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PTS and PTSO, two organosulfur compounds from onion by-products as a novel solution for plant disease and pest management

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Abstract

Background Over the past decade, the great impact of agricultural crop diseases has generated considerable economic losses and has compromised the production of edible crops at a time when the world population is only expected to rise, leading to the search for new pest management strategies. Besides that, the environmental impact resulting from the continued use of chemical pesticides has led to the search for natural and sustainable alternatives. One of the existing solutions that currently stands out for its effectiveness is the use of bioactive plant extracts. This study aims to evaluate the antimicrobial activity of propyl propane thiosulfinate (PTS) and propyl propane thiosulfonate (PTSO), two organosulfur compounds (OSCs) derived from *Allium cepa*, against a wide range of target bacteria and fungi. To this end, various in vitro procedures were conducted as well as soil sanitization tests using sterile substrate inoculated with soil-borne pathogens. In addition, this study also evaluates the pesticidal activity of both compounds through in vitro mortality and repellence tests.

Results PTS and PTSO revealed inhibition activity on all the pathogens tested, belonging to different taxonomic groups. Moreover, both significatively reduced the population of bacteria and fungi in soil. The quantification of active substances in soil carried out in parallel to the microbial quantification showed that their use reduces the risk of residue accumulation since they break down quickly when applied. The set of antimicrobial tests performed demonstrated that the antifungal effect of both compounds is higher than the bactericidal effect. Lastly, PTS and PTSO showed a concentration-dependent significant biocidal and repellent effect against aphids.

Conclusions The results presented in this work demonstrate that both PTS and PTSO have a significant antimicrobial and pesticidal activity against the great majority of phytopathogens tested, being a promising tool to improve pest management in crops.

Keywords Propyl-propane-thiosulfinate, Propyl-propane-thiosulfonate, *Allium cepa*, Phytopathogens, Antimicrobial, Soil sanitization, Botanical pesticide, Insecticidal activity, Integrated pest management

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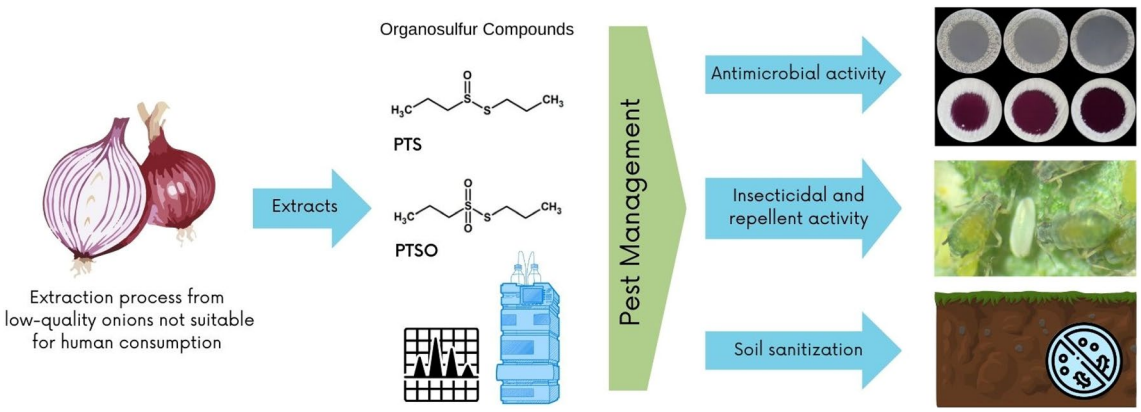
Graphical Abstract



Background

The global human population is predicted to number between 9.4 and 10.1 billion in 2050, an increase by about 1.5 billion compared to 2020 [80]. Around 80% of agricultural production is dedicated to human nutrition [24]. This includes not only the direct use of agricultural products as food, but also the use of crops and other vegetal matter to feed animals, which are in turn intended for human consumption. Keeping in mind the estimated population growth, the production of edible crops might need to increase by up to 119% [20]. This imposes a serious challenge, which involves adopting changes to ensure the transformation of agricultural and food systems toward greater sustainability, and to reduce waste and spoilage [54].

Plant diseases caused by biotic factors are the main responsible for the decrease in crop productivity; in fact, pests and pathogens bring about 40 billion dollars losses a year worldwide [77], which means reductions between 21 and 30% globally in major crops [68]. Plant pathogen control will be even more challenging as climate change conditions progress [11]. Since the environment has a great impact on plant pathogenesis [70], global warming is directly related to disease incidence and severity [81]. Higher temperatures are correlated with soil degradation and less water availability, and foster the emergence of new pathogens and a changeable geographic distribution [67].

Over the last several decades, synthetic agrochemicals have contributed to increase food production worldwide through controlling crop diseases, but with a severe environmental impact [65]. Their application has not only gradually disrupted biological control by natural enemies, but also caused disease outbreaks and the development of resistance [57]. Moreover, synthetic pesticides severely damage non-target organisms, such as pollinators, and human health [73].

In this regard, plant-derived secondary metabolites are receiving increasing attention and gradually replacing synthetic biocides and soil disinfectants from disease management protocols [85]. Many products based on antimicrobial phytochemicals isolated from plants have been developed over the past few years as novel eco-friendly non-synthetic plant protection measures [55]. The extraction of phytochemicals from medicinal and fragrant plants is quite common [35, 58]; however, the extraction of bioactive compounds from by-products from the food industry or second-class plant material, such as grape cane waste [63] and pepper leaves [56], through clean extraction methodologies has become an innovative strategy that contributes to the revaluation of agricultural waste and support circular economy [71].

In recent years, the functional properties of organosulfur compounds (OSCs) obtained from onion (*Allium cepa*) and garlic (*Allium sativum*), such as antioxidative, immunomodulatory and antimicrobial activity, have been deeply studied [61]. OSCs are secondary metabolites that are biosynthesized by the plant as a defence mechanism against biotic and abiotic stressors [61]. Garlic bulbs are rich in alliin (S-allyl cysteine sulfoxide) and in a lower degree methiin (S-methyl-L-cysteine sulfoxide), while onion bulbs contain methiin but also isoalliin (S-propenyl-L-cysteine sulfoxide) and propiin (S-propyl-L-cysteine sulfoxide) [61]. Cysteine sulfoxides are natural constituents of fresh bulb tissue, non-volatile and odourless [10, 66]. The disruption of the bulb tissue triggers an enzymatic reaction carried out by alliinase, that catalyses the conversion of these

precursors to thiosulfinates [62], volatile compounds to which the antimicrobial activity of *Allium* genus plants are mainly attributed and bear the primary responsibility for their organoleptic properties [41, 60].

According to the existing literature, the antimicrobial effect of thiosulfinates is primarily due to their ability to inhibit thiol-containing enzymes by oxidizing protein cysteine or glutathione residues [8]. Enzymes containing thiol include the main enzymes of microbial metabolism as well as bacterial enzymes of the acetyl-CoA-forming system, and RNA polymerase [8]. In onion, propiin turns into propyl-propane thiosulfinate (PTS), a labile compound that changes into dipropyl disulphide and propyl-propane thiosulfonate (PTSO) through dismutation or disproportionation reactions [27].

Whereas bioactive properties of Allicin—that represent about 75% of thiosulfinates in garlic—has been thoroughly investigated in several fields of study, from antimicrobial therapy for human infections to integrated pest management [15, 19], information regarding the antimicrobial activity of PTS and PTSO from onion is limited and focused on the potential of thiosulfinates against human and animal infections. Recent studies have shown broad-spectrum antibacterial activity of PTS and PTSO and its gaseous form against clinical isolates of bacteria and *Candida* species that are resistant to at least one group of antibiotics [74, 75]. In a previous study we demonstrated the in vitro and in planta antifungal activity of volatile organosulfur compounds PTS and PTSO from onion against *Verticillium dahliae* [23], the most devastating soil-borne pathogenic fungi affecting olive trees [51]. Moreover, the same study showed the potential of both compounds as soil sanitizers, as they reduced *V. dahliae* population in an artificially infested substrate. As previously mentioned, PTS and PTSO are volatile compounds [42]. Owing to their low molecular weight (< 300 g/mol), they can diffuse through plant cell membranes and soil, playing a key role in the functioning of the whole ecosystem [34]. The study of the active properties of their gaseous phase is thus of interest to back up their use against phytopathogens and in pest management systems, especially in the present context in which the search for alternatives to conventional pesticides has become one of the main focuses of modern agriculture research [33].

Within this context, the aim of the present study was to evaluate the bactericidal, fungicidal, pesticidal and repellent activity of PTS and PTSO obtained from low-quality onions not suitable for human consumption, through in vitro methodologies and performing soil sanitization trials.

Materials and methods

Compounds and reagents

Standardized fractions of PTS and PTSO at 20% were supplied by DOMCA SA (Granada, Spain). Both compounds were obtained from onions that had been discarded as they were not suitable for human consumption, following the methodology described by Hu et al. [30] to obtain the allyl derivatives form garlic. Summarizing, onions were chopped and immersed in a solution of Ethanol (70%) in a percentage equivalent to four times their weight. The extraction was carried out for 2 weeks at room temperature, then the mixture was filtered, and the solution was concentrated and extracted with Ethyl acetate (EtOAc). The EtOAc extract was concentrated and fractionated by 2 sequential column chromatography's, taking the trichloromethane ($CHCl_3$) fraction from the first column, and then using EtOAc/hexane as mobile phase in the second column to obtain purified PTS and PTSO. All reagents were purchased from Sigma-Aldrich Química S.L. (Spain), unless otherwise stated.

