Sodium N-methyldithiocarbamate impact on soil bacterial diversity in greenhouse tomato (*Solanum lycopersicum* L.) crop

Impacto de metam sodio en la diversidad bacteriana de un suelo cultivado con tomate (*Solanum lycopersicum* L.) en invernadero

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Abstract

The constant use of sodium N-methyldithiocarbamate (metam sodium: MS) in protected agriculture in México has attracted the attention of researchers and producers on their effects on the environment. The objective of this study was to evaluate the impact of MS on the bacterial community structure in an agricultural soil with tomato crop (*Solanum lycopersicum* L.) considering the different phenological stages of the crop. The experiment was carried out in a greenhouse, with a completely randomized block design with two treatments: 1) without MS and 2) with application of 400 L·ha^{-1} of MS. For the determination of the bacterial structure, the biodiversity indexes of richness (S), diversity (H') and equity (J'), identification of operational taxonomic units (OTU) were used through the T-RFLP technique. Application of MS in soil showed no significant effect on bacterial richness. However, the application of MS does alter the structure of the bacterial community (H' and J') in each of the tomato phenological stages. Finally, future studies which include the evaluation of the effects of MS on the physiology of intensive crops and functions in the different soil types are need.

Keywords
biodiversity indexes • soil • T- RFLP • bacterial community

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Resumen

El uso constante del N-metil ditiocarbamato de sodio (metam sodio: MS) en la agricultura protegida en México ha atraído la atención de investigadores y productores sobre sus efectos en el medio ambiente. El objetivo de este estudio fue evaluar el impacto del MS en la estructura de la comunidad bacteriana en un suelo agrícola con cultivo de tomate (*Solanum lycopersicum* L.) considerando las diferentes etapas fenológicas del cultivo. El experimento se llevó a cabo en un invernadero, con un diseño de bloques completamente al azar y dos tratamientos: 1) sin MS y 2) con aplicación de 400 $L \cdot ha^{-1}$ de MS. Para la determinación de la estructura bacteriana, se utilizaron los índices de biodiversidad de riqueza (S), diversidad (H') y equidad (J'), identificación de unidades taxonómicas operacionales (UTO) mediante la técnica T-RFLP. La aplicación de MS en el suelo no mostró un efecto significativo sobre la riqueza bacteriana. Sin embargo, la aplicación de MS altera la estructura de la comunidad bacteriana (H' y J') en cada una de las etapas fenológicas del tomate. Finalmente, se necesitan estudios futuros que incluyan la evaluación de los efectos del MS sobre la fisiología de los cultivos intensivos y las funciones en los diferentes tipos de suelos.

Palabras clave
índices de biodiversidad • suelo • T-RFLP • comunidad bacteriana

Introduction

Sodium N-methyldithiocarbamate (metam sodium: MS) is a disinfectant for farming soil which belongs to the thiocarbamates group. MS is applied to the tomato crops in greenhouse conditions. The use of MS in Mexico has increased due to the expansion of protected agricultural surface. The current surface of this system is over 23000 hectares (28). Greenhouse tomato growth is done through intensive monocultivation, which is what places most of the application of MS during the beginning of every agricultural year, in order to prevent any fungal diseases caused by *Fusarium* spp., *Phytophthora* spp., *Pyhlum* spp., *Rhizoctonia* spp., *Verticillium* spp., and *Sclerotinia* spp., among other species (10, 17). These applications affect the native populations of microbes and the non-target populations of the soil (9). The diversity of the bacteria populations in the soil, according to the impact of the MS, can be estimated through the biodiversity indexes, richness (S), diversity (H') and equity (J') (28), obtained from the operational taxonomical units (OTU), utilizing assigned determined phylogenetic through a molecular technique of T-RFLP (Terminal restriction fragment length polymorphism) (30), this technical molecular has advantage with independent culture methods. Where less than 1% of soil microorganisms can be grown (31). When considering the limitations of microcosmic experiments in the laboratory, it is suggested (21) that the composition and behavior of the microbes that inhabit the soil after fumigation should be evaluated more accurately by field trials. The objective was to evaluate the impact of MS application on the soil microbial diversity at different tomato phenological stages in a greenhouse cropping system.

