

Mycoremediation of an Agricultural Salty Soil Contaminated With Endosulfan by *Penicillium Crustosum*

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Abstract

This is the first report about the application of *Penicillium crustosum* isolated from *Citrus sinensis* peel for endosulfan biodegradation and improvement of soil characteristics of a salty agricultural soil contaminated with endosulfan; getting a degradation of $93 \pm 4.7\%$, after one month of treatment. Metabolites production was also evaluated, finding that sulphate endosulfan and mono alcohol endosulfan were the main compounds produced for that reason an oxidative pathway is suggest. Degradation was enhanced by the biological surface active agent produced by the fungus during soil mycoremediation, where a DST up to $17 \pm 0.58 \text{ mN m}^{-1}$, was determined, being the first time that the *in situ* production for this kind of application was studies. Additionally, an improvement in soil quality (reduction of clay and salinity, and an increase of soluble phosphorus, carbon content and organic matter) was observed during the mycoremediation process. The phytotoxicity of the pesticide on *Phaseolus leptostachyus* crop was evaluated; in the soil without the fungus the pesticide translocated into the crop, presenting a negative effect in germination index, root length and weight, aerial weight and proline content. This contrasted with the crop develop in the soil treated with *P. crustosum*, which had better agronomic characteristics. In general, was possible appreciated that the addition of *P. crustosum*, present a lot of benefits for agricultural applications.

Introduction

Worldwide, there is an average per capita of bean (*Phaseolus* genus) consumption equivalent to 2.5 kg per person per year; for that reason, around 33.8 million hectares of this crop are cultivated (FIRA, 2019). México became one of the six countries with the highest production of this legume in 2017, behind India, Myanmar, Brazil, the United States and China.

Therefore, in 2019, beans become the third most planted vegetable at a national level, occupying 1,411,550 planted ha, of which 5,077 belonged to the agricultural zone of Mexico City (SIAP, 2019). The agricultural zone of Mexico City is integrated by Xochimilco, Tláhuac and Milpa Alta, which is performed in floating gardens called 'chinampas', considered a Natural and Cultural World Heritage Area Site (FAO 2017).

However, the soils of this zone present high salinity and low fertility. For these reasons it has low crops productivity, having in 2018, 1 693 ha harvested, which represents only 17% of the total area (SIAP 2018). Agrochemicals and untreated dung have been added to provide a solution, but it has led to an over-fertilisation and an increase of salinity. An alternative to improve the soil quality could be through the addition of agro-industrial wastes, which can be used as nutrient sources and as a support for microbial growth with the capacity to remove the agrochemicals and excess salt (Barahona et al. 2007).

In 2018, 8.13 million tonnes of citrus fruit were produced in Mexico, representing a similar amount of wastes, where typical disposal is by composting. Citrus waste has been used as a support for microbial growth, with the capacity of removing cyanide from water treatment plant sludges (Ntwampe and Santos

2013) and hydrocarbons degradation (Roldán-Martín et al. 2006), as well as providing a nutrient source for surface active agents production by microorganisms (George and Jayachandran 2009).

Penicillium crustosum can grow on this kind of waste. This fungus is known to usually produce penitrem, which is a neurotoxin for some mammals, including dogs (Mantle et al. 1984), but there are no reports of this fungus being used for biotransformation of organochlorine pesticides (OCPs). These hydrophobic compounds have been restricted or removed worldwide, particularly endosulfan, which has been restricted in Europe because it is an acute neurotoxin for insects and mammals, including humans (Arellano-Aguilar and Rendón-Von Osten 2016). Endosulfan is classified by the World Health Organization (WHO) as moderately toxic and by the American Conference of Governmental Industrial Hygienists (ACGIH) as A4 (not carcinogenic to humans). Unfortunately, it does not have a classification for the IARC, due to the lack of data on its carcinogenic potential (National Institute of Health 2015).

This paper shows the contribution of *Penicillium crustosum* isolated from *Citrus sinensis* peel (CsP) for endosulfan biodegradation in a highly saline agricultural soil quantified the main compound and its metabolites showing the degradation pathway and the benefit of bioremediation process in the development of a model crop (*Phaseolus leptostachyus*) evaluating its phytotoxicity by morphological test and the compounds translocated into the crop.

