International Journal of
Plant Biology

MDPI

Article

Inhibitory Effect of CUSTOS, a Formulated Allium-Based Extract, on the Growth of Some Selected Plant Pathogens

Oluwashina Oladejo and Jafargholi Imani *

Institute of Phytopathology, Research Centre for BioSystems, Land Use and Nutrition, Justus Liebig University Giessen, D-35392 Giessen, Germany; oluwashina.oladejo@agrar.uni-giessen.de
* Correspondence: jafargholi.imani@agrar.uni-giessen.de

Abstract: Plants are in intimate association with a great diversity of pathogenic and mutualistic microbes that use host plants for proliferation. Plants, in turn, have evolved mechanisms that are contingent upon their innate immune system to resist perceived biotic stresses. The objective of this work is to determine the antimicrobial properties of an allium-based antimicrobial formulation named CUSTOS on the growth of plant pathogenic microorganisms such as fungi, oomycetes, and bacteria. Two anthracnose-related species of the fungal genus *Colletotrichum*, *Colletotrichum gloeosporioides*, the oomycete *Phytophthora cactorum*, and the bacterium *Xanthomonas fragariae* associated with strawberry plants were tested in vitro. Furthermore, two fungi *Alternaria dauci* and *Botrytis cinerea*, associated with carrot plants, were tested in planta. CUSTOS inhibited the growth of all plant pathogens tested. We found that both curative and preventive planta treatments with CUSTOS inhibited the growth of *Alternaria dauci* and *Botrytis cinerea* in carrots. Furthermore, the differential expression levels of the PR 10 genes were correlated with the magnitude of infection. We also found that the field application of CUSTOS on strawberry plants results in a reduction of fungal pathogens on strawberry fruits stored under refrigeration. In summary, CUSTOS may induce pathogen resistance in fruit and vegetable plants and can be used as both a curative and a preventive against rotting and disease.

Keywords: plant extract; CUSTOS; antimicrobial effects; curative and preventive treatment

Citation: Oladejo, O.; Imani, J. Inhibitory Effect of CUSTOS, a Formulated Allium-Based Extract, on the Growth of Some Selected Plant Pathogens. *Int. J. Plant Biol.* **2022**, *13*, 44–54. https://doi.org/10.3390/ijpb13020006

Academic Editor: Adriano Sofo

Received: 23 March 2022
Accepted: 11 April 2022
Published: 13 April 2022

1. Introduction

Plant pathogens and pests account for severe and increasing crop losses worldwide, amounting to $30–$50 billion annually [1,2]. Fungal pathogens quickly develop a resistance to fungicides, increasing the cost of chemical plant protection. Moreover, public concerns about the hazards of agrochemicals on human health and the environment are growing. The development of resistance to common fungicides and increasing restrictions on the use of toxic material in the environment has given an impetus to the search for novel plant protectants that interfere with fungal pathogenicity factors. Natural products with fewer negative environmental impacts can replace synthetic fungicides. Several non-phytotoxic plant extracts with a proven effect on germination and fungal spore viability have been used successfully for plant disease control, with no harm to the environment [3,4]. Edible, medicinal, and spice plants contain a large number of secondary metabolites that retard or inhibit the growth of pathogenic microorganisms such as bacteria and fungi [5,6]. These secondary metabolites are referred to as allelochemicals [7], support the plant's innate immune response elicited by microbial pathogens and pests, and are produced in roots, seeds, flowers, stems, and leaves, highlighting their physiological importance [8]. The antimicrobial compounds in plant materials are found in the essential oil isolated from leaves (rosemary, sage, basil, oregano, thyme, and marjoram), flowers or buds (clove), bulbs (garlic and onion), seeds (caraway, fennel, nutmeg, and parsley), rhizomes (asafoetida), fruits (pepper and cardamom), or other parts of plants [9]. In the context of agricultural pest management, botanical biopesticides are best suited for use in organic food production

in industrialized countries and could also play a greater role in the production and post-harvest protection of food products in developing countries [10]. Compounds of plant origin are best suited for controlling plant diseases because of their low risk of health and environmental hazards with reduced possibility of resistance development by pathogens and pests [11]. They may have less detrimental effects on seed viability, plant growth, and food quality with the application of the right concentrations [12].