Materials and methods

This study was carried out at the Faculty of Agronomy and Veterinary of the Autonomous University of San Luis Potosi, Mexico (22°13'48" N and 100°51'35" W, 1834 m a. s. l.). Soil characteristics were: silt 20%, sand 61%, clay 19%, organic matter 0.15%, interchangeable potassium 1.2 $C^{+}mol \cdot kg^{-1}$, extractable phosphorous 6 $mg \cdot kg^{-1}$, inorganic nitrogen 10 $mg \cdot kg^{-1}$, pH 8.17, electrical conductivity 0.86 $dS \cdot m^{-1}$ (23). Soil moisture at the time of the MS application was at holding capacity.

The soil was prepared a week previous to applying the MS with two steps of dredge in addition at a dose equivalent to 4 $t \cdot ha^{-1}$ of poultry manure of the brand Vertia® (20). The treatments involved in the study were: 1) without MS application and 2) with a single application of 400 $L \cdot ha^{-1}$ of MS. The MS was applied manually according the farmer practice.

The experimental design was performed by blocks at random with three replicates and the experimental units were plots of 2.2 m^2. Twenty-two days after applying of MS, the seedlings of Hannibal tomato variety (Harris Moran, USA) were transplanted. Mineral fertilizers were applied along the crop cycle (table 1) (5).

Samples were taken at four soil sampling dates: 1) 15 days before MS application (0 day); 2) 22 days after MS application (at transplanting); 3) 40 days after MS application (at flowering); and 4) 70 days after MS application (fructification).

The soil sampling procedure consisted on taking three samples from each plot at a depth of 0-25 cm (30 g per sample) and by mixing them, a compound sample was created for each plot.

Table 1. Tomato crop fertilization chronogram.

Tabla 1. Cronograma de fertilización del cultivo de tomate.

Fertilizer	8 a 25 DAT	26 a 45 DAT Kg $ha^{-1}day^{-1}$	46 a 70 DAT
N	1.0	2.0	6.0
P_2O_5	1.0	1.5	1.5
K_2O	2.0	4.0	10.0
Ca	1.8	3.0	3.5
Mg	0.6	1.0	2.0

DNA extraction - T- RFLP Analyses

The total DNA was extracted from samples composed of 10 g of soil with the DNA Power Soil kit (MoBio, Carlsbad, California, USA) following the manufacturer's instructions. The genomic DNA extracted was purified by the Clean DNA and Concentrator kit (Zymo Research, Irvine, California, USA), following the manufacturer's instructions.

The total DNA extracted from the soil samples was used as a mold for the amplification through PCR from a fragment of 1.5 kb of the ribosomal DNA region 16S, with a pair of universal bacterial (F27, 5'-AGA GTT TGA TCM TGG CTC AG- 3' y R1492, 5' TAC GGY TAC CTT GTT ACG ACT-3') (17), were digested with the endonuclease restriction *NdeI* (thermos Fisher Scientific).

The size of the final restriction fragments (T-RF) was determined in an automatized capillary sequencer ABI 3130 (Applied Biosystems, Foster City, CA) generating the electropherograms, with an internal size pattern of BTO 550 (Qiagen, USA), within a range of 50 to 550 pairs of bases (pb) (3).

The genetic profile was expressed in terms of the peak intensity and size of the T-RF, and was analyzed with the Genemapper software V3.7 (Applied Biosystems. Inc., USA) with a peak detection hight of 50 fluorescent units, Assignment Tool (PAT) (15). Found in the Microbial Community Analysis webpage (MICA). Considering as a basis the Silva data (R106) 16/18S rRNA (22). To compensate for differences in the PCR product quantity and T-RFLP profile intensity among samples, the peak relative heig of heach sample (OTU) divided by the sum of all peak heights from the corresponding sample (29).