Materials And Methods

2.1 Isolation and identification of the fungus The microorganism used in this study was obtained from *Citrus sinensis* peel and was identified based on the frequency of the *16S-ITS1-5.8S-ITS2-26S* ribosomal segment, amplifying the oligonucleotides *ITS4* and *ITS5* to obtain two sequences. These were edited in the Chromas 2.4 program and compared to the BLAST nucleotide in the National Center for Biotechnological Information (NCBI). Sequences were aligned to those obtained in the BLAST search with a Clustal W program to perform a dendrogram by the neighbour-joining method.

2.2 Culture medium The 250 mL Erlenmeyer flasks contained the following modified Wunder medium (Luna-Velasco et al. 2007): Glucose 9.5, NH_4NO_3 1, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.5, MnSO_4 0.03, KH_2PO_4 , 0.875, K_2HPO_4 0.125, CaCl_2 0.1, NaCl 0.1, FeSO_4 0.01 g L^{-1} . All chemicals were ACS grade and purchased from Sigma-Aldrich, México. The fungus *Penicillium crustosum* was grown for 5 days at 30°C and 120 rpm, and pH 5 buffered with 10% tartaric acid. The pellets were filtered through a Whatman 540 filter paper and used for biodegradation assays.

2.3 Physicochemical characterisation of the soil A saline agricultural soil from the 'Chinampas' of Xochimilco zone located in Mexico City (19° 16' 24.9" N, 99° 05' 29.9" W) was used in all treatments.

The physicochemical characterisation of soil was performed according to the Mexican Standard Norm NOM-021-SEMARNAT-2000. A 1:5 dilution (soil: deionised water) was prepared to measure the pH and conductivity of soil with a multiparameter HANNA meter (Model HI9828).

The carbon and organic matter content were analysed by calcination of soil at 350°C for 8 hours in a Sola Basic flask. Nitrogen (%) content was determined by the micro-Kjeldahl method in a FOSS (Model TECATOR 20) digester and a BÜCHI distiller (Model K-314). Soluble phosphorus (%) was analysed by the Olsen spectrophotometric method, using a Shimadzu spectrophotometer (Model UV-1800). The texture was determined by the Bouyoucos method with a hydrometer (Boyoucos 1936).

2.4 Mycoremediation assessment Endosulfan degradation was performed in 125 mL serologic bottles with 10 g of soil, then incubated at environmental conditions, at a moisture content of 80%, with daily aeration of 130 mL min⁻¹ for 5 min over 2 months. Two systems were considered; the fungal bioremediation treatment (FBR) with one mg kg⁻¹ of endosulfan and 1% (w w⁻¹) of *P. crustosum* inoculum with the native microorganisms and one mg kg⁻¹ of endosulfan. Sampling was performed on the initial day and then at 7, 14, 30 and 60 days later.

Endosulfan and its metabolites (endosulfan a and b, endosulfan sulphate, mono alcohol, lactone and ether) were extracted according to EPA 3546 method in a CEM MARS-Xpress microwave. Then, quantification was done in a Gas Chromatography (GC) Varian (Model CP-3380) with a power source of 63Ni, a Varian capillary column (15 m long, 0.25 mm internal diameter and 0.25 mm cap thickness, CP-Sil 5CB) and nitrogen was used as a carrier gas at a 1 mL min⁻¹ flow rate. The GC temperature conditions were: injection 200°C and detector 300°C, and then the oven was programmed increasing from 100 to 176°C at 4 °C min⁻¹, from 176 to 192 at 2 °C min⁻¹ and from 192 to 230°C at 4 °C min⁻¹.

2.5 Evaluation of the production of biological surface active agents The production of biological surface active agents was evaluated by the change in surface tension (DST) and emulsifying activity (E₂₄) on a crude extract of soil.

On days 7, 14, 30 and 60; 10 g of soil were taken out and dissolved in deionised water in an orbital shaker at 180 rpm for 30 minutes, and further filtered to obtain the crude extract.

The crude extract of day 14 was purified by a modified method (Pornsunthorntawee et al. 2008), acidified with 6 M HCl until pH was decreased to 2, and it was left at 4 °C for 18 h for stabilisation. Afterwards, an equal amount of 0.1 M NaHCO₃ was added to the extract, followed by extraction with a chloroform:ethanol solution (2:1). The aqueous phase of the extraction was then placed in an evaporator (at 60 °C to eliminate the solvent) and the resulting liquid was used to evaluate the surface active properties. All the solvents employed were ACS grade and purchased from JT Baker.

The surface tension of the extract was measured in a Krüss tensiometer (Model K6) and E₂₄ was evaluated with diesel, according to Pruthi and Cameotra, 1997.