Phytopathogens strains and growth media used

Bacteria and fungi used in this study were obtained from the Spanish Collection of Type Cultures (CECT), the German Collection of Microorganisms and Cell Cultures (DSMZ), the plant pathogen collection of DMC Research and the culture collection of the Department of Crop Protection, Institute for Sustainable Agriculture, Spanish National Research Council (Córdoba, Spain), which are listed in Table 1. Each phytopathogen grew on a specific culture medium and time, indicated by the corresponding culture collection. For the antimicrobial activity tests against pathogenic bacteria, Mueller–Hinton Agar and Mueller–Hinton broth supplied by Scharlau (Barcelona, Spain) were used as culture media [18]; for the in vitro antimycotic test, Rose-Bengal agar supplied by Scharlau and RPMI-1640 medium with L-glutamine [17] supplied by Labclinics (Barcelona, Spain) were used.

Insects

Adult individuals of the cotton aphid, *Aphis gossypii* Glover (Hemiptera: Aphididae) were supplied by TECNOVA Technological Center (Almería, Spain). The individuals were reared on courgette leaves at the TECNOVA Experimental Center greenhouse for future experiments.

Soil

The soil used in this study was superficially collected from an olive grove in Linares, Jaen (30U 444908.38 4209274.77 UTM WGS84), owned by the cooperative DCOOP, the world's largest producer of olive oil. This soil

Table 1 Bacterial and fungal strains used along with their references and source of isolation

	Reference	Isolation
Bacterial strain		
Erwinia persicina	DSM 19328	Tomato plant (*Lycopersicon esculentum*)
Xanthomonas campestris	CECT 97	Brussels sprout (*Brassica oleracea* var. *gemmifera*)
Pseudomonas savastanoi	CECT 5023	Olive tree (*Olea europaea*)
Pseudomonas syringae	DMC 15	Peach (*Prunus persica*)
Clavibacter michiganensis sp. *michiganensis*	CECT 790	Tomato plant (*Lycopersicon esculentum*)
Agrobacterium tumefaciens	CECT 4119	Crown gall of apple seedling (*Malus* spp)
Fungal strain		
Geotrichum candidum	DSM 1240	Tomato plant (*Lycopersicon esculentum*)
Alternaria alternata	CECT 2662	*Lycopersicon* spp
Fusarium oxysporum f. sp. *cubense*	DMC 02	Banana tree (*Musa paradisiaca*)
Fusarium graminearum	DSM 1095	Maize (*Zea mays*)
Phytophthora cinnamomi	CECT 20186	Avocado pear root (*Persea americana*)
Penicillium expansum	DMC 01	Apple (*Malus domestica*)
Penicillium digitatum	DMC 07	Sweet orange (*Citrus sinensis*)
Phyllosticta spp	DMC 10	Olive tree (*Olea europaea*)

was chosen because no biocide product had been applied on the farm in the last two years, as it was the control farm of an experimental field trial.

The soil was dried in an oven at 50 °C and passed through a 2 mm pore sieve to remove plant material, soil macrofauna and stones [9]. Then, it was stored in polyethylene bags for future analysis and characterized according to the procedures previously described. Soil pH, which was determined in a 1:1 water suspension according to the international standard (International Society of Soil Science, ISSS), was 8.71 ± 0.01, that is to say, moderately basic according to the criteria established by the United States Department of Agriculture [48]. Moreover, the soil, classified as sandy loam, contained 6.5 ± 0.22% fine silt, 4.9 ± 0.85% coarse silt, 21.5 ± 0.07% clay, 67.1 ± 0.72% sand (determined through Robinson pipette method [64]), and 1.13% organic matter (soil organic matter fractionation was measured according to [79]). Lastly, this soil presented a maximum Water Holding Capacity (mWHC) of 0.414 g H_2O per g soil dry matter (determined by the Keen—box method [37]). Based on this parameter, it was determined that the soil had 95.46% dry matter of field-moist soil and a water content of natural moist soil of 0.04 g water/g dry matter.

In vitro antimicrobial activity against pathogenic bacteria

The antibacterial activity of organosulfur compounds PTS and PTSO was evaluated by performing different testing procedures. The disk diffusion method proposed by Bauer et al. [1] and modified by Calvo and Asensio [14] was used to evaluate the antibacterial activity. Agar plates were inoculated using bacterial suspension adjusted to 10^6 CFU/ml, so that the growth after incubation was confluent. Sterile 6 mm cellulose disks (Whatman® antibiotic test discs, Buckinghamshire, UK) impregnated with 20 µl of PTS or PTSO at 5, 10 and 25 µg/µl were placed in the centre of inoculated agar plates. The inhibition zone of bacterial growth was measured after 48 h incubation.

Determination of the minimum bactericidal concentration (MBC) was performed by the broth microdilution method, following the guidelines of the Clinical and Laboratory Standards Institute (CLSI) collected in the standard Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically [18], to establish the lowest concentration of each antimicrobial agent that reduces the viability of the initial inoculum by 99.9%. 1:2 decreasing dilution were prepared from an initial solution of each compound at 10,000 µg/ml so that the following concentrations were obtained: 5000; 2500; 1250; 625; 312.5; 156.25; 78.125; 39.06; 19.53; and 9.76 µg/ml. Each dilution was inoculated with bacterial suspension so that the final concentration in each well was 10^5 CFU/ml, and incubated overnight at room temperature. As positive control, a mix of ampicillin and streptomycin (100,000 and 25,000 µg/ml, resp.) was used. As negative control, liquid media without antimicrobial agent was inoculated with bacteria. Bacterial growth was tested by culturing in agar plates, and the lowest concentration of PTS/PTSO in which no growth was observed was established as the MBC.

The antibacterial activity of the gaseous phase of PTS and PTSO was assessed through a previously described procedure [23]. Bacterial suspensions adjusted to 10^6 CFU/ml were spread on agar plates. Sterile 6 mm

cellulose disks were placed, not in the centre of the plate, but in the centre of the lid of the petri dish, and they were impregnated with 20 µl of PTS or PTSO solutions. The same PTS and PTSO concentrations as in the disk diffusion assay were used, i.e., 5, 10 and 25 µg/µl. Plates were incubated for 48 h and subsequently growth inhibition zones were measured. All in vitro assays were performed in duplicate.

In vitro antimicrobial activity against pathogenic fungi

The antifungal activity of PTS and PTSO was evaluated following the same methodology described for bacteria, using the appropriate liquid and solid media indicated in section "Phytopathogens strains and growth media used". The disk diffusion method and the gas phase activity test were carried out with no modifications, with the exception of the incubation time, which was 5 days. Regarding the determination of the Minimum Fungicidal Concentration (MFC), the broth microdilution method was also carried out according to the standard reference method for broth dilution antifungal susceptibility testing of filamentous fungi of the CLSI, which does not differ from that described for the determination of MBC [17]. For the positive control, natamycin (50,000 µg/ml) was used instead of ampicillin and streptomycin.

Moreover, in a fourth trial, the influence of both organosulfur compounds on mycelial growth was determined. Different volumes of PTS and PTSO at 20% were added to Rose-Bengal medium to obtain supplemented agar plates at 25, 50, 100, 250 and 500 µg/ml. Each fungal strain was grown on Rose-Bengal agar for 3 days. From these cultures, agar plugs of 5 mm diameter were obtained, which were distributed among the supplemented agar plates [47]. In addition, non-supplemented plates with 5 mm agar plugs from each strain tested were incubated as control of fungal growth. For 17 days of incubation the diameter of the mycelium over time was measured, compared to the mycelial growth of each fungus when growing on non-supplemented Rose Bengal agar plates. Each experiment was repeated twice.

In vitro activity against aphids

In this study, the contact toxicity and repellent activity were evaluated for a liquid blend of PTS and PTSO in proportion 1:1 (w/w) at different concentrations (5000; 2500; 1000; and 500 µg/ml). Since the treatments were prepared in water, blank control included only water.

The contact toxicity of PTS and PTSO was assessed by the leaf immersion method, as previously reported [69]. Circular cuttings of courgette leaves of 55 mm diameter were immersed in the treatment and control solutions for 5 s, air-dried and placed on 60 mm diameter petri dishes. Twenty-four adults were transferred to each treated leave cutting in petri dish using a brush. Decis® Protech, a deltamethrin-based pyrethroid insecticide purchased from Bayer CropScience S.L. (Barcelona, Spain) (Ref 84942464) was used as positive control at the dose indicated on the label. The plates were wrapped with Parafilm® purchased from amcor (Valencia, Spain) to prevent the aphids to scape, and maintained in a climate chamber at 25 ± 1 °C, 75 ± 5 relative humidity and Light:Dark photoperiod of 14:10 [89]. Mortality was recorded after 24 h. An aphid was considered dead if it did not move its legs when touching its abdomen with a brush and if the body turned black [86, 87].

The repellent activity was assessed by a choice assay in 90 mm diameter petri dishes [72]. N, N-diethyl-meta-toluamide (DEET), an active ingredient used in many repellent products, was purchased from Sigma–Aldrich at 97% (Ref. D100951) and diluted to 2000 µg/ml as positive control [32]. Circular cuttings of courgette leaves of 25 mm diameter were immersed in the treatment and control solutions for 5 s and dried at room temperature, as in the previous trial. A treated leaf and a negative control leaf were placed in each petri dish on a moist filter paper disk to maintain humidity [88]. Then, 24 adults were introduced into each petri dish using a brush. The parafilm-sealed petri dishes were maintained in the conditions previously indicated. The repellent effect was observed after 24 h and expressed as percentage of repellence according to the following formula [59]:

$$\%\,\text{repellence} = [(C - T)/(C + T)]\,x\,100$$

where C is the number of aphids on the control leaf, and T is the number of aphids on the treated leaf. Each experiment was performed in triplicate.