Diversity indices calculations

The microbial genetic diversity was estimated by mean of three indices, which reflex the bacterial structure:

Richness (S) represented by the number of bacteria species. Was calculated by the presence or absence of T-RF band electropherrograms present in the samples, represented in operational taxonomical units (OTU) (30). An OTU is a group of phylogenetically related organisms without specifying a taxonomical range (24).

Shannon index (H'), which represents the level of bacteria population diversity in the soil, was calculated as:

$$H' = -\Sigma(p_i)\ (\ln p_i)$$

where:

p_i = relative abundance of each OTU in relation to the total population, which was in turn calculated based on the peak area of each T-RF divided by the sum of the total areas of T-RF in the corresponding samples (9).

The H' values range from 0 to 5, which are generally between 1.5 y 3.5, it is highly uncommon that they exceed 4.5. If H' presents a value of 0, then it was have one OTU present, which is interpreted as low biodiversity (24).

Evenness index (J'), which represents the distribution of the abundance of the distinct OTUs in the soil, was calculated as J' = H'/ ln S. The values of J' vary from 0 al 1 (maximum value) (4).

Biodiversity indices were evaluated ($p \leq 0.05$), the richness data were logarithmically transformed (13), then ANOVA ($p \leq 0.05$) was performed to compare treatments, phenological stages and principal components analysis (PCA), using SAS program (version 9.0, USA).

Results and discussion

The genetic profile can be seen in terms of the intensity and size of the T-RF, which is the sequential longitude of the bases of paired units (50-500 pb) (8), just as indicated by the electropherograms for each of the treatments (figure 1, page 337). Each T-RF comes from a particular sequence of 16S rDNA, to which each T-RF is assumed as operational taxonomical unit (OTU) (28).

The results of the richness (S) in the flowering stage (40 days) a reduction in S is observed with respect to the previous stage, to increase again in the fructification stage, in both treatments. The richness was not affected by the MS application within the each phenological stage ($p \leq 0.05$) (table 2, page 337).

The bacterian richness of the soil (number of different species), cultivated with tomato in intense conditions was able to overcome the initial disturbance caused by the application of the MS. This phenomenon demonstrates an elevated soil resilience, *i.e.* the capacity of a community to try and recover its original state before a disturbance (2).

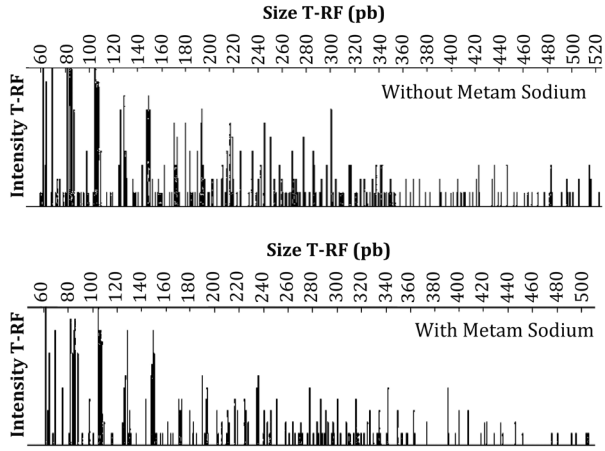


Figure 1. Soil bacterial genetic profile obtained by molecular technique T-RF (size and intensity) in without MS and with MS treatments, considering all sampling dates (0, 22, 40 and 70 days after transplanting). MS: sodium N-methyldithiocarbamate.

Figura 1. Perfil genético bacteriano obtenido por la técnica molecular T-RF (tamaño e intensidad) de suelo sin tratamiento con MS y suelo tratado con MS, al considerar todas las etapas del experimento (0, 22, 40 y 70 días después del transplante). MS: N-metil ditiocarbamato de sodio.