2.6 Phytotoxicity study Commercial seeds (La Merced, México) of 'Bayo' beans (*Phaseolus leptostachyus*), were employed for this assay.

Seeds were germinated in three different treatments: the 1st one was the saline agricultural soil of the 'Chinampa' (control); the 2nd treatment was the control soil amended with one mg kg⁻¹ of endosulfan (endosulfan treatment) and the 3rd one was the soil treated with *P. crustosum* (section 2.4) (FBR treatment).

All assays were performed in a greenhouse, at an environmental temperature of 32–35°C and relative humidity of 80–85% during the day for one month. The plants were grown in 30 cm tall pots with 50 grams of soil where a seed was added to each one. Plants were watered three times a week with 20 mL of water.

After a month, the physiological parameters (root and aerial lengths, number of leaves and biomass) were measured, and the germination index was calculated by dividing the number of germinated seeds over the number of those cultivated.

A signal molecule of stress conditions (proline) was evaluated, to determine the effect of endosulfan and salinity on the crop. Proline content was assayed with dry material according to Bates et al. (1973). Extracts were done in 3% aqueous sulfosalicylic acid and centrifuged at 13 000 rpm for 15 min. Then, the supernatant was mixed with ninhydrin and acetic acid, incubated for 1 h at 95°C, cooled on ice and extracted with toluene. After centrifugation, the absorbance of the organic phase was read at 520 nm using toluene as a blank. Proline (2 mM) was used as a standard.

In addition, endosulfan translocation was measured in roots, stems, and leaves for each assay. The endosulfan extraction was the same as used in section 2.4 for soil, using the EPA 3546 method in a CEM MARS-Xpress microwave and quantification with GC Varian (Model CP-3380).

2.7 Statistical analyses The SAS software was used for the multiple comparison of means ($p < 0.05$), including their standard deviation. Every soil experiment had 3 replicates and every crop had 5 replicates.

Results And Discussion

3.1 Identification of the fungus The microorganism isolated from the *Citrus sinensis* peel was identified as *Penicillium crustosum*, based on the frequency of segment *16S-ITS1-5.8S-ITS2-26S* with a 99% similarity with *Penicillium crustosum* (Fig. 1). The nucleotide sequence was deposited in the GenBank under registration number MG009431.

This fungus is known for its development in foods, such as vegetables, meats, cheese and fruits, among others (Rundberget et al. 2004). It produces metabolites as penitrems, roquefortine and viridicatin (Sonjak et al. 2005). However, this specie has not been used for OCPs biodegradation, nor applied to improve soil quality.

3.2 Endosulfan biodegradation Endosulfan degradation was determined after one month of treatment with *Penicillium crustosum* (FBR) and the native microorganism of the soil (control) (Fig. 2). It is showed

a higher (27%) endosulfan degradation in the FBR than with the native microorganisms, as well as 55% less endosulfan sulphate (more toxic metabolite).

The median lethal dose (LD_{50}) is an important parameter to know the toxicity of a compound. For endosulfan a, the value is 14 mg kg^{-1} , and for b, it is 17 mg kg^{-1} . Meanwhile, for endosulfan sulphate, it is 8 mg kg^{-1} , which is half of the original substance, for this reason, it is important to quantify it. In addition, endosulfan sulphate could remain for more time in the environment (up to 392 days) (Betancur et al. 2015).

Due to a higher concentration of metabolites of the oxidative pathway (endosulfan sulphate and mono alcohol), this degradation pattern is suggested for both treatments (Thangadurai and Suresh 2014). Comparing the rate obtained in this work with another fungus, it was found that *Aspergillus sydoni* could degrade 90% of endosulfan in 60 days with an initial concentration of 100 mg mL^{-1} of the pesticide, one-tenth of the present work, and they also found 8% more endosulfan sulphate in their bioremediation treatment (Goswami et al. 2009)

With the *Penicillium* genus, endosulfan degradation has been worked in a liquid medium having a rate of 94.87% at 6 days (Romero-Aguilar et al. 2014); however, is important to notice that the transport phenomena are different in liquids than in soil, and for that reason, the removal could be in less time.

It's important to highlight that this is the first report about the use of *P. crustosum* for biodegradation in a saline soil having a higher transformation rate.

3.3 Biological surface active agent property evaluation During the mycoremediation process, the production of biological surface active agents (BSAA) was found, quantified by the change in surface tension (DST) and emulsifying activity (E_{24}) characteristic of them. The production of BSAA could be associated with the improved bioavailability of the hydrophobic compound during the biodegradation process (Odukkathil and Vasudevan 2013).