Soil sanitization

The study of the persistence of phytopathogenic microorganisms in soil was carried out by microcosm systems [22]. The ability of a powder blend of PTS and PTSO in proportion 1:1 (w/w) to reduce the population of a pathogenic microorganism artificially inoculated in soil was determined against the bacterium *A. tumefaciens* and the fungus *F. oxysporum*, both pathogens that inhabit the soil, where they can survive for long periods [49, 90]. Two different concentrations of active substances, 100 µg/g (50 µg/g of each one) and 500 µg/g (250 µg/g of each one), were tested; and the efficacy of a treatment based on a single application was compared with the efficacy of a treatment consisting of 3 applications of the same dose separated in time. In addition, non-inoculated soil was used as sterility control, while untreated inoculated soil was used to follow up microbial growth. Finally, as positive control, the assay included a study group of inoculated soil that was treated with soil fumigant Metam

sodium ($C_2H_4NNaS_2$) (EPA Reg. No. 45728-16) [76]. Metam sodium 42.1% aqueous solution was purchased from Eastman Chemical Company (Madrid, Spain), diluted in sterile water and set to 60 µg/g, in accordance with the recommended application rates [44]. Each study group consisted of four replicates. Table 2 shows the experimental design of the assay and details of the different groups of study.

The experimental microcosm unit consisted of a polypropylene box with a drainage system, of 28 cm length × 5 cm width × 17 cm height and 1.75 l capacity. The soil was autoclaved through 4 cycles of 20 min at 121 °C in a steam sterilizer (Raypa, Terrasa, Spain) for 4 successive days [38], interspersed with incubations at 4 °C, to eliminate vegetative forms by heat shock [45]. After drying in an oven at 50 °C, 700 g of sterile soil were introduced into each microcosm unit on a bed of sterile gravel to facilitate drainage and prevent soil compaction. Subsequently, soil was inoculated with 140 ml of microbial suspension previously adjusted to 10^9 CFU/ml, so that the soil moisture was adjusted to 60% of the water holding capacity (WHC) [82]. The negative control group was inoculated with 140 ml of distilled water. Microcosms were then placed in a room at 25 °C (optimal growth temperature of the two phytopathogens used), where they were kept until the end of the trial. Four days after inoculation, every study group was sampled to establish the starting microbial population. Next, the treatments were applied to the corresponding group at the appropriate dose. Microbial population was quantified 1, 2, 4, 7, 11, 15, 31 and 45 days after treatment. The second and third application of the PTS/PTSO powder treatment was added to the corresponding microcosm units 10 and 30 days after the first application. At each enumerated date, 25 g of soil were diluted in 225 ml of buffered peptone water (Scharlau). A lab paddle blender (MASTICATOR, UIL, Barcelona, Spain) was used to homogenize the samples. Serial dilutions were prepared from the supernatant, cultured in the appropriate solid medium, and incubated at 25 °C for 3 days [21]. In the cases in which no microbial growth was observed on the plate, to confirm the absence of microorganisms a pre-enrichment step was carried out in a non-selective nutrient medium. Microorganism population was expressed as Log 10 CFU/g soil.

PTS and PTSO concentrations achieved by each application protocol were assessed by High-performance liquid chromatography using a UV detector (HPLC–UV). Fifty grams of soil were mixed with 100 ml of acetone, homogenized with vortex for 1 min and extracted in a sonication bath for 10 min. Supernatant was separated from the soil by filtration and the process of extraction was repeated adding 20 millilitres of acetone to the solid residue. Then, the supernatant from both extractions was evaporated until dryness in a vacuum rotator and reconstituted with 10 ml of methanol (MeOH) vortexing for 30 s. Finally, the extract was filtered through a nylon filter of 0.2 µm (Sigma–Aldrich, Darmstadt, Germany) and injected into the HPLC system. For the PTS and PTSO determination, an Agilent 1260 Infinity LC (Agilent Technologies, Santa Clara, CA, USA) system was used. The separation of the compounds was accomplished using a Zorbax Eclipse Plus RRHD (50 × 2.1 mm, 1.8 mm) column at 25 °C, and the gradient and mobile phases described by Sorlozano-Puerto et al. [75]. Wavelength of detection was set at 200 nm. A calibration curve using PTS and PTSO standards was made for the quantification.

Table 2 Experimental design of soil sanitization assay through microcosm system

Group	Treatment dose	Number of treatment applications	Microorganism concentration
Negative control	–	–	–
Positive control	–	–	10^7 CFU/g soil
Metam Sodium	60 µg/g	1 application: 4 days after inoculation	10^7 CFU/g soil
PTS/PTSO	100 µg/g	1 application: 4 days after inoculation	10^7 CFU/g soil
PTS/PTSO	100 µg/g	3 applications: 4 days after inoculation 10 days after 1st application 30 days after 1st application	10^7 CFU/g soil
PTS/PTSO	500 µg/g	1 application: 4 days after inoculation	10^7 CFU/g soil
PTS/PTSO	500 µg/g	3 applications: 4 days after inoculation 10 days after 1st application 30 days after 1st application	10^7 CFU/g soil

Statistical treatment

GraphPad prism 8.0 software (GraphPad Software Inc., San Diego, California) was used for statistical analysis. The data obtained in the in vitro antimicrobial activity assays were analyzed using descriptive statistics. Shapiro–Wilk normality tests were used to determine normal distribution of all data subjected to ANOVA. A one-way ANOVA test supplemented with Tukey's post hoc test was used to compare every treatment and control of the in vitro assays against aphids with each other. Repeated measures two-way ANOVA test supplemented with Dunnett's post-hoc test was used for evaluation of statistically significant inhibition of mycelial growth and to establish significant differences between microorganism survival in treated soil and the positive control, considering different treatments and time points. Differences were considered statistically significant when $p < 0.05$.

Results

In vitro assessment of antibacterial activity

The antibacterial activity of PTS and PTSO was tested against different bacteria involved in infectious processes of agricultural crops. As shown in Table 3, both compounds displayed antimicrobial activity against all the bacterial strains included in the study in various degrees. Moreover, in most cases the bacteriostatic effect rises as the concentration of the product increases, being considerably more modest in the case of *P. syringae*. Regarding the volatility-linked activity assay, PTS and PTSO inhibited growth of all bacteria tested without coming into direct contact with either the medium or the microorganism, but rather by diffusion of their gas phase, as shown in Fig. 1. *Xanthomonas campestris*, *C. m. michiganensis* and *A. tumefaciens* were the most sensitive, showing growth inhibition zones of at least 40 mm in all cases (Tables 3 and 4). *Erwinia persicina* and specially *P. syringae* were, on the other hand, the most resistant strains to PTS and PTSO in both tests, with the smallest inhibition zones among the strains studied. Furthermore, in both agar tests PTSO displayed a greater capacity to inhibit the growth of *P. savastanoi*, *P. syringae*, *C. m. michiganensis* and *A. tumefaciens* than PTS. Whereas the results of the diffusion and volatility tests in agar appear to suggest that PTSO may have higher antibacterial capacity, the results obtained in the MBC test indicated that it was significantly more active than PTS. As shown in Table 5, the lower MBC of PTS is 156.25 µg/ml (Me = 312.5 µg/ml), while MBC data for PTSO ranges from 156.25 to 19.53 µg/ml (Me = 78.125 µg/ml).

In vitro antimycotic activity

As for antifungal activity, all phytopathogenic fungi used in this study were sensitive to both organosulfur compounds in a dose dependent manner according to the results of the disk-diffusion method, presented in Table 6. Table 7 details the results obtained in the MFC determination. In all cases, the MFC of PTSO for each strain was at least one dilution lower than the corresponding concentration of PTS. The highest values obtained, which ranges from 625 to 156.25 µg/ml, correspond to the two *Penicillium* species used. On the other hand, *A. alternata*, *F. oxysporum* and *F. graminearum* were the most sensitive, displaying PTSO MFC values of 39.06, 19.53 and 9.76 µg/ml, respectively. The fungicidal activity of the gas phase of the compounds was also demonstrated, since the volatility test generated growth inhibition halos whose diameters were similar to the halos obtained by the agar diffusion test (Table 8 and Fig. 2).

