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Effect of the Pesticide Endosulfan and Two Different Biostimulants on the Stress Responses of *Phaseolus leptostachyus* Plants Grown in a Saline Soil

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Abstract: Soil salinity and the indiscriminate use of agrochemicals has significantly reduced the productivity of the 'Chinampas' agroecosystem in Mexico City. Crop improvement under these stressful conditions may be achieved by soil bioremediation. In this study, we checked the effects of the organochlorine pesticide endosulfan and bioremediation with *Penicillium crustosum* or a citric waste on the growth of *Phaseolus leptostachyus* plants in saline soil from the Chinampas area. Biochemical markers associated with specific stress responses were also determined after one month of growth in the different substrates. Plant growth was stimulated by bioremediation of the soil. Both biostimulants reduced the degree of stress affecting the plants, as shown by the increase in photosynthetic pigments and the reduction of proline, malondialdehyde (MDA), and H_2O_2 contents, and the activation of antioxidant systems. However, the biostimulants appeared to mitigate oxidative stress through different mechanisms. Endosulfan contamination inhibited seed germination—which was reverted to control values in the presence of the biostimulants—and further decreased plant growth. No clear patterns of variation of biochemical stress markers were observed combining endosulfan and the biostimulants. In any case, bioremediation with *P. crustosum* and/or citric waste is recommended to improve the germination and growth of *P. leptostachyus* plants.

Keywords: chinampas agroecosystem; organochlorine pesticides; seed germination; salt stress; oxidative stress; oxidative stress markers; antioxidant systems; proline

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1. Introduction

Xochimilco is an area southeast of Mexico City, a remnant of an ancient wetland region consisting of five lakes that has suffered anthropogenic alterations over the last 2000 years. Pre-Columbian intensive agriculture was practiced in this area on narrow raised platforms surrounded by canals and ditches, known as 'chinampas', a term that also refers to this type of agriculture [1,2]. Chinampas soils are unique in the world, representing a cultural heritage from the pre-Hispanic period; they are still used as a distinctive agroecosystem, with high sustainability and supporting a large biodiversity. The Chinampas zone of Xochimilco allows a type of urban farming with great potential for a modern megalopolis, and still provides a source of income for a significant part of the Mexico City population [2]. The area has been used for horticulture for thousands of years, but it has experienced several environmental problems since the last century [2]. Due to overexploitation of water resources, the water level decreased. To mitigate this problem, since 1959, the water level has been raised using wastewater, which contains a high concentration of salts, toxic

metals, and endocrine disruptors such as organochlorinated pesticides [3,4]. These actions resulted in a sharp increase in water salinity in the canals, which was often reported to be around 0.5 g per liter [5]. The salinity of the soils varied considerably according to their location, but the concentration of salts in the upper layers during the dry season can reach values above 16 dS m^{-1}, indicating severe salinity, reaching in some cases values as high as 45–50 dS m^{-1} [6]. Salinity is already one of the major constraints for agriculture, and salinised areas are increasing continuously at a global scale [7,8]. Salinity is becoming more extensive because of land clearing, unsustainable irrigation practices, and bringing marginal land into production [9]. Because of this situation, only 1693 ha were harvested in 2018 in Xochimilco, representing 17% of the total cultivation area [10]. Plant growth on saline soils is directly affected by the osmotic stress and ion toxicity components of salt stress, to which most plant species, including all standard crops, are susceptible [11].

An additional environmental problem in the 'Chinampas' area is the use of pesticides, which increased steadily from the 1940s until the last decade of the 20th century [12]. The polluting effect of chemical pesticides is now well known; only a small fraction of the product applied reaches the pest, whereas the rest remains in the environment, contaminating soil, water, and biota [13]. One of the pesticides commonly used in the area is endosulfan, a broad-spectrum organochlorine insecticide, highly soluble in fats, with slow biodegradability and long persistence in the environment; in the soil, the half-life of endosulfan under aerobic conditions can be up to six years [14]. Endosulfan is transformed into more toxic and more bioaccumulative derivatives through biological oxidation and enzymatic oxidative processes [15]. For this reason, this pesticide has been banned in more than 50 countries, including the EU; in Central and South America, however, endosulfan is one of the most widely acquired pesticides and is still used in several countries, including Mexico [16]. Due to the long-lasting effects and slow biodegradability of organochlorine insecticides, environmental problems may remain several years after the ban. These compounds can cause serious health problems, including endocrine disruption, adverse effects on the nervous and reproductive systems, shortness of breath, decreased respiration, convulsions, physical deformities, mental disorders, abortions, cancer, and delayed sexual maturity [17,18]. One efficient way of solving this type of soil contamination could be based on bioremediation. This term is used for the process of cleaning up contaminated areas using biological organisms such as bacteria, molds and yeasts, algae, and some plants, in the presence of oxygen and nutrients to accelerate the natural biodegradation processes. Different fungi species can be included among the microorganisms used for bioremediation. The genus *Penicillium* stands out, being present in a wide range of habitats, soil and vegetation, indoor environments, and various foodstuffs. These are non-ligninolytic fungi, tolerant to pesticides and with the ability of biodegradation of such chemical compounds [19]. In the area of study, bioremediation with *Penicillium* has proved efficient to remove 4,4,-DDT (4,4′-Dichlorodiphenyltrichloroethane) and endosulfan sulphate in laboratory setups and in the field [12].

Phaseolus leptostachyus Bentham belongs to a small monophyletic group within its genus, characterised by a dysploid karyotype with 20 chromosomes, instead of the 22 chromosomes general in the genus *Phaseolus* [20], and distributed from Mexico to Costa Rica [21]. The species is a short-lived perennial with a tuberous root, surviving up to 4 years, reported to be tolerant to salinity [22], drought, and heat [21]. It can withstand moderate grazing or mowing for road maintenance, which often contributes to its persistence on roadsides, but usually grows in open natural areas such as slopes and landslides [21], and also in open forests [23]. It is an edible species, occasionally consumed and cultivated in Mexico [24]. Due to its relative stress tolerance, *P. leptostachyus* seems to be suitable for growth in areas with salinity problems, where more salt susceptible crops would not survive. Legume species can contribute to sustainable cropping systems by fixing nitrogen, increasing soil fertility, and supporting biodiversity [25].

Phaseolus leptostachyus has been losing agricultural importance in Mexico over the years; from 2016 to 2018, its production decreased, down to 0.5% of the total *Phaseolus* production [26], and now is not included in the national economic indicators.