Table 1 shows the values of DST and E_{24} in the FBR treatment every 7 days for one month. The highest activity was found on the fourteenth day, for that reason this product was purified, and a higher decrease in DST was obtained ($16.67 \pm 0.58 \text{ mN m}^{-1}$). The control treatment was also quantified but no activity was presented.

There are some reports about the use of BSAA to enhance endosulfan biodegradation (Odukkathil and Vasudevan 2013; Mani et al. 2011; García-Reyes et al. 2017); however, these works are in a liquid media and the BSAA are added to the media to improve the degradation rate. In addition, there are some reports of the use of BSAA in OCPs degradation on soils (Manickam et al. 2012; Awasthi et al. 1999), however, in these works, the BSAA are added to the treatment; so, we can conclude that this is the first report about BSAA *in situ* by *P. crustosum* production for a remove of an hydrophobic compound like the endosulfan.

3.4 Soil quality improvement The physicochemical characterisation of soil showed improvement after 1 month of bioremediation (Table 2). There was a decrease up to 20 units in salinity, as well as an increase in the concentrations of phosphorus (41 mg kg⁻¹), carbon and organic matter (23% and 40%, respectively). In addition, there was a decrease in the percentage of clay (32%); all these parameters represent an improvement in soil fertility and permeability.

This result could be associated with the phosphate solubilisation previously reported for the *Penicillium* genus. When the phosphorus content is increased, the clay content is reduced. Also, the formation of organic acids may be acting as chelating agents or sequestrants of Al⁺³, Ca⁺², Fe⁺³, Mg⁺², Na⁺ and K⁺, among others, leaving phosphorus complexes free to be assimilated (Romero-Fernández et al. 2019). The increase in sand provides greater permeability and better nutrient transport, which may be related to the release of sulphur compounds, favouring the exchange of sodium for calcium, improving physicochemical properties of the soil and taking advantage of the sodium salts that could be causing salinity (Zúñiga-Escobar et al. 2011). This is the first report where it was possible to appreciate an improvement in physicochemical properties of the soil, while an OCPs is removed.

3.5 Endosulfan translocation in *Phaseolus leptostachyus* crops Endosulfan translocation was evaluated in the crops develop on the soil with endosulfan and in the FBR treatment (Fig. 3).

The crop in the soil with endosulfan present secondary metabolites of oxidative and hydroxidative pathways, mainly sulphate endosulfan was the one most abundant (0.57 mg kg⁻¹) in the stem. This part of the crop is associated with the transport of nutrients, and for that reason, a higher value of endosulfan sulphate in this part could be associated to the physiological damage and death induction (Pfender, 2009). On the other hand, the highest concentration of endosulfan a was found in leaves (0.53 and 0.18 mg kg⁻¹, respectively) in the control and FBR treatment, which could be related to the volatilization of this compound as a defence mechanism of the plant (Mitton et al. 2014). The FBR presented a minimal or null production of the secondary metabolites, which could be due to low stress and, therefore, better growth of the crop.

3.6 Phytotoxicity in *Phaseolus leptostachyus* seeds The morphological parameters of *Phaseolus leptostachyus* were evaluated in the control, control soil amended with endosulfan and in the bioremediated soil with *P. crustosum*.

Table 3 shows 13.5 % more aerial part length in the FBR treatment, which could be associated with two conditions; the first one is related to the improvement of the available phosphorous in the soil, which was three times higher than in controls (section 3.4). Phosphorus plays an essential role in the development of the crops; it is related to photosynthesis and the structure of the plant at the cellular level, in addition to faster growth (Richardson et al. 2009).

The second condition for a better crop development is associated with endophytes characteristic of the *Penicillium* genus, which is related to a higher supply of nutrients into the crop (Elias et al. 2016). Elsharkawy et al. (2012) and Hossain et al. (2007) reported an increase in the biomass of *Arabidopsis*

thaliana, *Nicotiana tabacum* and *N. benthamiana* treated with *P. simplicissimum*; this increase could be associated to the increase in accessibility of minerals to the crops and the hormones produced by the fungus that stimulate growth.

The statistical analysis of phytotoxicity assays showed a significant difference ($\alpha < 0.05$) in most of the parameters evaluated in *Phaseolus* crops, mainly between the FBR and the control without the fungus. This behaviour could be explained by the high toxicity of the pesticide or the endosulfan sulphate metabolite, which were detected in a relatively higher amount in the stem (section 3.5) of the endosulfan treatment.