Table 2. Richness (S), Shannon (H') and Shannon evenness (J') indices (mean ± SD) for with MS and without MS treatments in each tomato phenological stage. MS: sodium N-methyldithiocarbamate.

Tabla 2. Índices de riqueza (S), Shannon (H') y Shannon evenness (J') (media ± DE) para los tratamientos sin MS y con MS en cada etapa fenológica del tomate. MS: N-metil ditiocarbamato de sodio.

Sampling	Richness		Shannon (H')		Shannon evennes (J')	
	without MS mean ±SD	**with MS** mean ±SD	**without MS** mean ±SD	**with MS** mean ±SD	**without MS** mean ±SD	**with MS** mean ±SD
0 Days	3.4 ± 0.42	-	2.92 ± 0.067	-	0.919 ± 0.047	-
22 Days (vegetative growth)	3.7 ± 0.30a	4.1 ± 0.16	3.7 ± 0.329b	3.74 ± 0.08a	0.887 ± 0.052b	0.911 ± 0.041a
40 Days (flowering)	2.7 ± 0.20a	2.3 ± 0.23	2.97 ± 0.071a	2.52 ± 0.212b	0.904 ± 0.007b	0.982 ± 0.011a
70 Days (fructification)	3.7 ± 0.41a	3.2 ± 0.44	3.46 ± 0.079a	3.82 ± 0.357b	0.890 ± 0.013a	0.851 ± 0.020b

Diverse causes could have contributed to maintaining the bacterian richness similar to farming soil after applying MS along the passing of time: a) Fertilization; the elevated nutrient levels applied to the tomato crop in an intensive system (1 to 6 kg $N \cdot ha^{-1} \cdot day^{-1}$, table 1, page 335). such nutrients could have been utilized by the bacterian community (12, 32); b) Root functions (14), which release chemical compounds in a secretion such as sugar, amino acids, flavonoids, proteins and so on throughout the different tomato crop phenological stages (6); c) The accelerated decomposition of the MS, even after one single fumigation increases decomposing bacteria in the soil (10); d) The application of a low dosis (400 $L \cdot ha^{-1}$), which according to the product data sheet, can be applied up to 1200 $L \cdot ha^{-1}$ (Buckman Lab., USA).

The result of a disturbance, such as fumigation, generates high reproduction rates of surviving bacteria (25). Contrarily to the richness (S), the Shannon (H') and the evenness (J') indices, showed a significant change between treatments in each vegetative stage ($p \leq 0.05$), (table 2), thus also the vegetative stages show significant differences with the application of the MS ($p \leq 0.05$), (figure 2, page 339). These results show changes in the structure of the community caused by the applying chemical fertilizers and pesticides (MS) to the soil, which provoke modifications in the nutrient contents, organic carbon in the soil, pH, and humidity amongst others (25). This could explain the results obtained where significant changes can be seen in the diversity and evenness of the OTU.

There were other two studies where there was also a change in diversity and evenness after fumigation (19, 32). Future studies must demonstrate if the changes of diversity found in this research (a fluctuation of H' between 10 and 18%; in J' they were of 3 and 8%), representing an effectuation over the crop soil sustainability in an intensive system (greenhouses).

Relative abundance in Operational Taxonomical Units (OTUs)

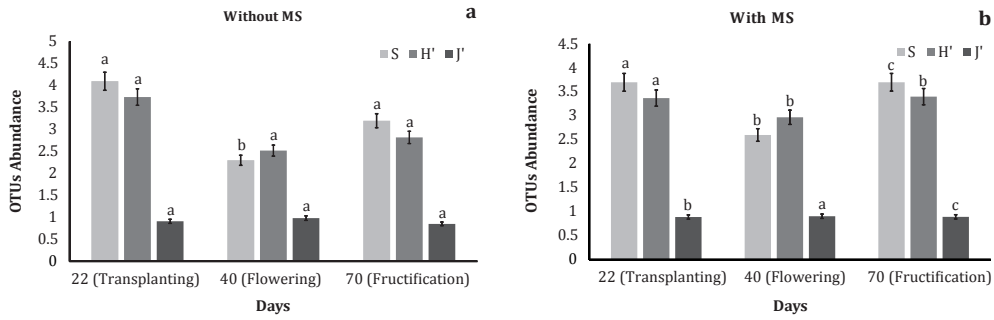
The Phylogenetic assigning of the OTU, was in this case, coincidentally carried out by the same MICA data base (27). There were eight phylogenetic groups of bacteria adjusted to the T- RFLP profiles generated by this research.