Plants grown in saline soils activate stress response mechanisms, including growth inhibition, accumulation of specific osmolytes for osmotic adjustment, or activation of antioxidant systems to counteract the secondary oxidative stress associated with salinity [27,28]. Endosulfan, like other pesticides, represents an additional stress factor for crops, which, combined with increased salinity, may have a strong inhibitory effect on plant growth. To overcome these limiting factors, biostimulants can be used. The primary aim of this work was to assess the effects of this pesticide on the plants cultivated in the Chinampas zone of Mexico City and the possibility to remediate the contaminated soil with biostimulants. Therefore, it was evident that natural saline soil from this area should be used as the substrate, even though we were aware that it would not provide the optimal conditions for plant growth. Indeed, even low soil salinity levels (below 2 dS m^{-1}) can significantly reduce crop productivity [29]. At a salinity equivalent to 100 mM NaCl, pod yield per plant decreased by 85% in the common bean [30]. Therefore, the study was performed on the salt tolerant, related species *Phaseolus leptostachyus*, which is locally cultivated in this area. With this objective in mind, we have analysed the responses of plants grown for one month in saline soils sampled from the natural Chinampas area, combined with endosulfan, citrus waste, and *Penicillium crustosum* (as biostimulants), or a combination of the pesticide and the biostimulants. To get insights into the physiological and biochemical mechanisms underlying those responses, growth parameters were correlated with the levels of photosynthetic pigments, osmolytes (proline and total soluble sugars), oxidative stress markers [malendialdehyde, (MDA) and H_2O_2], and non-enzymatic (phenolic compounds) and enzymatic [superoxide dismutase (SOD), catalase (CAT), aspartate peroxidase (APX), and glutathione reductase (GR)] antioxidants.

2. Materials and Methods

2.1. Physicochemical Characterization of the Soil

A saline agricultural soil from the 'Chinampas' area of Xochimilco, Mexico City, with the following geolocation: N 19°16′24.9″, W 99°05′29.9″, was used as the substrate for the potted plants in the greenhouse. The soil was physico-chemically characterised, using five randomly collected samples, according to the Mexican standard norm NMX-021 [31]. Soil pH and conductivity were measured using a 1:5 dilution (soil: deionised water), and the extract was analysed with a multiparameter equipment HANNA, model HI 9828 (Hanna Instruments Mexico, Mexico City, Mexico). Carbon and organic matter content were determined by calcination of the soil at 350 °C for 8 h in Sola Basic flasks. The nitrogen content (%) was determined by the Microkjendalh method with a FOSS brand digester, model TECATOR 20 (FOSS Mexico, Mexico City, Mexico), and a BÜCHI distiller, model K-314 (Buchi AG, Flawil, Switzerland). Soluble phosphorus (%) was quantified according to Olsen methodology in a Shimadzu model UV-1800 spectrophotometer (Shimadzu, Kyoto, Japan). Soil texture was determined by the Bouyoucos method with a hydrometer (Kimble Chase Life Science, Rockwood, TN, USA) [32].

The electrical conductivity in the pot substrate was measured at the end of the treatments, when the biological material was sampled, in 1:5 (soil: water) suspension with a Crison 522 Conductivity-meter (Crison Instruments SA, Barcelona, Spain).

2.2. Plant Material and Treatments

Bayo beans (*Phaseolus leptostachyus*) brand 'La Merced', México, were cultivated in six types of substrates, all based on the saline soil collected in the field mentioned above: the saline soil without any treatment (Control, C); the saline soil with 1% of *Penicillium crustosum* (Control Pc, CPc), the saline soil with 1% of citric waste (Control CW, CCW), the saline soil with 1 mg kg^{-1} of endosulfan (CE), and two CE-bioremediated soils, one with the inoculum *P. crustosum* (EPc) and the second with citric waste (ECW). The bioremediation with EPC and ECW was performed by pre-treatment of the different substrates for one month, before sowing the bean seeds (data not shown).

The pesticide used in this study was commercial granulated endosulfan (Gowan Mexico, Mexicali, Mexico), diluted in hexane to prepare a stock solution with a concentration of 10 mg L^{-1}, which was added to the soil to have a final concentration of 1 mg kg^{-1}. Once the solution was added to the soil, it was stirred vigorously for 18 h to obtain a homogeneous distribution of the endosulfan. This concentration is above that measured in the soils of the study area (from 0.013 to 0.025 mg kg^{-1} [33]), but values up to 6.7 mg kg^{-1} have been reported in Mexico [34]; therefore, an intermediate concentration of the pesticide was chosen for the present study.

The citric waste, consisting of orange (*Citrus sinensis*) peels, was washed and the bagasse (fruit residue) was removed. Once cleaned, the peel waste was cut into 2 mm-disks, and incubated for five days at 30 °C, keeping the relative humidity at 80%. After this time, the formation of a green layer was appreciated (CW sample), from which the fungus *Penicillium crustosum* was isolated, as described [35]; *P. crustosum* was identified based on the sequence of the ribosomal RNA genes, which were submitted to GeneBank (accession number MG009431).

The microorganism *P. crustosum* was incubated for five days in a 500 mL Erlenmeyer flask containing 250 mL of Wunder mineral medium at pH 5.0 (buffered with 10% tartaric acid), shaken at 120 rpm and 30 °C. After this incubation period, the pellets were collected by filtration through a Whatman 540 filter, and the wet biomass was determined; finally, *P. crustosum* was added to the soils corresponding to Control PC and EPC substrates, at 1% (*w*/*w*) concentration. The soils corresponding to Control CW and ECW, contained 1% (*w*/*w*) of the CW sample.

The seeds were individually germinated and grown in small, 10 cm-high pots, with 20 g of soil each. The pots were incubated in a growth chamber with a photoperiod of 14 h of light, at 28 and 18 °C (day/night temperatures), and relative humidity between 60 and 65% during the day and 95–100% at night. Watering of the plants was performed twice a week with 10 mL of deionised water per pot for one month. Five plants were used as biological replicas per treatment.

2.3. Seed Germination and Plant Growth Parameters

The germination percentages of the seeds in the different substrates were recorded. One month after seedling emergence, the following growth parameters were measured for each plant: root length (RL), stem length (SL), and fresh weight of roots (RFW), stem (SFW) and leaves (LFW). A fraction of the leaf material was weighed (FW), dried at 65 °C for 72 h, and weighed again (dry weight, DW) to calculate the leaf water content percentage according to the formula: WC (%) = [(FW−DW)/FW] × 100. The remaining plant material was flash-frozen in liquid N_2 and stored at −75 °C for the biochemical analyses.