To complete the in vitro assessment of antimycotic activity, the influence of both organosulfur compounds on the mycelial growth was studied. The results of the mycelial growth inhibition test, represented in Figs. 3 and 4, also indicates that most fungal species were sensitive to at least the two highest concentrations evaluated (250 and 500 µg/ml), showing significant inhibition ($p < 0.05$). In the case of *F. graminearum*, which, as in the MFC test, turned out to be the most sensitive, all concentrations of both products completely inhibited the mycelial growth ($p < 0.0001$), with the exception of PTSO at 25 µg/ml. Moreover, the treatments of 50 and 100 µg/ml of PTS

Table 3 Antimicrobial activity of PTS and PTSO against phytopathogenic bacteria by disk-diffusion method, expressed as the average diameter ± standard deviation of inhibition zone (mm)

Species	PTS (µg/µl)			PTSO (µg/µl)		
	5	10	25	5	10	25
E. persicina	18.0 ± 0.71	23.0 ± 1.87	29.5 ± 1.12	17.5 ± 2.06	20.3 ± 1.79	29.0 ± 0.71
X. campestris	31.5 ± 1.12	36.3 ± 1.09	47.3 ± 1.48	41.0 ± 1.58	48.5 ± 2.06	61.3 ± 1.48
P. savastanoi	12.8 ± 1.30	22.0 ± 1.58	25.3 ± 1.48	25.5 ± 2.06	38.0 ± 1.87	45.5 ± 1.66
P. syringae	11.3 ± 1.09	11.8 ± 1.48	14.5 ± 1.12	19.3 ± 1.09	19.5 ± 1.50	29.8 ± 1.09
C. m. michiganensis	38.0 ± 1.58	46.3 ± 0.83	59.3 ± 1.09	51.5 ± 1.12	56.0 ± 1.58	75.0 ± 1.41
A. tumefaciens	27.5 ± 1.12	36.5 ± 2.18	48.8 ± 2.38	54.3 ± 0.83	64.0 ± 1.58	75.3 ± 2.68

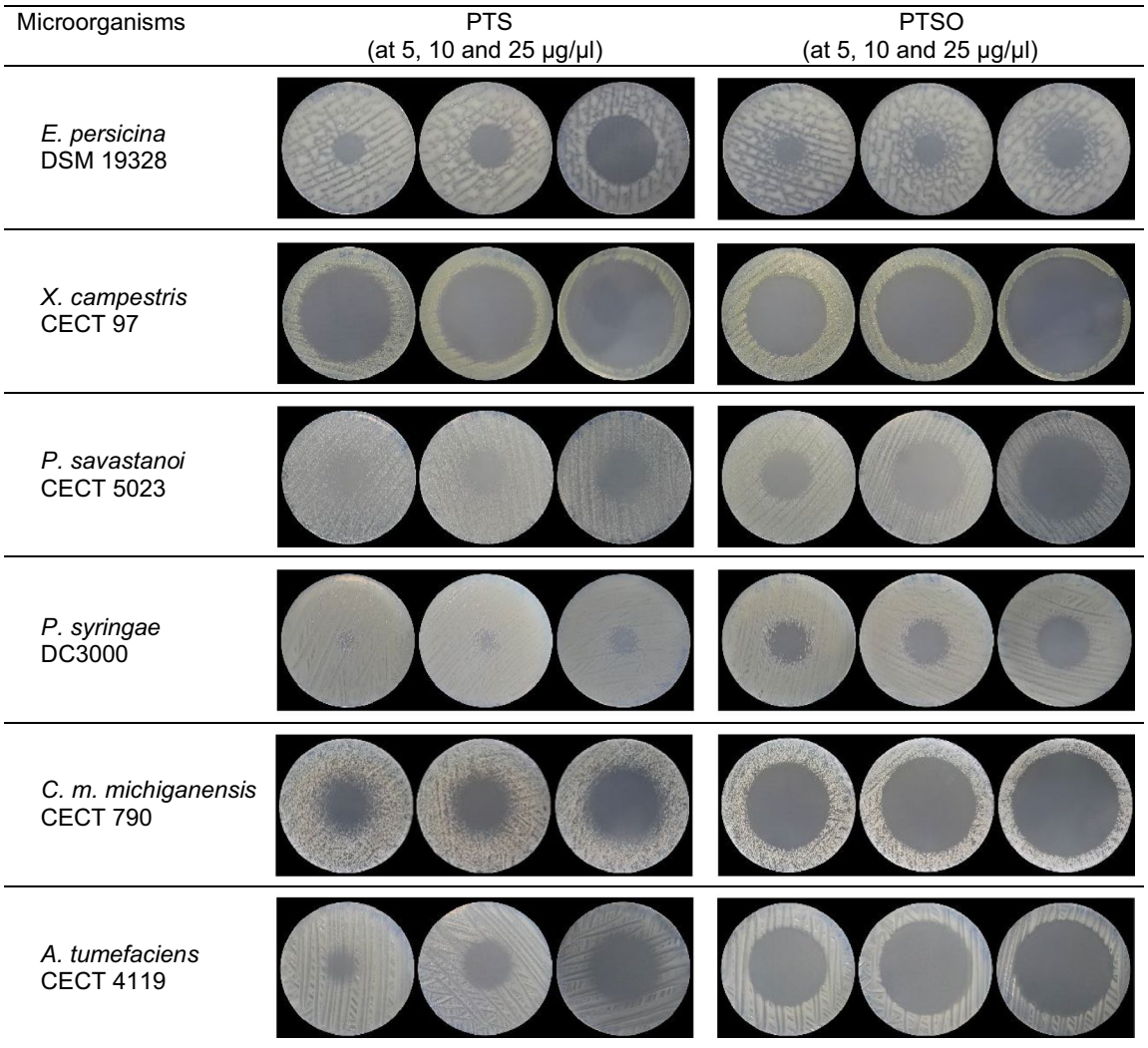


Fig. 1 Antibacterial activity of the gaseous phase of PTS and PTSO against phytopathogenic bacteria

Table 4 In vitro antimicrobial activity of PTS and PTSO against phytopathogenic bacteria via the gas phase, expressed as the average diameter ± standard deviation of inhibition zone (mm)

Species	PTS (µg/µl)			PTSO (µg/µl)		
	5	10	25	5	10	25
E. persicina	19.0 ± 1.41	25.0 ± 3.16	40.8 ± 2.59	15.8 ± 2.59	22.3 ± 2.17	28.5 ± 2.69
X. campestris	42.8 ± 3.96	50.8 ± 2.59	59.3 ± 3.67	44.3 ± 3.11	54.5 ± 3.84	67.3 ± 3.03
P. savastanoi	16.3 ± 0.83	19.5 ± 1.66	26.8 ± 1.09	26.5 ± 1.12	36.3 ± 1.92	47.0 ± 1.41
P. syringae	0.0 ± 0.00	7.5 ± 1.66	13.3 ± 1.48	20.0 ± 1.41	23.5 ± 1.12	27.8 ± 1.48
C. m. michiganensis	15.0 ± 3.36	25.0 ± 1.41	40.5 ± 1.12	44.8 ± 2.86	55.0 ± 3.39	65.8 ± 1.92
A. tumefaciens	15.5 ± 2.18	26.5 ± 1.12	41.0 ± 2.55	49.5 ± 1.80	57.5 ± 2.69	60.5 ± 2.69

and PTSO successfully controlled the development of *P. cinnamomi*, *Phyllosticta* spp and *F. graminearum* in agar plates. On the other hand, only the exposure to PTS and PTSO at 500 µg/ml achieved a significant reduction of *P. digitatum* and *G. candidum* growth ($p < 0.05$). *Penicillium expansum* was the most resistant fungal strain, since

Table 5 Minimum bactericidal concentration (MBC) of PTS and PTSO against phytopathogenic bacteria

Species	MBC (µg/ml)	
	PTS	PTSO
E. persicina	312.5	156.25
X. campestris	156.25	19.53
P. savastanoi	312.5	39.06
P. syringae	156.25	78.125
C. m. michiganensis	312.5	78.125
A. tumefaciens	625	78.125

none of the evaluated concentrations of PTS and PTSO significantly inhibited mycelial development. Although in the case of the other fungal strains no differences were observed between compounds, the effectiveness of PTSO was lower against *Phyllosticta* spp, *F. oxysporum* and *F. graminearum*.

In vitro activity against aphids

Insecticidal and repellent capacity of PTS and PTSO were assessed against adult aphids and presented in Fig. 5. In the contact toxicity assay, the mortality rate of the aphid population after 24 h of exposure to impregnated leaves was investigated. We found that the exposure of *A. gossypii* to both compounds significantly reduced the population at all concentrations evaluated with respect to the negative control ($p < 0.05$). The average mortality of aphids treated with 500 and 1000 µg/ml of PTS and 500 µg/ml of PTSO were 17, 19 and 19% respectively, which is lower than the mortality rate of the population treated with the insecticide used as positive control (28%). On the other hand, the death of individuals treated with PTS ≥ 2500 µg/ml and PTSO ≥ 1000 µg/ml equalled or exceeded the mortality rate of the positive control. However, none of the treatments showed significant differences compared to the positive control, except for the group of aphids treated with PTSO at 5000 µg/ml which registered a significant increase of mortality, reaching 42% after 24 h of exposure ($p < 0.05$).

Moreover, whereas the insecticidal capacity of PTSO was found to be higher than that of PTS, the behaviour of both compounds was quite homogeneous in terms of repellent activity. At concentrations of 500 and 1000 µg/ml, PTS and PTSO demonstrated a repellent action of 53–56% and 39–47%, respectively. Even though these treatments showed no significant differences, neither between them nor with respect to the positive control (39% repellence), the responses were stronger with higher concentrations, displaying a significant increase of the repellent activity with respect to the control ($p < 0.05$).