Several plant species contain antimicrobial components such as flavonoids, phenolics, and triterpenoids in abundant amounts that exhibit a significant protective effect on H_2O_2-mediated injury in rat adrenal medulla PC12 cells at low concentrations and can also prevent pathogenic growth when applied to infected crops [13]. The extract of *Glycyrrhiza glabra* inhibits the growth of some Gram-negative bacteria such as *Salmonella* species, *Shigella* species, and *E. coli* [14]. Extracts from *Yucca schidigera* also have antibacterial activity, which was attributed to the presence of saponin, a compound found to inhibit microbial growth through hemolytic activity [15]. The control of Bacterial Leaf Spot (BLS) caused by seed-borne xanthomonads using biopesticides in tomatoes has provided an alternative control approach against copper-resistant strains present in Tanzania [16]. Moreover, plant extracts from *Aloe vera, Coffea arabica*, and *Yucca schidigera* are potential candidates for seed treatment against seed-borne Xanthomonads in tomatoes [17]. The most commonly and sustainably used biopesticides in agricultural practice are extracts of the Neem tree (*Azadirachta indica*, A. Juss) and garlic (*Allium sativum*, L.), essential oils from Nettle (*Urtica* spp.), rue (*Ruta graveolens*), thyme (*Thymus vulgaris*), and tea tree (*Melaleuca alternifolia*) [10,18]. In particular, compounds with phenyl groups in their structures, such as carvacrol, eugenol, and thymol, are highly active against plant pathogens [19].

An Allium-based antimicrobial formulation from garlic and onion was recently tested for antimicrobial properties in several applications. The volatile antimicrobial substance allicin, a thiosulfinate with two alkyl groups as carbon chains (diallylthiosulphinate), is synthesized in garlic [20] when the tissues are damaged and the substrate alliin (S-allyl-L-cysteine sulfoxide) is converted by the enzyme alliin lyase. Allicin is readily membrane-permeable and undergoes thiol-disulfide exchange reactions with free thiol groups in proteins. Allicin applications reduced seed-borne *Alternaria* spp. on the carrot, *Phytophthora* leaf blight on tomato tuber blight on potato, *Magnaporthe* on rice, and downy mildew (*Peronospora parasitica*) on *Arabidopsis thaliana* [21,22]. The product named CUSTOS (former name VEG'LYS) was developed as an antimicrobial product for surface sterilization of seeds and was reported to be an effective antimicrobial product for use on seeds of bitter gourd and globe artichoke [23,24]. The product was also used to maintain nutrient quality and extend the shelf life of strawberries, with no effect on the taste [25]. The antimicrobial effects of CUSTOS were demonstrated against *Fusarium graminearum*, the causal agent of Fusarium head blight and root rot of cereals as well as powdery mildew caused by the ascomycete fungus *Blumeria graminis* [26].

In this study, the antimicrobial properties of CUSTOS were further evaluated against some of the most devastating phytopathogenic microorganisms in vitro and applied in planta. The effect of CUSTOS against pathogens was analyzed by in vitro assays and the number of pathogens was determined by qPCR after CUSTOS treatment on the experimental plants. Additionally, the role of CUSTOS as a potential systemic resistance inducer was determined by the evaluation of the *PR* gene expression upon curative and preventive CUSTOS treatment on plants.

2. Materials and Methods

The commercial product CUSTOS was obtained from D2Bio, Inc. (http://d2bioinc.com/ accessed on 22 March 2022). CUSTOS components, eg., dialylsulfid; dialyldisulfid, and dialyltrisulfide are from onion, leek, and garlic, and extracted, concentrated, and formulated. Licitin serves as a carrier. Strawberry (*Fragaria ananassa* var. cherry) and carrot (*Daucus carota ssp. sativus*, var. Rotin) plants were investigated following inoculation by the two fungal pathogens *Alternaria dauci* and *Botrytis cinerea*. Carrot plants were grown

in pots in vermiculite with a grain size of 0–3 mm in a plant-growing chamber under 16 h light/8 h dark intervals (light intensity approx. 160 µmol m^{-2} s^{-1}) at 24 °C with 60% relative humidity.

2.1. In Vitro Assays of CUSTOS Effects on Pathogen Growth

Three primary growth media were used, namely a malt extract peptone agar for *Colletotrichum gloeosporioides*, V-8 Juice agar for *Phytophthora cactorum*, and nutrient agar for *Xanthomonas fragariae*. Different concentrations of CUSTOS (0%, 0.01%, 0.05%, and 0.1% (*v*/*v*)) were added to the media when allowed to cool down after autoclaving. Four plates of 25 mL medium in 100 mm-diameter petri dishes were prepared for each CUSTOS concentration. The inoculation of microbes onto the respective media was conducted using agar blocks with the help of a sterilized cork-borer and a needle. Fungal and oomycete growth was quantified using ImageJ software (http://imagej.nih.gov/ij/ accessed on 22 March 2022). The control plate served as a 100% growth area. For the bacterial growth assay, a 10 mL LB medium (Luria-Bertani broth: tryptone 1% (*w*/*v*); sodium chloride (NaCl) 1% (*w*/*v*); yeast extract 0.5% (*w*/*v*) was pipetted into four different 25 mL flasks containing four different concentrations of CUSTOS, namely; 0.0%, 0.01%, and 0.02%, and was inoculated with a small colony of the bacterium, *Xanthomonas fragariae*, using a sterilized inoculation loop. The 25 mL flask containing 0% served as the control experiment. The bacterial activity was measured by the optical activity (OD_{600}) 48 h post-treatment.