2.4. Quantification of Photosynthetic Pigments

Chlorophylls a (Chl a) and b (Chl b) and total carotenoids (Caro) contents were determined following the Lichtenthaler and Wellburn method [36], using fresh leaf material. The extraction was performed from 0.1 g of fresh leaves, ground in the presence of 30 mL of 80% (*v*/*v*) ice-cold acetone. The samples were mixed overnight in an orbital shaker and centrifuged at 13,300× *g* for 15 min at 4 °C. The absorbance of the supernatants was measured at 663, 646, and 470 nm with a UV-Vis spectrophotometer, VWR model UV 1600 PC (VWR lnternational Eurolab, Llinars del Vallès, Barcelona, Spain). The concentration of each compound was calculated according to the equations in the original protocol [36] and expressed as 'mg g^{-1} DW'. Additionally, the ratio Chl a/Chl b was calculated.

2.5. Osmolyte Quantification

Proline (Pro) content was determined using fresh leaf material, according to the ninhydrin-acetic acid method [37]. Extracts were prepared in 3% aqueous sulphosalicylic acid and centrifuged at 13,300× *g* for 15 min. The supernatant was mixed with ninhydrin and acetic acid, incubated for one h at 95 °C, cooled on ice and then extracted with toluene.

After centrifugation, the absorbance of the organic phase was read at 520 nm using toluene as the blank. Reaction mixtures containing known Pro concentrations were run in parallel to obtain a standard curve. Pro concentration was expressed as µmol g^{-1} DW.

Total soluble sugars (TSS) were extracted and quantified according to the classical method of Dubois et al. [38]. Leaf extracts, prepared in 80% (*v*/*v*) methanol, were shaken on a rocker shaker for 24 h and centrifuged at 13,300× *g* for 10 min. The supernatants were collected and mixed with concentrated H_2SO_4 and 5% phenol. The absorbance of the resulting solution was determined at 490 nm. TSS content was expressed as 'mg equivalent of glucose', used as the standard (mg eq. gluc g^{-1} DW).

2.6. Oxidative Stress Markers and Total Phenolic Compounds

Malondialdehyde (MDA) and hydrogen peroxide (H_2O_2) contents were measured in the plants to assess their level of oxidative stress under different experimental conditions. MDA concentration was determined according to Hodges et al. [39], with the modifications introduced by Taulavori et al. [40], using the same methanol extracts prepared for TSS determination. The extracts were mixed with a solution of 0.5% thiobarbituric acid (TBA) in 20% trichloroacetic acid (TCA) (or with 20% TCA without TBA, for the control), and the samples were incubated at 95 °C for 15 min, cooled on ice and centrifuged at 13,300× *g* for 10 min at 4 °C. The absorbance of the supernatants was measured at 532, 600, and 440 nm, and the MDA content was calculated with the equations previously described [40], based on the molar extinction coefficient at 532 nm of the MDA-TBA adduct (155 mM^{-1} cm^{-1}). MDA contents were expressed as 'nmol g^{-1} DW'.

H_2O_2 content was calculated according to Loreto and Velikova [41], with minor modifications. Extracts of fresh leaf material were prepared in 0.1% TCA and mixed with TRIS buffer and 1 M KI. Samples with known amounts of H_2O_2 were assayed in parallel to obtain a reference curve. The absorbance of the samples was measured at 390 nm, and H_2O_2 concentration was expressed as 'nmol g^{-1} DW'.

Total phenolic compounds (TPC), taken as representative non-enzymatic antioxidants, were quantified in 80% (*v*/*v*) methanol extracts according to Blainski et al. [42]. The extracts were mixed with the Folin-Ciocalteu reagent and Na_2CO_3; after incubation for 90 min at room temperature in the dark, the absorbance of the samples was measured at 765 nm. TPC contents were expressed as gallic acid equivalents (mg eq GA g^{-1} DW), used as the standard.

2.7. Antioxidant Enzyme Activities

Four antioxidant enzymes were evaluated: superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), and glutathione reductase (GR). The preparation of the protein extracts followed Gil et al. [43], and protein quantification in the crude extracts was according to Bradford [44] using the BioRad commercial reagent and bovine serum albumin (BSA) as the standard.

SOD activity was calculated according to Beyer and Fridovich [45] by measuring spectrophotometrically, at 560 nm, the inhibition of nitroblue tetrazolium (NBT) photoreduction in a reaction mixture containing riboflavin as the source of superoxide radicals. One SOD unit was defined as the amount of enzyme required to cause 50% inhibition of the NBT photoreduction under the assay conditions. CAT activity was determined according to Aebi [46], following the consumption of H_2O_2 added to the extract by the decrease in absorbance at 240 nm. One CAT unit was defined as the amount of enzyme necessary to decompose one mmol of hydrogen peroxide per minute at 25 °C, the assay temperature. APX activity was measured as described by Nakano and Asada [47], following, at 290 nm, the oxidation of ascorbate by the plant extract. One APX unit was defined as the amount of enzyme required to catalyse the consumption of one mmol of ascorbate per minute at 25 °C. GR activity was determined according to Connell and Mullet [48], following, at 340 nm, the oxidation of NADPH during the GR-catalysed reduction of oxidised glutathione (GSSG) to its reduced form (GSH). One GR unit was defined as the amount of enzyme to oxidise one mmol of NADPH per minute at 25 °C.

2.8. Statistical Analyses

The program IBM SPSS Statics 25 (IBM Armonk, NY, USA) was used for the statistical analysis of the experimental data. Normality of the data was checked by the Shapiro–Wilk test and the homogeneity of variance by the Levene's test. One-way ANOVAs were performed, at the 99% confidence level, independently for each experiment, using the Tukey HSD test to make post-hoc comparisons. All mean values mentioned in figures and tables include the standard deviation (SD). A principal component analysis (PCA), based on the mean values of all analyzed growth and biochemical parameters, was performed using the statistical package Stagraphics Centurion, v. XVIII (Statpoint Technologies, Warrenton, VA, USA).

3. Results

3.1. Physicochemical Characterisation of the Soil

Table 1 shows the physicochemical characterisation of the soil collected in the Chinampas area of Xochimilco, Mexico City, according to the NMX-021 norm. The soil has a clay-loam texture, with a high percentage of clay, which implies a low permeability that can favor pesticide accumulation. The soil is also characterised by high total nitrogen and organic matter contents. Nevertheless, the most relevant property, which can adversely affect plant growth, is that the soil is saline, with an electrical conductivity of more than 12 dS m^{-1}.