Antimicrobial effect and PTS/PTSO quantification in soil

When non-treated, *A. tumefaciens* and *F. oxysporum* generated growth curves according to what was expected, reaching the concentration of 10^8 CFU/g of soil at the end of the study. Although Metam sodium and the two concentrations of PTS and PTSO tested significantly reduced the population of *A. tumefaciens* and *F. oxysporum* when compared to the untreated soil ($p < 0.05$), the behaviours of the bacterium and the fungus in the presence of the antimicrobials were very unlike. As shown in Fig. 6, within 24 h of exposure *A. tumefaciens* density were narrowed 3 logarithmic units (from 10^7 to 10^4 CFU/g) and, surprisingly, thereupon the population remained steady till the end of the assay. In contrast to the bacterial response, *F. oxysporum* was drastically affected by all the treatments during the first 11 days of sampling. As illustrated in Fig. 7, from day 15, the progressive recovery of the fungus was observed in soil treated with Metam sodium and a single application of PTS and PTSO at 100

Table 6 Antimicrobial activity of PTS and PTSO against phytopathogenic fungi by disk-diffusion method, expressed as the average diameter ± standard deviation of inhibition zone (mm)

Species	PTS (µg/µl)			PTSO (µg/µl)		
	5	10	25	5	10	25
G. candidum	15.5 ± 1.80	26.0 ± 3.16	36.8 ± 3.34	27.3 ± 2.86	34.8 ± 3.11	44.8 ± 2.59
A. alternata	38.5 ± 3.35	44.8 ± 2.17	53.3 ± 2.05	30.8 ± 0.83	42.0 ± 2.12	56.3 ± 1.64
Phyllosticta spp	64.0 ± 1.41	68.8 ± 0.83	75.5 ± 1.12	55.0 ± 1.63	64.5 ± 2.05	79.5 ± 1.25
P. cinnamomi	28.5 ± 1.12	42.5 ± 3.64	54.0 ± 1.41	39.5 ± 2.69	52.0 ± 1.41	66.3 ± 2.59
F. oxysporum	26.5 ± 2.18	46.5 ± 1.66	60.5 ± 2.69	41.8 ± 3.03	51.3 ± 2.59	54.0 ± 1.41
F. graminearum	51.5 ± 2.06	58.0 ± 1.22	65.3 ± 2.17	50.8 ± 2.59	57.8 ± 0.83	69.0 ± 2.12
P. expansum	34.3 ± 3.03	39.8 ± 1.64	48.5 ± 1.50	35.8 ± 3.34	46.3 ± 0.83	54.8 ± 1.48
P. digitatum	15.8 ± 0.83	20.5 ± 0.50	34.0 ± 1.22	12.0 ± 2.55	18.5 ± 1.12	26.0 ± 1.22

Table 7 Minimum fungicidal concentration (MFC) of PTS and PTSO against phytopathogenic fungi

Species	MFC (µg/ml)	
	PTS	PTSO
G. candidum	156.25	78.125
Alternaria spp	78.125	39.06
Phyllosticta spp	156.25	39.06
P. cinnamomi	156.25	78.125
F. oxysporum	78.125	19.53
F. graminearum	39.06	9.76
P. expansum	625	312.5
P. digitatum	312.5	156.25

and 500 µg/g, being less pronounced with 500 µg/g. During the 45 days of the assay, *F. oxysporum* could not be isolated from the soil treated with 3 applications of PTS and PTSO at both 100 and 500 µg/g, which suggests a greater efficacy of the treatments based on repeated lower doses compared to those including a single initial dose at a higher concentration.

Figure 8 shows the concentrations of PTS and PTSO detected in soil by HPLC–UV. In each of the treatments, both compounds evolved in the same way over time. Due to their volatility, neither PTS nor PTSO remained at the established concentration in soil 1 h after being applied, time at which the first determination was made ([PTS] = 16.04 ± 0.36 and 112.20 ± 0.1168 µg/g instead of 50 and 250 µg/g) ([PTSO] = 37.02 ± 0.54 and 159.20 ± 2.11 µg/g instead of 50 and 250 µg/g). The steady reduction in PTS and PTSO concentration explains why the treatment based on 3 applications of 100 µg/g of active compounds obtained better results than those based on 1 single dose of 500 µg/g for both, *A. tumefaciens* and *F. oxysporum.*

Additionally, although the doses administered of PTS and PTSO for each application were equal (50 µg/g or 250 µg/g of each, depending on the total concentration of active substance), PTSO was shown to be somewhat more stable since its concentration decreases less drastically.

Discussion

In this work, PTS and PTSO have demonstrated broad antimicrobial activity against phytopathogens that represent a significant threat to many economically important crops, including 6 bacterial and 8 fungal strains.

Organosulfur compounds PTS and PTSO antimicrobial activity had been previously studied against a wide range of human and animal pathogens, including those affecting the poultry industry and aquaculture [2, 12, 74]. Despite of its instability, allicin significant microbicidal effect has been demonstrated on soil-borne plant pathogenic fungi [4] and, to a lesser extent, on phytopathogenic bacteria [84]. However, there is a very little information available that refers to the bioactive properties of PTS and PTSO against phytopathogens and plant pests, and that supports their application within the framework of plant health. Hayat et al. [28] found that potent broad-spectrum biofungicidal effect of *Alliaceae* extracts might not only be attributed to allicin but also to other organosulfur compounds, stressing the importance of identify such compounds and their bioactivity. Previous reports have suggested that onion essential oils, and particularly PTS and PTSO, have potential applications in post-harvest preservation [7, 53]. The present investigation is so far the first study to characterize the activity of these compounds in vitro against a wide range of target organisms implicated in plant disease breakouts.

Our results indicate that PTSO is more active than PTS against bacteria, what was also reported by Sorlozano-Puerto et al. [74]. Similarly, the higher fungicidal activity of PTSO was also observed in comparison to PTS but,

Table 8 In vitro antimicrobial activity of PTS and PTSO against phytopathogenic fungi via the gas phase, expressed as the average diameter ± standard deviation of inhibition zone (mm)

Species	PTS (µg/µl)			PTSO (µg/µl)		
	5	10	25	5	10	25
G. candidum	28.0 ± 3.16	32.0 ± 3.16	39.0 ± 2.24	34.8 ± 3.49	41.3 ± 3.96	47.8 ± 2.49
A. alternata	27.0 ± 2.12	36.3 ± 2.59	42.5 ± 3.28	26.5 ± 1.50	34.3 ± 2.68	37.0 ± 1.22
Phyllosticta spp	38.8 ± 3.03	49.8 ± 1.92	57.0 ± 1.87	46.3 ± 2.17	64.8 ± 2.38	70.3 ± 1.09
P. cinnamomi	25.3 ± 2.17	33.3 ± 1.92	47.8 ± 1.48	39.0 ± 3.08	52.0 ± 1.22	57.3 ± 1.30
F. oxysporum	50.0 ± 1.58	59.8 ± 2.38	68.8 ± 2.86	37.5 ± 1.80	50.8 ± 3.49	57.0 ± 3.39
F. graminearum	49.5 ± 3.64	55.5 ± 2.50	65.8 ± 1.92	26.0 ± 1.58	31.5 ± 2.29	46.3 ± 1.92
P. expansum	34.3 ± 0.83	39.3 ± 0.83	47.5 ± 2.06	34.3 ± 3.49	45.3 ± 0.83	49.5 ± 1.80
P. digitatum	12.8 ± 1.92	16.5 ± 1.12	22.3 ± 3.34	10.8 ± 0.83	14.5 ± 1.12	21.8 ± 1.09

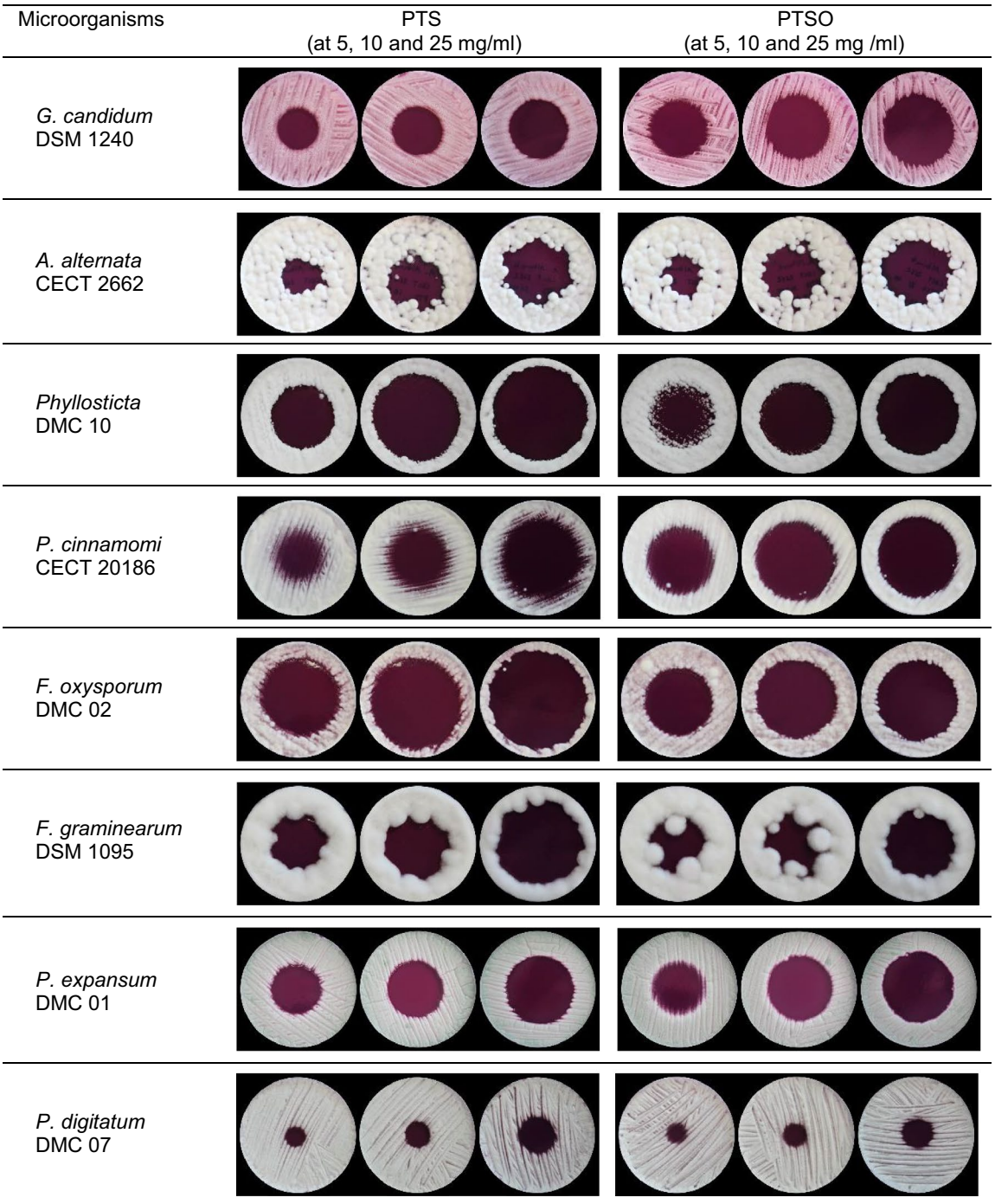


Fig. 2 Antifungal activity of the gaseous phase of PTS and PTSO against phytopathogenic fungi

(See figure on next page.)