Plants development in the endosulfan treatment shows a lower germination index, root and aerial weight, and root length, as well as higher proline levels. However, in this treatment, the aerial part length and the number of leaves does not have a negative effect, which may be related to a defence mechanism of the vegetable to translocate the xenobiotic, as was discussed in section 3.5.

Proline is a signal molecule that increases under stress conditions; higher values of this molecule were found in the control and endosulfan treatments, which could be associated with the oxidation of the crop by the pesticide and the abiotic stress for the salinity of the soil. The function of proline is to balance the water deficit and acts as a carbon and nitrogen source in plants (Hoai et al. 2003). Previous works of *Phaseolus* genera under saline conditions demonstrate that salinity is an abiotic stress to the plants and for that reason tend to increase the proline as a self-regulation mechanism (Al Hassan et al., 2016).

Finally, the lower value of proline and the benefits to the morphological parameters of *P. leptostachyus* crops in the FBR treatment with *P. crustosum* demonstrate that at the same time than a xenobiotic like endosulfan in bio transformed the soil properties improve, which brings a better crop development.

Conclusions

This is the first report about the use of *Penicillium crustosum* fungus for endosulfan biodegradation and the *in situ* production of a BSAA to improve the biodegradation process, at the same time that the pesticide was removed an improvement on a saline soil quality was appreciated and a better develop in a model crop (*Phaseolus leptostachyus*).

Bioremediation of the soil with the fungus decreased the salinity, the clay portion and increased phosphorus, carbon and organic matter. The *Phaseolus leptostachyus* grown in the treated soil with the fungus presented a higher germination index, root and aerial weight and root length as well as lower proline accumulation in contrast with the control and endosulfan treatment, thus demonstrating the benefits of the use of *P. crustosum* for an agricultural applications.

Abbreviations

OCPs: Organochlorine pesticides; FBR: Fungal bioremediation treatment; DST: Change in surface tension; E₂₄: Emulsifying activity; BSAA: Biological surface active agents.

Declarations

Ethics approval and consent to participate

Not applicable.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Experimental part: Landa-Faz A. Writing and result discussion: Landa-Faz A., Rodríguez-Vázquez R., Roldán-Carillo T. Supervision and sources: Rodríguez-Vázquez R.

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Tables

Table 1 Evaluation of surface active agents properties in FBR extracts

Day	ST (mN m ⁻¹)	E ₂₄ (%)
7	68 ± 0.47	9 ± 0.16
14	64 ± 0.47	17 ± 0
21	70 ± 0	0
30	67 ± 0.47	0

Values shown are the means (n=3) with standard deviation; ST= Surface Tension; E₂₄: Emulsifying activity

Table 2 Physicochemical characterization of soil according to Mexican Norm NMX-021.

Characteristics	Control	FBR
pH	7.4 ± 0.8 ^b	6.8 ± 0.6 ^a
Conductivity (dS m ⁻¹)	27 ± 2.4 ^b	8 ± 1.2^a
Humidity (%)	91 ± 5.43 ^b	86 ± 0.75 ^a
C organic (%)	12 ± 0.15 ^a	23 ± 0.83^b
Organic matter (%)	22 ± 0.25 ^a	40 ± 1.43^b
Total nitrogen (%)	0.8 ± 0.06 ^b	0.4 ± 0.02 ^a
Phosphorus (mg kg ⁻¹)	11 ± 0.53 ^a	41 ± 2.1^b
Sand (%)	33 ± 2.3 ^a	37 ± 1.5 ^b
Clay (%)	36 ± 0.3 ^b	32 ± 0.7 ^a
Silt (%)	31 ± 2 ^a	31 ± 1.2 ^a

Sampling zone at 15 cm depth. Values shown are the means (n=3) with standard deviation of every parameter. Same letters indicate there are no significant statistical difference with LSD test ($\alpha = 0.05$). FBR = Fungal bioremediation treatment.