Table 1. Physicochemical characterisation of the soil collected in the Chinampas area of Xochimilco, Mexico City, according to the Mexican NMX-021 norm. Mean values ± SD (n = 5) are shown for each soil parameter.

Soil Characteristics	Value	Classification NMX-021
pH	7.35 ± 0.76	Neutral
EC (dS m^{-1})	12.8 ± 3.53	Saline soil
Moisture (%)	90.7 ± 5.43	N/A
C organic (%)	12.5 ± 0.15	N/A
Organic matter (%)	21.6 ± 0.25	Very high
Total nitrogen (%)	0.80 ± 0.06	Very high
Phosphorus (mg kg^{-1})	10.7 ± 0.53	High
Sand (%)	33.0 ± 2.28	N/A
Clay (%)	36.2 ± 0.28	N/A
Silt (%)	30.7 ± 2.00	N/A

N/A: not applicable.

Soil samples were also collected from the pot substrates, together with the plant material, at the end of the experiments; that is, after one month of growing the plants in the different tested substrates. The electrical conductivity data, shown in Table 2, indicate that the use of biostimulants had a statistically significant effect, reducing the salinity of the control soil. The same is true for the soil contaminated with endosulfan, which also showed a significantly reduced salinity with respect to the control; however, treatment with *P. crustosum* or the citric waste did not reduce further the electrical conductivity of the contaminated soil (Table 2).

Table 2. Electrical conductivity (EC) of the pot substrates after one month of plant growth. Soil sampled in the Chinampas area was used as control (C); soil contaminated with endosulfan (CE); soil bioremediated with *Penicillium crustosum* (CPc); endosulfan-contaminated soil, bioremediated with *P. crustosum* (EPc); soil bioremediated with citric waste (CCW); endosulfan-contaminated soil, bioremediated with citric waste (ECW). Values represent the means of five samples per treatment, followed by SD. Different letters indicate significant differences between treatments, according to the Tukey test, at the 99% confidence level.

Substrate Type	EC (dS m^{-1})
C	11.1 ± 2.88^{c}
CE	10.0 ± 3.40^{b}
CPc	9.23 ± 2.76^{a}
EPc	9.88 ± 3.29^{b}
CCW	9.35 ± 3.03^{a}
ECW	10.2 ± 3.38^{b}

3.2. Effect of the Treatments on Plant Growth

Phaseolus leptostachyus plants were subjected to six treatments, by growing them in the different types of soil defined above. Seed germination rates were determined and, one month after seedling emergence, the plants were harvested, and several growth parameters were measured (Table 3). Germination percentages were high and not significantly different in the control and the substrates treated with the biostimulants. Contamination of the soil with endosulfan had a negative effect, significantly reducing germination rates from more than 90% in the control to ca. 67%. Bioremediation of the contaminated soil with *P. crustosum* or citric waste restored the germination capacity of the seeds to control percentages. Considering that seed germination is generally the most stress-sensitive phase of the life cycle of plants, and the electrical conductivity of the substrates, these germination percentages close to 100%, show the high salt tolerance of *P. leptostachyus*.

Table 3. Effect of soil treatments on plant growth parameters. Soil sampled in the Chinampas area was used as control (C); soil contaminated with endosulfan (CE); control soil bioremediated with *Penicillium crustosum* (CPc); endosulfan-contaminated soil, bioremediated with *P. crustosum* (EPc); control soil bioremediated with citric waste (CCW); endosulfan-contaminated soil, bioremediated with citric waste (ECW). Growth parameters abbreviations: germination rate (GR); root length (RL); stem length (SL); root fresh weight (RFW); stem fresh weight (SFW); leaf fresh weight (LFW); leaf water content (LWC). Values represent the means of five replicas (five individual plants) followed by SD. Different letters in each row indicate significant differences between treatments, according to the Tukey test at the 99% confidence level.

Trait	C	CE	CPc	EPc	CCW	ECW
GR (%)	91.7 ± 15.1^{b}	66.7 ± 13.6^{a}	100.0 ± 0.0^{b}	100.0 ± 0.0^{b}	85.0 ± 24.1^{b}	91.7 ± 15.1^{b}
RL (cm)	12.4 ± 2.70^{c}	3.99 ± 0.90^{a}	10.8 ± 1.25^{c}	7.68 ± 1.70^{b}	7.94 ± 1.53^{b}	6.28 ± 1.34^{b}
SL (cm)	$36.0 \pm 10.5^{a,b}$	39.4 ± 6.29^{b}	$34.8 \pm 3.80^{a,b}$	30.8 ± 8.79^{a}	$35.9 \pm 4.81^{a,b}$	39.5 ± 7.98^{b}
RFW (g)	0.11 ± 0.02^{a}	0.14 ± 0.03^{a}	0.43 ± 0.15^{b}	0.12 ± 0.03^{a}	0.51 ± 0.17^{c}	0.14 ± 0.05^{a}
SFW (g)	$0.82 \pm 0.40^{a,b}$	0.94 ± 0.14^{b}	1.16 ± 0.38^{b}	$0.88 \pm 0.21^{a,b}$	1.07 ± 0.20^{b}	0.73 ± 0.22^{a}
LFW (g)	0.80 ± 0.28^{c}	0.50 ± 0.121^{b}	1.43 ± 0.36^{d}	0.38 ± 0.16^{a}	1.41 ± 0.33^{d}	0.50 ± 0.19^{b}
LWC (%)	88.2 ± 4.20^{b}	78.4 ± 3.60^{a}	89.5 ± 0.88^{b}	80.4 ± 3.85^{a}	88.4 ± 1.08^{b}	80.9 ± 3.14^{a}

Plant growth is generally inhibited under salt stress conditions; therefore, it could not be optimal in the saline soil used in our experiments. Still, a stronger negative effect was observed when the plant substrate had been treated with the pesticide, reflected mainly on a considerable reduction of the root length, down to 32% of the control, and a decrease of almost 40% in the leaf FW; endosulfan addition to the substrate also caused a slight, but statistically significant, reduction in leaf water content (Table 3).

The addition of the biostimulants stimulated plant growth in the absence of endosulfan; the most relevant parameters, the FW of roots, stems, and leaves, increased significantly, about 3.9-, 1.3-, and 1.8-fold, respectively, in the presence of *P. crustosum*, and 4.6-, 1.4-, and 1.8-fold in citric waste-treated soil. Contrary to their positive effect on seed germination, remediation with the biostimulants of endosulfan-contaminated soil did not show any substantial effect on plant growth, except for an increase in root length; however, root length did not reach the value of the control not treated with the pesticide. All other growth parameters did not change significantly, or even decreased slightly, by adding *P. crustosum* or the citric waste (Table 3).