Fig. 3 Mycelial growth of fungi on agar plates supplemented with PTS (left column) and PTSO (right column) at 25, 50, 100, 250 and 500 µg/ml over time compared to control, using the Dunnett test at a 95% confidence level. Values are means with SD in bars. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$ respect to control. **1** PTS **2** PTSO **a** *G. candidum* **b** *A. alternata* **c** *Phyllosticta* spp. **d** *P. cinnamomi*

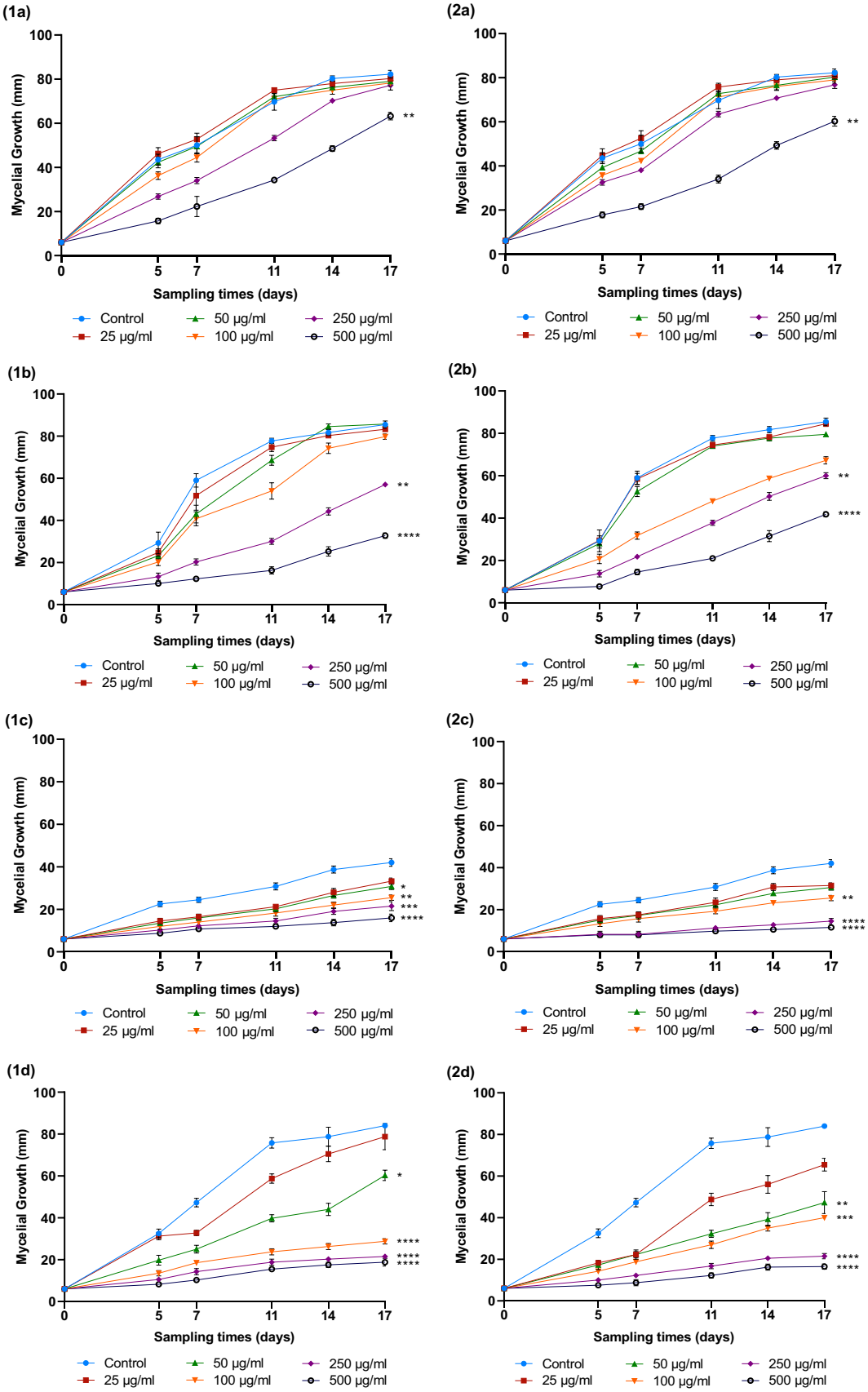


Fig. 3 (See legend on previous page.)

interestingly, no differences were appreciated between both compounds with regard to the fungistatic effect, which was assessed through the mycelial growth inhibition method. This has already been described by our group in previous research on the antifungal activity of PTS and PTSO against the soil-borne pathogenic fungus *V. dahliae* [23]. Additionally, according to the results presented in this study, both compounds preserve the antimicrobial activity in their gas phase. However, when compared the MFC and MBC values of PTS and PTSO, it was found that their antifungal effect was higher than their antibacterial effect. These results are consistent with the data obtained in previous in vitro investigations in which the antimicrobial capacity of PTS and PTSO was examined against yeasts and bacteria for their potential use in human therapy [75].

On the basis of our findings, the persistence of a soilborne pathogenic bacterium and fungus in soil when treated with PTS and PTSO was assessed. As acknowledged by Arnault et al. [4], the potential of thiosulfinates contained in *Allium* species to be employed in pest management and, in particular, to serve as biofumigants, has yet to be investigated. From the results obtained in this study it can be concluded that PTS and PTSO have a significant effect on soilborne pathogens. Furthermore, we have established a correlation between the antimicrobial effect of both compounds and their persistence in soil. PTS and PTSO quantification in soil by HPLC–UV demonstrated that both rapidly volatilize in varying degrees, being PTSO more stable than PTS. Due to their high volatility, the concentration of PTS and PTSO in soil decreases immediately after the application, which allows the microorganism to recover. It can therefore be concluded that their antimicrobial activity in soil is not only linked to the concentration applied initially but, more importantly, to their persistence in soil, which highlights the importance of establishing an optimal application protocol based on repeated doses over time. What is more, despite not being the objective of this study, the evaluation of the antifungal activity of PTS and PTSO in olive plantlets carried out in a previous work of our group evidenced the absence of phytotoxicity, not observing any effect on the appearance or development of the plants [23]. The absence of phytotoxicity and the non-permanence of the compounds in soil supports the safety profile of PTS and PTSO as well as their use as a sustainable alternative for pre- and post-plant soil fumigation. However, further studies on how these compounds influence plant physiology and stress response mechanisms are needed.

Additionally, the results obtained in the soil sanitization assay indicate that PTS and PTSO antifungal activity is higher than their antibacterial activity, which had previously been suggested according to the in vitro antimicrobial activity tests performed in this investigation. While the high permeability of volatile organosulfur compounds through the phospholipid membranes and their ability to interact with thiols containing compounds may explain their antimicrobial activity [28, 50], the higher permeability of the fungal chitin cell wall compared to the peptidoglycan cell wall of bacteria support our findings that PTS and PTSO influence fungal growth to a greater extent [39, 43]. Another explanation might be the interaction of PTS and PTSO with small secreted cysteine-rich proteins (SSCPs), specific of the secretomes of filamentous and dimorphic fungi [25]. These molecules present a broad functional versatility and, although most of them remain unclassified, it has been demonstrated that SSCPs have an essential role in fungal reproduction and dispersal [13, 86, 87]. They are also involved in environmental interactions, fungi-plant interactions and substrate colonization [26, 83]. Lu and Edwards [46] functionally characterized SSCPs of *F. oxysporum* by their detection in the secretome by nano LC–MS/MS and the subsequent identification in infected wheat heads through gene expression profiling, thus demonstrating the role of SSCPs in the pathogenesis of the disease caused by *F. oxysporum* in wheat.

According to several studies, antimicrobial plant derived compounds might also possess insecticidal and repellent activity within their bioactive properties [72]. Jiang et al. [32] found that linalool is responsible for the insecticidal and repellent activities of *Cinnamomum camphora* essential oil against cotton aphids. Similarly, other authors showed that glucosinolates extracted from *Tropaeolum tuberosum* and capsaicinoids extracted from *Capsicum Chinese* can successfully control *Aphis cytisorum* [16]. Insecticidal and repellent activity of volatile compounds of onion and garlic had already been established against aphid *Myzus persicae* by contact toxicity test and choice test using commercial essential oils [29]. In addition, a non-standardized blend of aqueous extracts of onion and garlic was effectively used as an alternative pesticide that was able to

(See figure on next page.)