Table 3 Phytotoxicity in germinated *Phaseolus leptostachyus* seeds

Parameter	Control	Endosulfan	FBR
Germination index (%)	87 ± 12.5 ^b	67 ± 4.30 ^a	93 ± 11.46 ^b
Root length (cm)	7.4 ± 1.27 ^b	5 ± 0.16 ^a	6.9 ± 1.56 ^b
Aerial length (cm)	18.6 ± 2.97 ^a	19.4 ± 1.49 ^{ab}	21.5 ± 2.04 ^b
Number of leaves	8 ± 2 ^a	10 ± 1 ^b	8 ± 3 ^a
Root dry weight (g)	0.5 ± 0.15 ^b	0.3 ± .007 ^a	0.5 ± 0.15 ^b
Aerial dry part weight (g)	1.8 ± 0.56 ^b	1.5 ± 0.47 ^a	1.8 ± 0.47 ^b
Proline (µmol DW ⁻¹)	15 ± 0.64 ^b	19 ± 0.2 ^c	6 ± 0.44 ^a

Plants were development for 1 month in a greenhouse average Temperature = 25 °C, Moisture content = 46 % . Values shown are the means (n=5) with standard deviation of every parameter. Same letters

indicate there are no significant statistical difference with LSD test ($\alpha = 0.05$). FBR = Fungal bioremediation treatment.

Figures

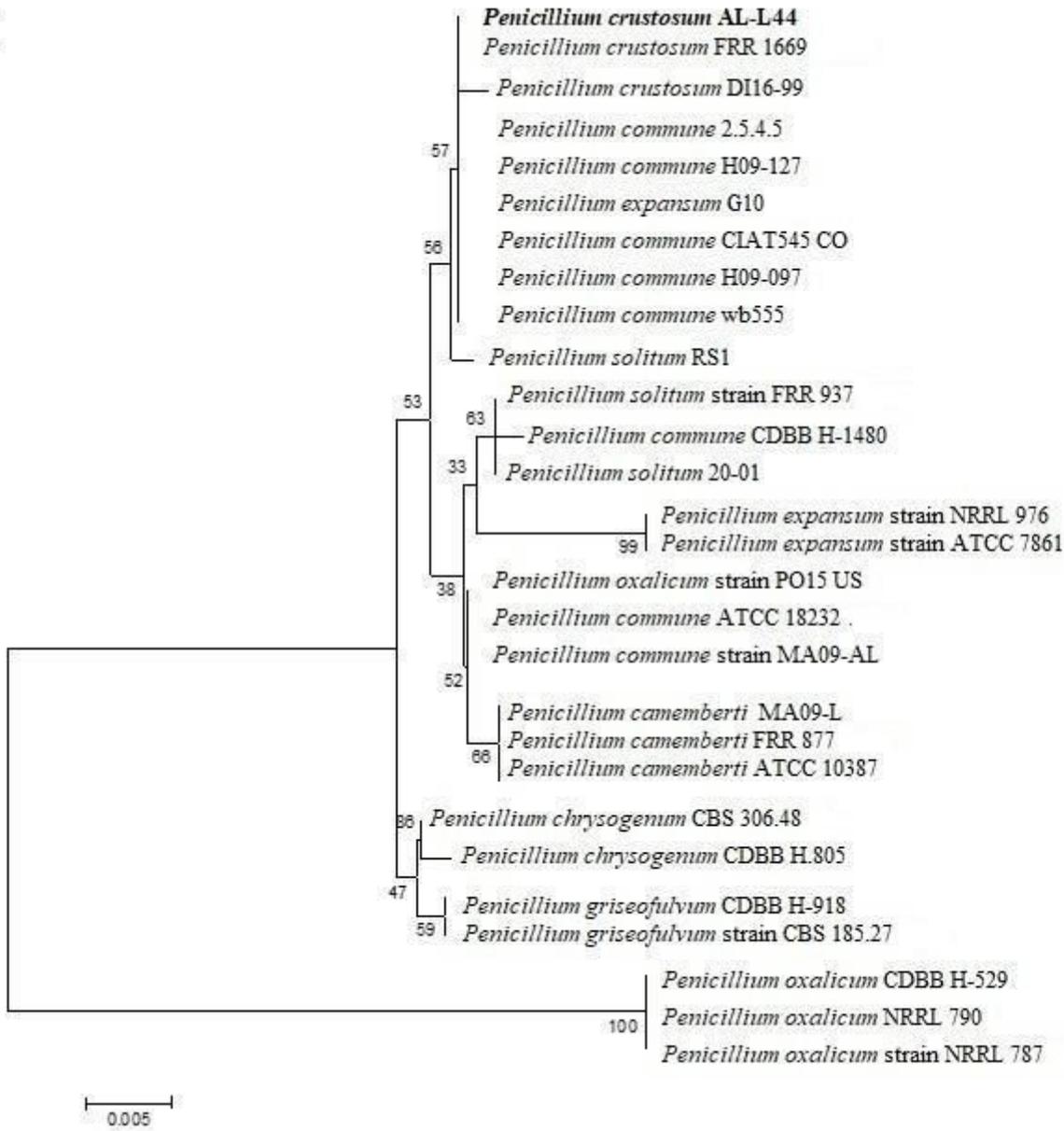


Figure 1

Phylogenetic dendrogram obtain with Clustal W program of the isolated microorganism from *Citrus sinensis* peel.