3.3. Photosynthetic Pigments Contents

Interestingly, all treatments resulted in an increase of Chl a, Chl b, and Caro contents in the plant leaves, with respect to the control soil, with similar qualitative patterns of variation for the three pigments (Table 4). Thus, in soil treated with the biostimulants, foliar Chl a concentration increased about three-fold, Chl b contents 2.7-fold in the presence of *P. crustosum*, and 1.9-fold in the citric waste-remediated soil, whereas carotenoids levels increased ca. 2.3-fold for both biostimulants (Table 4). The increase of the means of pigments concentrations was less pronounced in endosulfan-contaminated soil, between 1.5- and 1.8-fold higher than in non-contaminated soil, but the differences with the corresponding controls were significant only for Chl a. Bioremediation with *P. crustosum* further increased pigment contents, reaching or even exceeding the values measured in non-contaminated soil supplemented with the fungus. On the contrary, bioremediation of endosulfan-contaminated soil with the citric waste did not have any additional effect on photosynthetic pigment levels. The effects of the treatments on the two chlorophylls, a and b, were similar since the ratios Chl a/Chl b did not vary significantly, except for the saline soil treated with citric waste, which increased compared to the non-bioremediated control (Table 4).

Table 4. Effect of soil treatments on photosynthetic pigments contents. Chl a: chlorophyll a, Chl b: chlorophyll b, Caro: total carotenoids. Treatments: Soil sampled in the Chinampas area was used as control (C); soil contaminated with endosulfan (CE); control soil bioremediated with *Penicillium crustosum* (CPc); endosulfan-contaminated soil, bioremediated with *P. crustosum* (EPc); control soil bioremediated with citric waste (CCW); endosulfan-contaminated soil, bioremediated with citric waste (ECW). Values represent the means of five replicas (five individual plants) followed by SD. Different letters in each column indicate significant differences between treatments, according to the Tukey test at the 99% confidence level.

Treatment	Chl a (mg g^{-1} DW)	Chl b (mg g^{-1} DW)	Ratio Chl a/Chl b	Caro (mg g^{-1} DW)
C	7.40 ± 2.36^{a}	$2.13 \pm 0.74^{a,b}$	3.65 ± 1.06^{a}	1.50 ± 0.26^{a}
CE	13.4 ± 2.05^{b}	$3.59 \pm 0.45^{a,b}$	3.71 ± 0.57^{a}	2.26 ± 0.57^{a}
CPc	22.7 ± 1.93^{c}	5.68 ± 0.59^{c}	$4.01 \pm 0.18^{a,b}$	3.45 ± 0.25^{b}
EPc	25.4 ± 1.99^{c}	6.73 ± 0.89^{c}	$3.83 \pm 0.61^{a,b}$	4.21 ± 0.29^{b}
CCW	22.0 ± 1.75^{c}	$4.09 \pm 0.88^{b,c}$	5.68 ± 1.67^{b}	3.32 ± 0.67^{b}
ECW	14.03 ± 1.90^{b}	$3.66 \pm 0.29^{a,b}$	3.82 ± 0.24^{a}	2.22 ± 0.26^{a}

3.4. Osmolyte Quantification

To compensate for the osmotic stress generated by salinity and other stress conditions, plants accumulate different compatible solutes or osmolytes to help cellular osmotic adjustments. Leaf concentrations of proline (Pro) and total soluble sugars (TSS), common plant osmolytes, were determined in plants grown in all selected substrates (Table 5). Pro concentrations decreased to 50–60% of the control in the presence of the biostimulants, whereas endosulfan alone or in combination with *P. crustosum* did not have any significant effect. On the other hand, plants grown in endosulfan-contaminated soil bioremediated with the citric waste showed a reduced Pro level, but not so pronounced as in the corresponding control, the non-contaminated soil with citric waste (Table 5). The mean TSS leaf levels

showed only small, in most cases, non-significant changes when comparing the different treatments (Table 5).

Table 5. Effect of soil treatments on osmolyte contents. Pro: proline, TSS: total soluble sugars; gluc: glucose, used as the standard in TSS determination. Treatments: Soil sampled in the Chinampas area was used as control (C); soil contaminated with endosulfan (CE); control soil bioremediated with *Penicillium crustosum* (CPc); endosulfan-contaminated soil, bioremediated with *P. crustosum* (EPc); control soil bioremediated with citric waste (CCW); endosulfan-contaminated soil, bioremediated with citric waste (ECW). Values represent the means of five replicas (five individual plants) followed by SD. Different letters in each column indicate significant differences between treatments, according to the Tukey test at the 99% confidence level.

Treatment	Proline (μmol g^{-1} DW)	TSS (mg eq. gluc g^{-1} DW)
C	18.2 ± 0.58^{c}	1.84 ± 0.10^{a}
CE	18.6 ± 0.52^{c}	$2.16 \pm 0.34^{a,b}$
CPc	8.53 ± 1.27^{a}	1.95 ± 0.18^{a}
EPc	17.4 ± 0.56^{c}	2.34 ± 0.34^{b}
CCW	10.1 ± 0.93^{a}	1.46 ± 0.21^{a}
ECW	13.6 ± 0.44^{b}	1.95 ± 0.17^{a}

3.5. Oxidative Stress Markers and Total Phenolic Compounds

Malondialdehyde (MDA) and hydrogen peroxide (H_2O_2) leaf contents were determined to evaluate the degree of oxidative stress affecting the plants after the different treatments; total phenolic compounds (TPC) were also quantified as representative non-enzymatic antioxidants (Table 6). The highest concentrations of MDA were measured in plants grown in the control and endosulfan-contaminated soils. Treatment with the biostimulants significantly reduced MDA levels, especially in the presence of the pesticide; the lowest value, of about 25% of the control, was observed in endosulfan-contaminated soil bioremediated with citric waste (Table 6). H_2O_2 concentrations, on the other hand, did not vary significantly after the different treatments, except for a small increase in endosulfan-contaminated soil bioremediated with *P. crustosum*. TFC contents showed a somewhat different pattern; a significant decrease was observed in the citric waste treatment (with and without endosulfan) but not in the *P. crustosum* treatments; as for MDA, endosulfan addition to the control soil did not affect the concentration of these compounds (Table 6).