2.2. Inoculum Preparation and in Planta Pathogen Assays

Alternaria dauci and *Botrytis cinerea* conidia were collected from sporulation medium (sucrose, 20 g; $CaCO_3$, 30 g; agar, 20 g; pH 7.4) and potato dextrose agar (dextrose, 15 g; potato extract, 4 g; water, 1 l; agar, 15 g; pH 5.6), respectively, by pouring Tween 20 water (0.05% *v*/*v*) and gently scrubbing with a sterile glass rod. Conidia suspensions were filtered through a single-layered, sterilized Miracloth and the remaining dislodged conidia were removed by flooding the media plates with sterilized distilled water into a 50 mL falcon tube and centrifuged at 3500 rpm for 10 min. The supernatant was removed and the pellet was re-suspended in 20 mL of sterile deionized water. Conidia suspensions were adjusted to 10^8 conidia mL^{-1} and 10^3 conidia mL^{-1}, respectively, by a hemocytometer (Fuchs Rosenthal). Conidia suspension from both pathogens was then sprayed on carrot plants (*Daucus carota* var. Rotin) with the plants incubated in white plastic boxes lined with papers and maintained under 100% relative humidity, with constant illumination from a white light tube (Philips TLD 36 W/830 HF) in a plant growth chamber. Infection was evaluated at 7 dpi for *Botrytis cinerea* and 29 dpi for *Alternaria dauci*.

2.3. Experimental Procedure for Carrot Plants and Pathogenic Fungi

This experimental procedure involved curative and preventive methods. In the curative method, 10 mL each of *Alternaria dauci* and *Botrytis cinerea* conidia suspension (10^8 conidia mL^{-1} and 10^3 conidia mL^{-1}, respectively) was sprayed on three carrot plants followed by 10 mL of 0.03% (*v*/*v*) CUSTOS exactly 48 h after pathogen inoculation on the plant materials and vice versa for the preventive method. The control plants were sprayed with sterilized distilled water. All carrot plants were put in white plastic boxes and maintained under 100% relative humidity by spraying the plastic boxes with sterilized water under constant illumination from a white light tube (Philips TLD 36 W/830 HF). The complete experimental setup was observed for *Botrytis cinerea* 7 dpi and for *Alternaria dauci* 29 dpi.

2.4. Treatment of Strawberry Fields with CUSTOS

The strawberry field (spring season 2018; Giessen, Germany) was divided into four rows with each row containing 25 strawberry plants. Different CUSTOS concentrations were used. The first row was sprayed with sterilized water, the second row with 0.1% *v*/*v*, the third row with 0.2% *v*/*v*, and the fourth row with 0.5% *v*/*v*. The application of CUSTOS

was conducted twice per week for one month. Nine ripe fruits were harvested separately from each row and stored in a refrigerator in an uncovered small white box and checked daily for the presence of fungal growth.

2.5. Fungal DNA Quantification in Plant

Genomic DNA was extracted from carrot callus and pure cultures of *Alternaria dauci* and *Botrytis cinerea* to test the specific primers with qPCR (Table 1). Samples from each experimental material (uninfected carrots, curative material, preventive material, and carrot plants infected with *Alternaria dauci* or *Botrytis cinerea*) were collected in 50 mL falcon tubes and immediately frozen by liquid nitrogen and then stored at −80 °C. Each sample was analyzed using DNA extraction for fungal gene quantification using the QIAGEN DNeasy plant mini kit, followed by polymerase chain reaction (PCR) for the amplification of the fungal gene of interest.

Table 1. List of all the primers used in this study.

Primer's Names	Sequence (5′->3′)
qpcr1.01_F	CTTGACATACGACTCCACCG
qpcr1.01_R	TATGGCCGTGGTCTTGGTA
qpcr1.02_F	TGAGGTTGAGGCTCCTTCC
qpcr1.02_R	CTGACGGTTCCAACACCAC
qpcr1.03_F	AGACAGCCTTGTGGTACTGGA
qpcr1.03_R	GCTTCCACTGTTGCACTCAA
Bc1-β-tubulin_F	GTTACTTGACATGCTCTGCCATT
Bc1-β-tubulin_R	CACGGCTACAGAAAGTTAGTTTCTACAA
qpcr-Ubiquitin_F	AAGCCCAAGAAGATCAAGCA
qpcr-Ubiquitin_R	TCAAAATGATTGGCCATGAA
Gpd_F	AAGCTTCCCCAAGCACTCACAA
Gpd_R	CTGCGTTCTGCAGCTGTAGAGA
qpcrActin_F	CCACTGAACCCAAAAGCAA
qpcrActin_R	CCGTGTGGCTAACACCATC