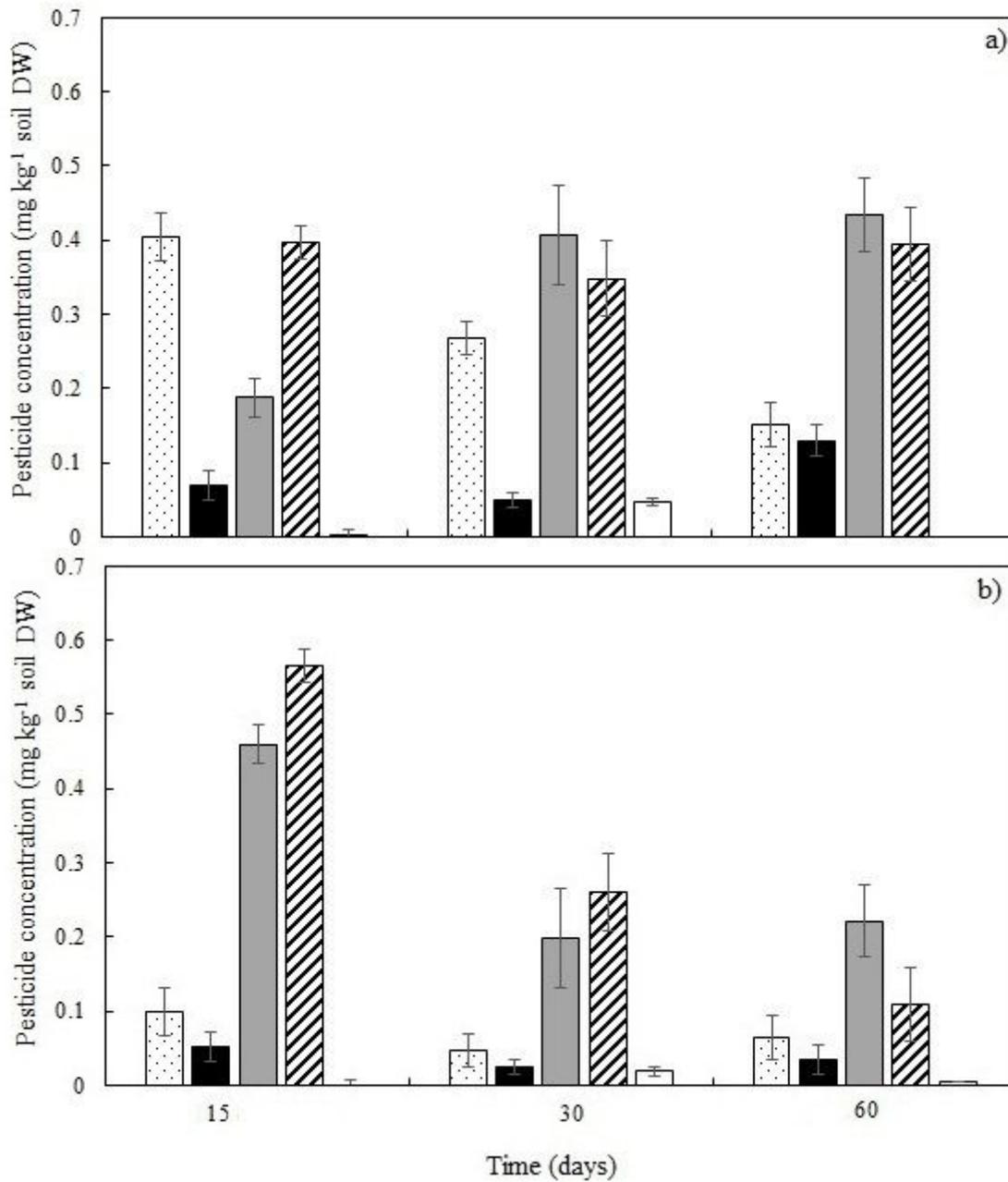


Figure 2

Concentration of endosulfan and its metabolites in soil treated at 15, 30, 60 days. a) Control (Native microorganisms) and b) *Penicillium crustosum* (FBR). Temperature = 25°C Moisture content = 65%. Endosulfan α ; Endosulfan and β ; Endosulfan sulphate ; Endosulfan mono alcohol ; Endosulfan ether . Bars represent means with SE (n=3).