Fig. 4 Mycelial growth of on agar plates supplemented with PTS (left column) and PTSO (right column) at 25, 50, 100, 250 and 500 µg/ml over time compared to control, using the Dunnett test at a 95% confidence level. Values are means with SD in bars. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$ respect to control. **1** PTS **2** PTSO **e** *F. oxysporum* **f** *F. graminearum* **g** *P. expansum*. **h** *P. digitatum*

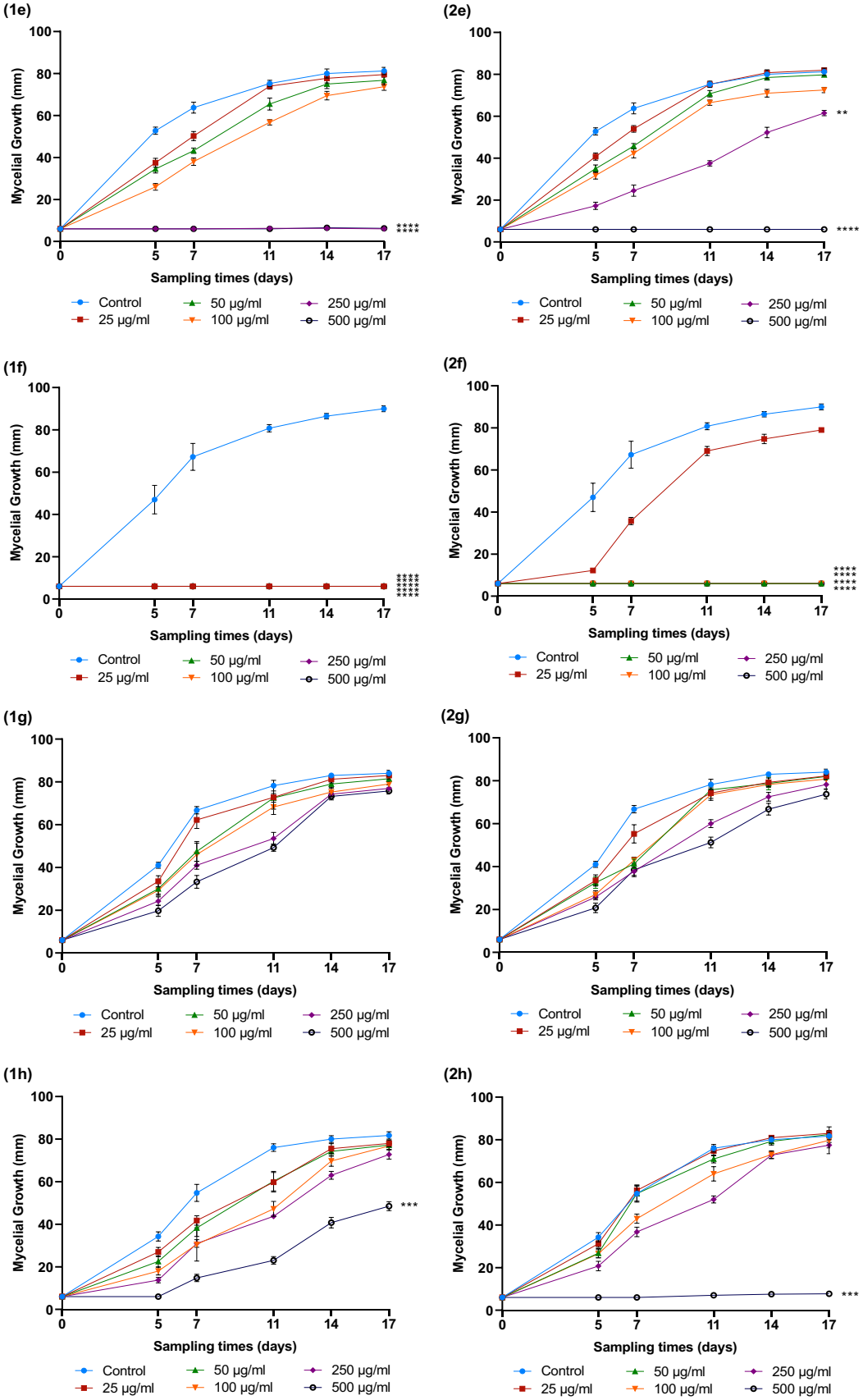


Fig. 4 (See legend on previous page.)

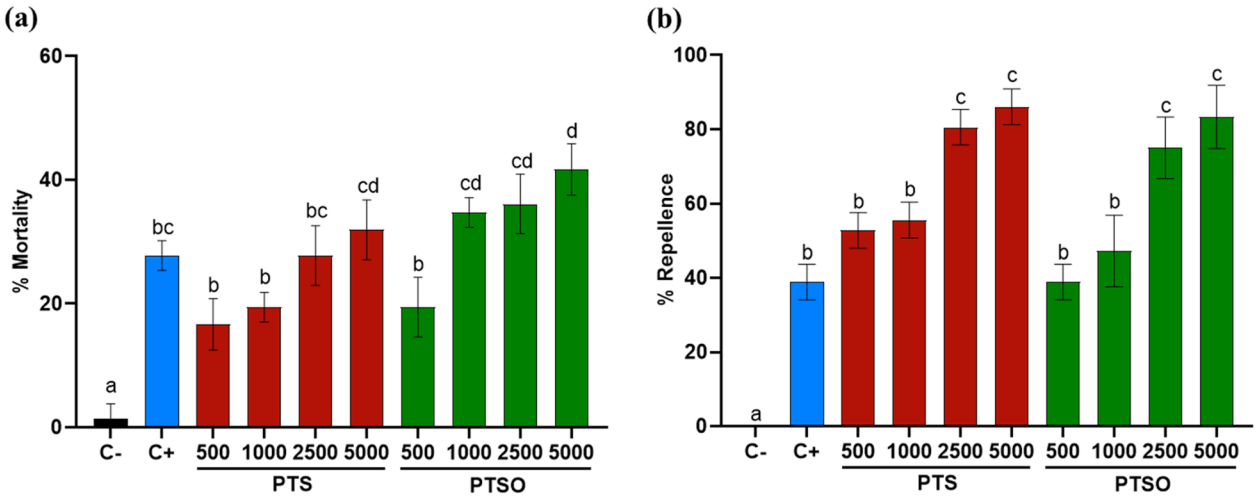


Fig. 5 In vitro activity of different concentrations of PTS and PTSO against *A. gossypii*: **a** Mortality rate due to contact toxicity expressed as percentage, **b** Repellent activity expressed as percentage. Each panel includes a group in which the leaves cuttings were immersed in water (C –), in a commercial biocide/pesticide (C +) and in PTS or PTSO at 500; 1000; 2500 and 5000 µg/ml. For both panels, bars with different letters indicate significant differences according to Tukey test ($p < 0.05$)

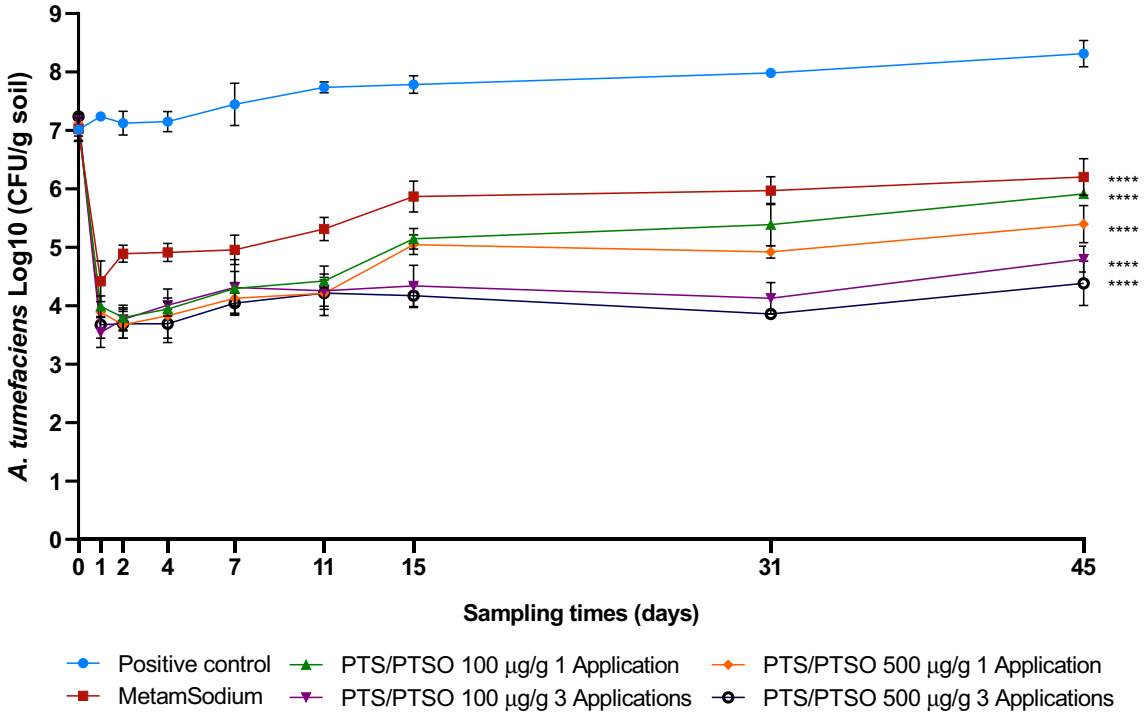


Fig. 6 Density of *A. tumefaciens* in soil expressed as Log10 CFU/g of soil over time. Significant reductions in the population of each treated microcosm in comparison with the non-treated group (control) were established according to the Dunnett test at a 95% confidence level. Values are means with SD in bars. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$ respect to control

significantly reduce aphids' population of different species infecting date palm trees, as reported by Ali Al-Shuraym [3]. In this study we have also demonstrated the effect of PTS and PTSO against the aphid species *A. gossypii*. Our in vitro results showed that the mortality rates achieved by both compounds equals that of commercial chemical insecticide from a concentration of 0.25% PTS and 0.1% PTSO. What is more, the repellent