Table 6. Effect of soil treatments on oxidative stress markers and non-enzymatic antioxidants. MDA: malondialdehyde, H_2O_2: hydrogen peroxide, TPC: total phenolic compounds; GA: gallic acid, used as the standard in TPC determination. Treatments: Soil sampled in the Chinampa area was used as control (C); soil contaminated with endosulfan (CE); control soil bioremediated with *Penicillium crustosum* (CPc); endosulfan-contaminated soil, bioremediated with *P. crustosum* (EPc); control soil bioremediated with citric waste (CCW); endosulfan-contaminated soil, bioremediated with citric waste (ECW). Values represent the means of five replicas (five individual plants) followed by SD. Different letters in each column indicate significant differences between treatments, according to the Tukey test at the 99% confidence level.

Treatment	MDA (nmol g^{-1} DW)	H_2O_2 (nmol g^{-1} DW)	TPC (mg eq. GA g^{-1} DW)
C	656.5 ± 58.4^{d}	362.4 ± 31.5^{a}	21.1 ± 2.68^{b}
CE	704.8 ± 54.5^{d}	$395.8 \pm 35.9^{a,b}$	19.6 ± 2.71^{b}
CPc	$460.4 \pm 64.8^{b,c}$	$399.2 \pm 17.0^{a,b}$	19.5 ± 1.91^{b}
EPc	328.3 ± 64.8^{b}	467.2 ± 52.1^{b}	19.0 ± 2.71^{b}
CCW	476.8 ± 37.4^{c}	331.7 ± 15.8^{a}	11.6 ± 1.75^{a}
ECW	165.1 ± 13.7^{a}	333.6 ± 25.7^{a}	13.3 ± 2.24^{a}

3.6. Activity of Antioxidant Enzymes

To counteract the oxidative stress generated, as a secondary effect, by high soil salinity and other stress conditions, plants activate their antioxidant enzymatic machinery. The specific activities of four relevant antioxidant enzymes—superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), and glutathione reductase (GR)—were determined in *P. leptostachyus* plants after concluding the different treatments (Table 7). The activity of the four enzymes was highest in the control saline soil in relation to most other treatments. Bioremediation of this substrate with *P. crustosum* resulted in a significant reduction of all activities, whereas the citric waste had the same effect on GR but did not affect SOD, CAT, and APX activities (Table 7). Endosulfan treatment caused a significant decrease in CAT, APX, and GR specific activities; however, SOD activity was not significantly different from the control value. The treatment with the biostimulants of endosulfan-contaminated substrates, in general, decreased further SOD, CAT, and APX mean activities, with respect to the corresponding controls, although the observed differences were not always statistically significant; on the contrary, GR activity increased when the endosulfan-treated soil was bioremediated with *P. crustosum* or citric waste (Table 7).

Table 7. Effect of soil treatments on the specific activity of antioxidant enzymes expressed in units mg^{-1} protein. SOD: superoxide dismutase; CAT: catalase; APX: aspartate peroxidase; GR: glutathione reductase. Treatments: Soil sampled in the Chinampas area was used as control (C); soil contaminated with endosulfan (CE); control soil bioremediated with *Penicillium crustosum* (CPc); endosulfan-contaminated soil, bioremediated with *P. crustosum* (EPc); control soil bioremediated with citric waste (CCW); endosulfan-contaminated soil, bioremediated with citric waste (ECW). Values represent the means of five replicas (five individual plants) followed by SD. Different letters in each column indicate significant differences between treatments, according to the Tukey test at the 99% confidence level.

Treatment	SOD (U mg^{-1} prot)	CAT (U mg^{-1} prot)	APX (U mg^{-1} prot)	GR (U mg^{-1} prot)
C	36.9 ± 7.87^{c}	10.6 ± 1.59^{b}	0.13 ± 0.02^{b}	2.89 ± 0.27^{e}
CE	46.5 ± 3.53^{c}	6.77 ± 1.41^{a}	0.02 ± 0.01^{a}	$1.47 \pm 0.13^{b,c}$
CPc	26.7 ± 4.74^{b}	6.05 ± 1.04^{a}	0.10 ± 0.01^{b}	1.20 ± 0.24^{a}
EPc	15.3 ± 1.99^{a}	7.34 ± 1.16^{a}	0.02 ± 0.01^{a}	$1.18 \pm 0.05^{a,b}$
CCW	44.4 ± 4.08^{c}	$8.33 \pm 1.33^{a,b}$	0.13 ± 0.03^{b}	1.67 ± 0.18^{c}
ECW	24.1 ± 4.24^{b}	6.21 ± 0.61^{a}	0.04 ± 0.02^{a}	2.19 ± 0.11^{d}

3.7. Principal Component Analysis

A principal component analysis (PCA) was performed, based on the means of all determined growth and biochemical parameters (Figure 1). Five components with Eigenvalues higher than one were detected, covering together 100% of the total variability. The first component explained 33.4% of the variability and showed highly significant positive correlations with the most relevant growth parameters, the fresh weight of leaves (LFW), stems (SFW), and roots (RFW) and leaf water content (%WC) and was negatively correlated mainly with the concentration of proline (Pro). These data agreed with the observation that Pro concentration was higher under conditions causing plant growth inhibition. The second component, explaining an additional 30.0% of the total variability, correlated with the variables related to oxidative stress and antioxidant systems, particularly (and negatively) with MDA levels and the activities of the assayed antioxidant enzymes. The applied treatments were separated along the first component axis. The control (saline) soil appears in the negative part of the graph, in agreement with the fact that soil salinity causes inhibition of growth. However, treatment with the biostimulants, *P. crustosum*, and citric waste, stimulates plant growth and, consequently, the CPc and CCW variables localised in the positive part of the axis. The strong negative correlation of the endosulfan-contaminated substrate (CE) with the growth parameters supports the adverse effect of the pesticide on plant growth, an effect that is not compensated by the biostimulants. Nevertheless, there

are differences between the two biostimulants, which are clearly separated with respect to the second axis of the figure, both in the presence and the absence of endosulfan (Figure 1).