The behavior of each group with relative abundance bases of the bacterian OTU showing changes in the different phenological stages, is illustrated in figure 3 (page 339): a-*Proteobacteria*. d-*Proteobacteria*, g-*Proteobacteria*, *Firmicutes*, *Cyanobacteria* and *Terrabacteria* were conspicuous in the treatment without MS.

And for treatment with MS: *Proteobacteria*, a-*Proteobacteria*, b-*Proteobacteria*, d-*Proteobacteria*, g-*Proteobacteria*, *Firmicutes*, *Cyanobacteria* and *Terrabacteria*. The *phylas* show changes in the relative abundance percentages of the operational taxonomical units (OTU) throughout the tomato crop phenological stages evidentiating changes in the bacterian communities for both treatments (without MS *vs* with MS), and were coincidental in the H' indicator (diversity).

The structural changes in the community were marked by the *phyla* a-*Proteobacteria*, g-*Proteobacteria*, *Firmicutes* and *Agrobacterium*, which were found within the most abundant in farming soils, during the whole evolutionary stages and are particularly known for their potentialities to promote plant growth (7).

From this *in situ* composition of the identified bacterian community, the most abundant *phyla proteobacteria,* which is a diversely metabolic group of four *subphylas* (α-, β-. γ- y δ-), was commonly reported in the soil (1).



Different letters indicate statistical differences ($p \leq 0.05$) between treatments.

Letras diferentes indicant diferencias estadísticas ($p \leq 0,05$) entre tratamientos.

Figure 2. Comparison between the phenological stages of the tomato crop with the biodiversity indices of richness (S), diversity (H') and equity (J') for without MS (a) and with MS (b) treatments. MS: sodium N-methyldithiocarbamate.

Figura 2. Comparación entre las etapas fenológicas del cultivo de tomate con los índices de biodiversidad de riqueza (S), diversidad (H') y equidad (J') para tratamientos sin MS (a) y con MS (b). MS: N-metil ditiocarbamato de sodio.

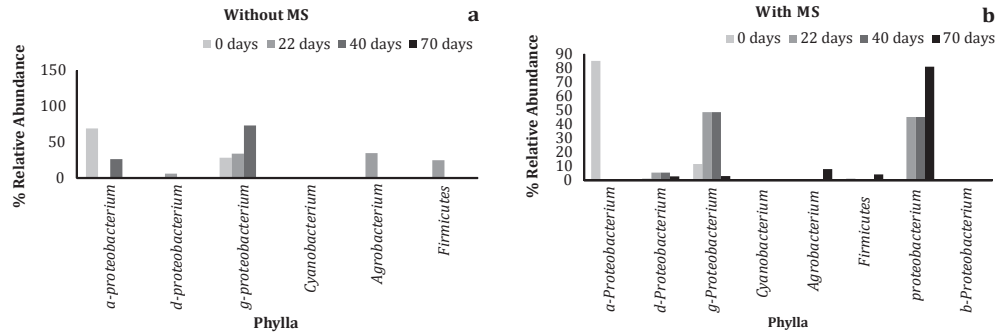


Figure 3. *Phylla* (phylogenetic assignment) *vs* % Relative abundance OTUs for treatments without MS (a) and with MS (b). MS: sodium N-methyldithiocarbamate. Within each sampling date.