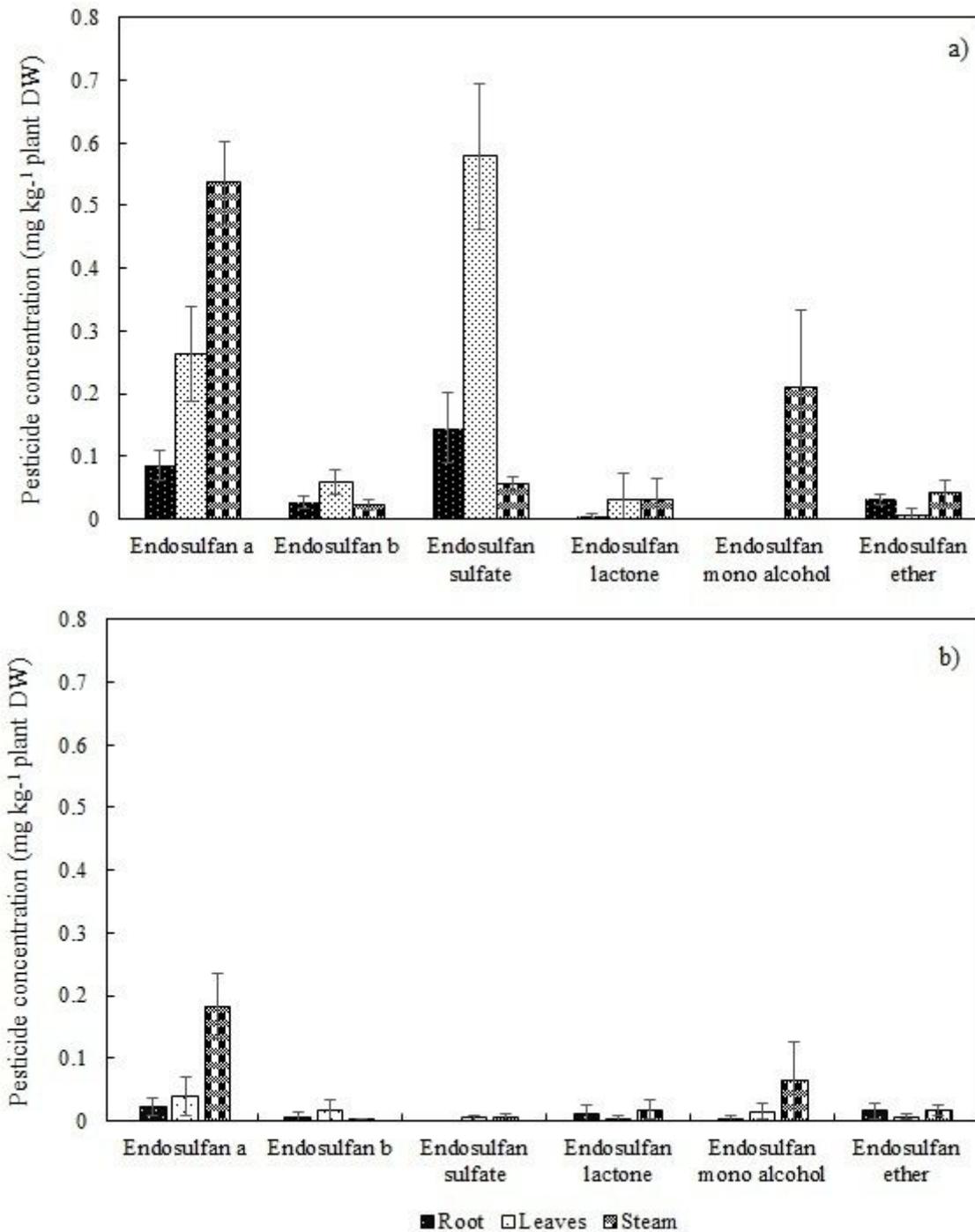


Figure 3

Concentration of endosulfan and its metabolites in *Phaseolus leptostachyus* development for 1 month in a greenhouse average Temperature = 25 °C, Moisture content = 46 % . a) Soil amended with endosulfan and b) Bioremediated soil with *Penicillium crustosum* (FBR). Root ; Stem ; Leaves . Bars represent means with SE (n=3).

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