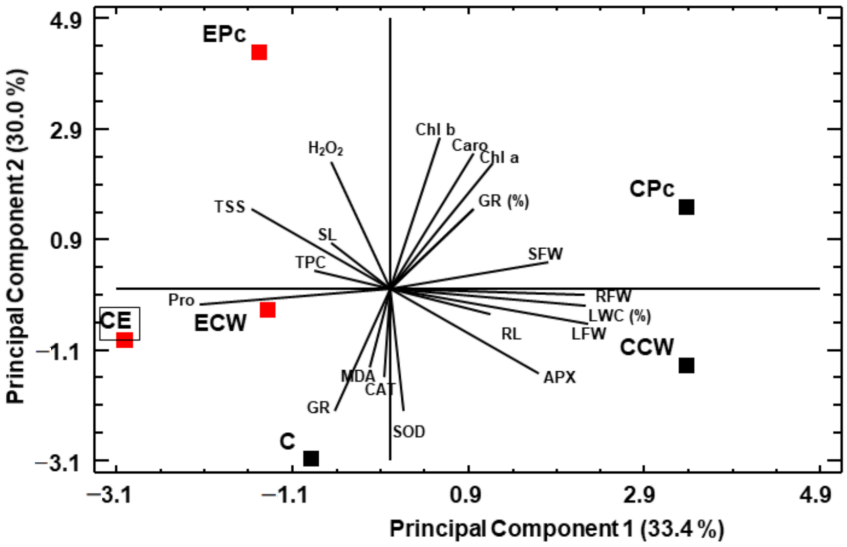

Figure 1. Principal component analysis (PCA). Changes in growth and biochemical parameters in plants grown on soil sampled in the Chinampas area used as control (C); soil contaminated with endosulfan (CE); control soil bioremediated with *Penicillium crustosum* (CPc); endosulfan-contaminated soil, bioremediated with *P. crustosum* (EPc); control soil bioremediated with citric waste (CCW); endosulfan-contaminated soil, bioremediated with citric waste (ECW). The percentages of the total variability explained by the first two components are shown (in parentheses) on the X and Y axes, respectively. Red squares correspond to treatments including endosulfan. Abbreviations: GR (%), germination rate; RL, root length; RFW, root fresh weight; SL, stem length; SFW, stem fresh weight; LFW, leaf fresh weight; LWC (%), leaf water content; Chl a, chlorophyll a; Chl b, chlorophyll b; Caro, carotenoids; Pro, proline; TSS, total soluble sugars; MDA, malondialdehyde; H_2O_2 hydrogen peroxide; TPC, total phenolic compounds; SOD, superoxide dismutase; CAT, catalase; APX, aspartate peroxidase; GR, glutathione reductase.

4. Discussion

The physicochemical characteristics of the Chinampas soils are related to their genesis and evolution in time. These soils were generated by the accumulation of organic matter, loamy lacustrine sediments, or other materials used to consolidate the elevated platforms, separated by a system of canals [6,49]. At present, the use of the Chinampas zone for agriculture faces a severe problem of soil salinisation, although there are large variations between different sites within this area. Pedologic analyses could not correlate salinity to the clay or organic matter content of the soil, so it was considered that the main reasons for salts accumulation were water evaporation in the canals, the use of urban wastewater to compensate for the reduced water level, illegal settlements, and the conversion of agricultural lands into urban areas [6,50]. The soil samples used in this work as the substrate for *P. leptostachyus* plants grown in pots, as control or for the different treatments—with the pesticide endosulfan and biostimulants—were moderately saline with a clay-loam texture and a high content in organic matter. Increased organic matter content is frequent in Chinampas soils and is related to the accumulation of the organic-rich materials, raised during the excavation of canals and enhanced due to manuring and warping, or related to natural humification processes [49,51].

The present findings indicate that *P. leptostachyus* is tolerant to salinity, mainly shown by its high seed germination capacity, close to 100%, on soil with salinity as high as 12 dS m^{-1}, as has been previously reported by Bayuelo-Jiménez et al. [22]. Seed germination itself, morphological parameters of plants grown in the same substrate and the relative

levels of several biochemical stress markers were determined to evaluate the effects of endosulfan and the biostimulants on plant development.

Regarding endosulfan, initial reports indicated that this pesticide is "very plant tolerant", producing little damage to the plants and having little effect on mineral tissue composition and yields of different crops [52]. However, more recent studies revealed its high phytotoxic effects, even at lower concentrations, on seed germination, plant growth, and nutrients uptake [53–57], and its adverse effects on soil and water biota [58–61] and human health [14,62]. In the present study, the deleterious effects of the pesticide were most clearly shown by the substantial inhibition of seed germination. This stage represents the bottleneck in the biological cycle of plants, being generally the most susceptible to environmental stressors, including pesticides [57]. Both tested biostimulants, *P. crustosum* and the citric waste, completely restored the germination capacity of the seeds to control percentages. The addition of endosulfan to the substrate also inhibited the growth of young plants, as reflected in the considerable reduction of root length and a significant drop in leaf FW and water content, and also in the results of the PCA, where the endosulfan treatment is strongly and negatively correlated with the growth parameters. The pesticide inhibitory effect would most likely have been more clearly observed in plants cultivated in non-saline soils since the salinity of the control substrate must also negatively affect plant growth in the absence of endosulfan. Treatment of the substrate with *P. crustosum* or the citric waste stimulated plant growth compared to the control, whereas the biostimulants did not have any relevant effect on endosulfan-contaminated soil.

Degradation of photosynthetic pigments is a typical response to salt stress and many other stressful conditions [63–65], also reported in common beans [66]. In our experiments, after all the applied treatments, the plants grown in the control substrate showed the lowest pigment contents, followed by those treated with endosulfan. In the absence of the pesticide, both biostimulants induced a two- to three-fold increase in the concentrations of the three pigments, indicating that previous bioremediation of the substrate with the fungus or the citric waste partly counteracted the negative effects of soil salinity on the *P. leptostachyus* plants. In endosulfan-contaminated substrates, however, only the fungus had a significant effect, increasing the three pigments contents between 1.8- and 2-fold.

Proline is one of the most common osmolytes in plants, accumulating under different environmental conditions that induce osmotic stress, such as drought and salinity [67–69]. Apart from its role in osmotic adjustment, Pro is involved in antioxidant mechanisms, acting as a free radical scavenger and contributing to cellular redox balance [70]. Furthermore, Pro can be considered a low-molecular-weight chaperon, which directly stabilises proteins and macromolecular structures, and is also a signalling molecule [68]. In common beans, Pro is a reliable stress biomarker, accumulating in direct proportion to the level of stress affecting the plants; that is, under the same stressful conditions, higher Pro concentrations are generally measured in the more sensitive genotypes [71–74]; this seems to be also the case in *P. leptostachyus*, according to the results of the PCA, where Pro levels are negatively correlated to plant growth parameters. In our experiments, the plants grown in the control soil, with and without endosulfan, appeared to be the most stressed as they showed the highest leaf Pro contents. In the absence of the pesticide, pre-treatments of the substrate with both biostimulants had a clear effect, reducing the concentration of Pro and, therefore, the degree of stress affecting the plants after one month of growth. In the pots with endosulfan-contaminated substrates, bioremediation with the citric waste had the same effect, reducing Pro contents with respect to the corresponding control; addition of *P. crustosum*, on the contrary, did not induce any significant change in foliar Pro levels.