Figura 3. Phylla (asignación filogenética) *versus* % Abundancia relativa UTOs. para tratamientos sin MS (a) y con MS (b). MS: N-metil ditiocarbamato de sodio. Dentro de cada fecha de muestreo.

Proteobacterias represent more than 40% of all the publicly validated prokariot genre and exhibit an extreme metabolic diversity (16).

Given the phylogenetic results and relative abundance, there is a possibility for an initial bacterian succession which usually occurs in disturbed ecological systems in as much as by natural as for anthropogenic causes (25). However, this must be corroborated in later studies with longer evaluation periods.

Principal components analysis (PCA)

The treatment without MS, the principal component analysis (PCA1), explained 26% of the relative abundance corresponding to 0 day. While, the PCA2 which corresponds to the transplanting stage explains 47.7% of the accumulated; the PCA3 the 60.54% of the variables corresponding to the flowering stage, and the PCA4 within the fructification stage explained 72.6%. An interaction rate of 56% between the OTUs and the phenological stages were detected.

In the with MS treatments, the PCA1 explained 24.45%, identified at 0 days; the PCA2 the 43.8% at the transplanting stage; PCA3 the 58% at the flowering; and the PCA4 corresponding to the fructification stage demonstrated a total accumulated in relative abundances of 70%.

Grouped OTU's for both treatment (without MS and with MS) at 0 days were identified by observing the highest relative abundance, considering that perhaps no disturbance had been caused by the MS application to the soil. All of the phenological stages have an accumulated of 72% in MS treatment and of 70% in without MS, indicating difference in the microbial community structures, which is in agreement with the results of H' and the OTUs relative abundances (table 2, page 337 and figure 4, respectively).

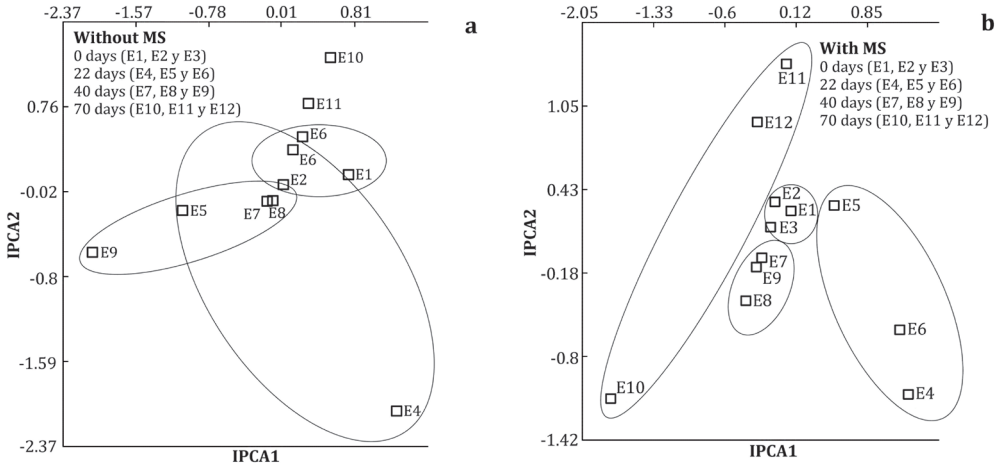


Figure 4. Principal component analysis in without MS (a) and with MS (b) treatments, discriminated by each sampling date. MS: sodium N-methyldithiocarbamate. E: replicates in each sampling date.

Figura 4. Análisis de componentes principales en tratamientos sin MS (a) y con MS (b), discriminados por cada fecha de muestreo. MS: N-metil ditiocarbamato de sodio. E: réplicas en cada fecha de muestreo.

Conclusion

The fact of that MS application modifies the biodiversity index (H' and J') of the soil bacterial community in all tomato phenological stages suggests that the use of this product in terms of dosage and frequency of application needs more attention. Moreover, future studies that consider the crop physiology and the effects on the basic soil functions are need.

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