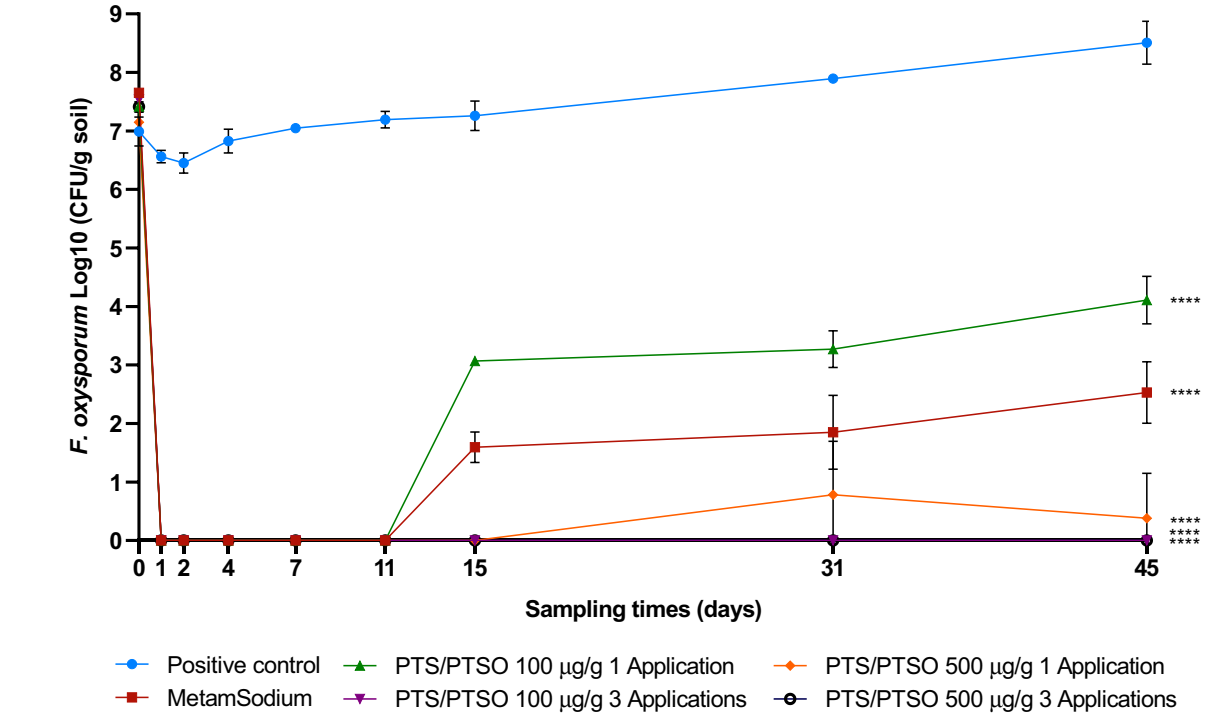


Fig. 7 Density of *F. oxysporum* in soil expressed as Log10 CFU/g of soil over time. Significant reductions in the population of each treated microcosm in comparison with the non-treated group (control) were established according to the Dunnett test at a 95% confidence level. Values are means with SD in bars. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$ respect to control

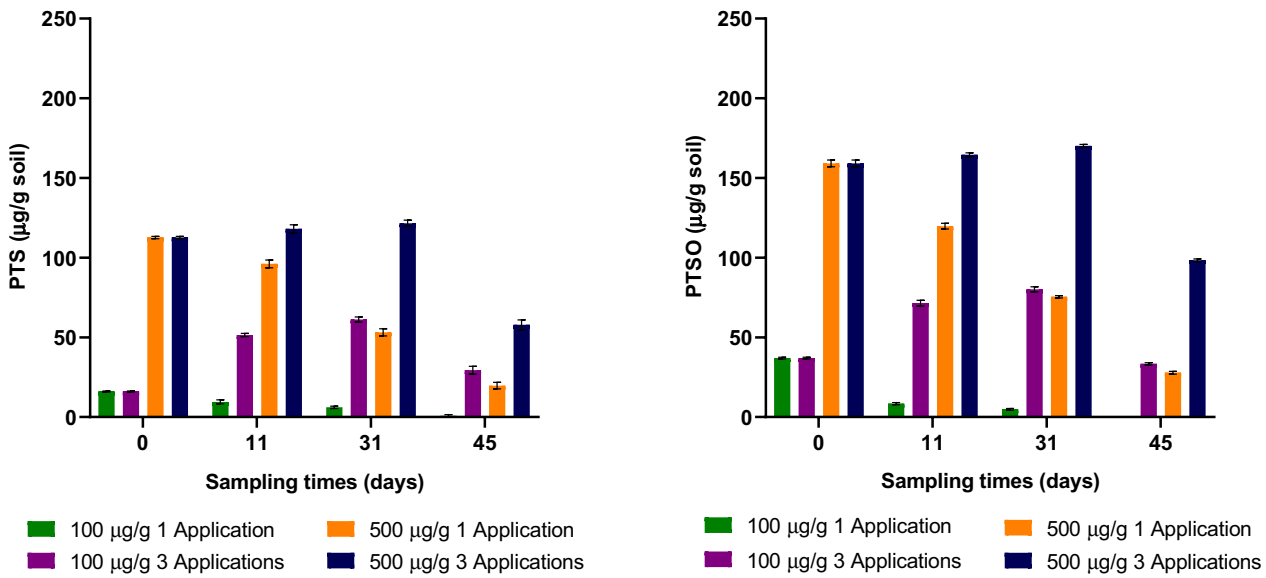


Fig. 8 Concentration of PTS (left) and PTSO (right) reached in soil 1 h (day 0), 11 days, 31 days and 45 days after the first application of the treatment

capacity exhibited by 0.1% PTS and PTSO significantly exceed the effect of DEET. While PTSO shows greater biocidal activity against *A. gossypii*, the greater volatility of PTS compared to PTSO, as demonstrated by HPLC–UV determination, explains why PTS has a greater repellent capacity.

Bioactive compounds from plant material with proven antibacterial, antifungal and insecticidal activity are an

important natural source for the development of new environmentally safe plant protection products [5, 31]. Onion is one of the most cultivated and consumed vegetables worldwide [78]. Taking into account those that do not reach the consumer due to their low quality and the inedible parts, onion processing creates massive wastes that are a rich source of bioactive compounds [6, 52]. Therefore, to orientate the valorisation of onion solid wastes towards the formulation of novel and sustainable products suitable for agricultural production could not only reduce the environmental impact but also cover important needs for the agrifood sector [36, 40].

Even thought our results suggest that PTS and PTSO from *A. cepa* display strong activity against pathogenic microorganisms and aphids, and provide useful information that support their use for crop disease control, the present work has not evaluated how these organosulfur compounds influence soil microbial populations, natural enemies or pollinators. Therefore, further studies should focus on the analysis of the effect of PTS and PTSO on soil microbiome and non-target species.

Conclusions

PTS, and specially PTSO, showed antimicrobial effect against a wide range of bacteria and fungi infecting plants. This study revealed that the degree of efficacy of PTS and PTSO depend on the target species, being more effective against the fungal strains evaluated. Despite their rapid volatilization from soil, the combination of these compounds successfully controlled soilborne microorganism population. Both of them had similar, if not more, biocidal and repellent effect than commercial fumigants. Although PTS and PTSO will be further evaluated in field experiments for potential control of pathogen populations in crops, these results encourage their use for the development of sustainable biopesticides that contribute to environmental health. Moreover, both metabolites are found to be promising candidates for Integrated Pest Management, whose bases include sustainable pest control, reduction of pesticide residues and the use of natural resources.

Abbreviations

PTS	Propyl-propane thiosulfinate
PTSO	Propyl-propane thiosulfonate
OSCs	Organosulfur compounds
EtOAc	Ethyl acetate
CECT	Spanish collection of type cultures
DSMZ	German collection of microorganisms and cell cultures
WHC	Water holding capacity
MBC	Minimum bactericidal concentration
CLSI	Clinical and Laboratory Standards Institute
MFC	Minimum fungicidal concentration
DEET	N, N-diethyl-meta-toluamide
HPLC–UV	High-performance liquid chromatography UV detector
MeOH	Methanol
SSCPs	Small secreted cysteine-rich proteins

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Author contributions

AB and DG designed the study. AF-P, AB and AKM conducted in vitro analyses. AF-P and DG-L conducted experiments with aphids. AF-P and MDC-Y performed soil sanitization assays. LG-M and JMdlT conducted HPLC–UV determination. BB-D and SL-P provided soil samples and data. AB supervised the experiments. AF-P analyzed data and wrote the manuscript. AB, DG and EG reviewed the manuscript. All authors read and approved the manuscript.

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Availability of data and materials

All data generated during this study are included in this manuscript.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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