Soluble sugars may also play a significant role in osmotic adjustment under stress conditions. Stress-induced changes in the leaf concentration of TSS or some specific carbohydrates, such as glucose and *myo*-inositol, have been reported in common beans [71,75]. However, soluble carbohydrates are the direct product of photosynthesis, energy sources, precursors in different metabolic pathways and signalling molecules involved in many biological processes, making it difficult to assess their participation in specific stress re-

sponse mechanisms [76,77]. In the present study, in general, TTS levels did not change significantly in the different treatments, suggesting that these compounds are not involved in the plants' reactions to endosulfan and biostimulants.

Under different stress conditions, all organisms increase the production of reactive oxygen species (ROS), including several free radicals and molecules such as O_2, O_3, or H_2O_2. ROS are by-products of aerial metabolism and play an essential function as secondary signalling messengers in many physiological processes. However, when produced in excess, they become toxic and negatively affect cell metabolism, even causing cell death at high concentrations [78,79]. Malondialdehyde (MDA), a product of membrane lipid peroxidation [80], is widely used as a biochemical marker to estimate the level of oxidative stress experienced by the plants under specific conditions and their degree of sensitivity to a particular type of stress [81,82]. MDA levels were highest, and not significantly different, in plants grown for one month in control and endosulfan-contaminated soils. In both cases, bioremediation with *P. crustosum* or citric waste induced a significant decrease in MDA concentrations and, consequently, a reduction in the degree of oxidative stress. Hydrogen peroxide, H_2O_2, formed by the dismutation of the superoxide radical, is a moderately reactive molecule that is also used as a marker of oxidative stress [83,84]. However, H_2O_2 measurements should be considered with caution because it is a relatively unstable compound and is involved in a wide variety of reactions and stress signalling cascades, which could mask its direct involvement in cellular redox balance [85,86]. In the present experimental conditions, H_2O_2 concentrations did not differ between the control and the endosulfan-contaminated soil and decreased, in both cases, by bioremediation with the citric waste. These results showed that the biostimulant caused a reduction in the levels of oxidative stress in the plants, as compared to the non-treated substrate, as was also indicated by the decrease in MDA levels. However, contrary to what was expected, treatment of the soil with *P. crustosum* did not alter H_2O_2 contents, either in control soil or in the presence of the pesticide. This contradiction may be explained by the ability of fungi of the genus *Penicillium* to generate free radicals, used to oxidise different types of xenobiotics [87], thus maintaining ROS levels in the plants. Changes in total phenolic compounds (TPC) contents followed the same pattern that those of H_2O_2, with higher and not significantly different values in plants grown in control and *P. crustosum*-treated soils, with and without endosulfan; significantly lower TPC concentrations were determined in the citric waste treatments. There is, therefore, a positive correlation between the level of oxidative stress affecting the plants in the different substrates and the accumulation of this group of antioxidant compounds.

Concerning the specific activities of antioxidant enzymes in the different treatments, a general positive correlation between the level of oxidative stress and the enzymatic activities was observed. Thus, for all four enzymes, the highest activities were measured in plants grown in the control saline soil and decreased significantly in the *P. crustosum*-bioremediated substrates; however, except for GR, this effect was not observed in the soils treated with citric waste. Regarding the endosulfan-contaminated substrates, no clear pattern could be established for the variation of enzyme activities as qualitative and quantitative differences were observed, depending on both the enzymes and the treatments.

It should be remarked that the determination of the biochemical parameters was carried out after one month of growth on the different substrates, when plants were already acclimated to the diverse experimental conditions. Therefore, the lower TPC contents and antioxidant enzyme activities in the bioremediated soils simply reflect a lower degree of oxidative stress in those plants, as compared to the controls. It is likely that different patterns of antioxidant activities, with higher levels in the bioremediated soils, would have been initially observed. However, we did not harvest the plant material at shorter times of growth since under those conditions, no differences in growth parameters between treatments could be detected, to be correlated with possible changes in the biochemical variables.

5. Conclusions

The 'Chinampas' zone of Xochimilco, in Mexico City, represents a unique, sustainable agroecosystem, worth to be maintained and protected for cultural, economic, and environmental reasons. The productivity of crops cultivated in this area is affected by the salinity of the soil and, presumably, also by the excessive use of agrochemicals, such as the organochlorine pesticide endosulfan. In the present study, we show that soil salinity negatively affects the growth of *Phaseolus leptostachyus* plants and that bioremediation of the saline soil with the fungus *Penicillium crustosum* or a citric waste stimulates plant growth. This stimulation effect, assessed after one month of growth in the different substrates, appear to be due, at least in part, to a reduction of the salt-induced oxidative stress in the plants, as shown by a general decrease in the levels of biochemical stress markers (Pro, MDA, H_2O_2), which was accompanied by a reduction in the activity of antioxidant compounds and enzymatic systems. The two biostimulants, however, seemed to exert their effects through different mechanisms. In the case of the fungus, the lower oxidative stress degree was associated with a reduction in the specific activities of antioxidant enzymes, whereas the levels of antioxidant phenolic compounds did not vary. The opposite is true for remediation with the citric waste, where reduced oxidative stress was marked by a less pronounced accumulation of phenolic compounds, whereas enzymes activities (except for GR) were not affected. Further studies performed in the field on a larger scale and including the reproductive phase will be needed to demonstrate that the biostimulants also improve crop yields. In any case, despite the relative salt tolerance of *P. leptostachyus*, the use of biostimulants is recommended to cultivate this species in the Chinampas, to partly compensate for the adverse effects of soil salinity on plant growth.

Soil contamination with endosulfan inhibited seed germination and further decreased plant growth with respect to the control saline soil. Both biostimulants increased germination percentages to control values, close to 100%, but their effects on plant growth and the assayed biochemical parameters did not follow a clear pattern, probably because they were masked by the salt stress simultaneously affecting the plants. Even if the application of this pesticide, already banned in many other countries, is stopped, the organochlorine compounds and/or their derivatives, all toxic, will remain in the soil for a long time. Therefore, soil remediation with biostimulants is also recommended to improve seed germination and plant growth in endosulfan-contaminated soils.

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