* with *M. symbioticum* and *X. autotrophicus* fed with 50% mineral solution in soil polluted by WMO remaining from biostimulation.

at 100%, sown in the soil not polluted by WMO used as a RC, shown in fig. 2. In terms of phenology and biomass of *P. vulgaris*, was enhanced due to *M. symbioticum* and *X.*

autotrophicus, which supports that, when both endophytic plant growth promoting bacteria, were able to colonizing the interior of the roots of *P. vulgaris*, to use compounds of the radical

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

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

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

metabolism, for transforming them into phytohormones, that induced a dense root system, then due to biostimulation with the mineral solution, *P. vulgaris* achieved a maximum mineral uptake and at the same time to mineralize aromatic hydrocarbons remaining from the WMO, that allowed it, to achieve the same root growth as was observed of *P. vulgaris* sown in unimpacted soil by WMO [21,22].

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 lower concentration than the maximum limit accepted by NOM-138-SEMARNAT/SSA1-2012. This numerical value, was statistically different compared to 34,949 ppm, in the same 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 [4], to allow seed sowing and growth of *P. vulgaris*, enhanced with *M. symbioticum* and *X. autotrophicus*, due that at interior of the roots of *P. vulgaris*, both endophytic plant growth promoting bacteria, were able to converted organic compounds from root metabolism into phytohormones, that increased the mineral uptake capacity, and the mineralization of aromatic hydrocarbons of WMO [23,5,17], to reduce the WMO concentration to a

value below the maximum established by NOM-138-SEMARNAT/SSA1-2012 to biorecover for agriculture aims [24-26].

4. CONCLUSION

The results of this research support, that an agricultural soil polluted 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*, enhanced with *M. symbioticum* and *X. autotrophicus*, for relative rapid soil fertility recovering.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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