2.6. RNA Extraction

For gene expression analysis, the plant sample materials were crushed into fine powder in liquid nitrogen and the total RNA was extracted using the TRIzol® Reagent. The RNA samples were all treated with DNAse to remove all the traces of remaining genomic DNA. The experiment was performed in PCR tubes with a total volume of 10 µL. Incubation followed immediately, as the DNAse was added to each sample for 30 min at 37 °C. Subsequently, 2 µL 0.25 M EDTA was added and incubated at 65 °C for 10 min. One µg of RNA (5 µL from the previous preparation) was used for cDNA synthesis with Quantiscript Reverse Transcriptase (Thermo Fisher Scientific Inc., Darmstadt, Germany), with a total volume of 20 µL. The quality of the cDNA synthesized was checked on an Agarose gel (1 µL from each sample) in a PCR reaction with the carrot *ubiquitin* gene (forward and reverse primers).

2.7. Quantitative PCR (qPCR)

Two hundred ng/µL of cDNA as a template and gene-specific primers (Table 1) were used for the quantitative real-time PCR in an Applied Biosystems 7500 Fast real-time thermocycler (ThermoFisher Scientific Inc., Darmstadt, Germany). A 96-well plate was used for the qPCR reaction with 20 µL of the reaction mixture pipetted into each well where the amplification process was performed with SYBR Green JumpStart TaqReadyMix

(Sigma-Aldrich GmbH, Taufkirchen, Germany). Each sample was performed with three repetitions. Each run of the qPCR reaction included a non-template control to detect an exclude potential test reagent contamination. Respective melting curves were determined at the end of each cycle to ensure the amplification of only one PCR product. C_t values were calculated with the 7500 Fast software supplied with the instrument. Transcript levels of the target genes were determined via the ΔCt method with a reference gene [27]. The relative expression of each pathogen gene versus the plant reference gene (carrot ubiquitin) was calculated, then the ratio of expression and the biomass of fungal DNA for each sample was determined against the control sample (carrot plants). All statistical calculations were made the via unpaired two-tailed Student's *t*-test.

3. Results

3.1. CUSTOS Has Strong Antimicrobial Effects against Various Microbial Pathogens in In Vitro Assays

First, we determined the antimicrobial effects of different CUSTOS concentrations in inhibiting the in vitro growth of microbial pathogens in axenic culture. CUSTOS (0.01% and 0.05% *v*/*v*) completely inhibited the axenic growth of the oomycete *Phytophthora cactorum* (Figure 1a,b). Moreover, 0.05% and 0.1% CUSTOS also completely inhibited the axenic growth of the ascomycete *Colletotrichum gloeosporioides*, while 0.01% CUSTOS strongly but not completely inhibited its growth (Figure 2a,b). The growth of the bacterium *Xanthomonas fragariae* in a liquid medium was also strongly inhibited by 0.01% and 0.02% CUSTOS, as shown by the significantly lower OD_{600} values as compared to the untreated control (Figure 3).

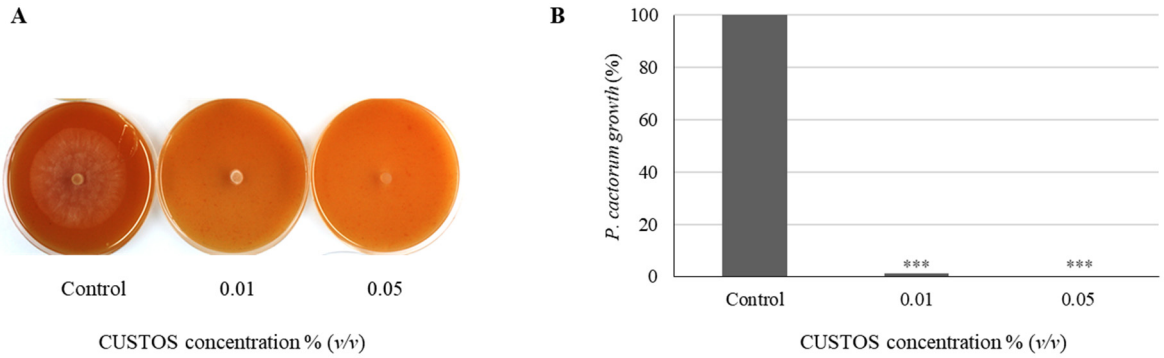

Figure 1. Inhibition by CUSTOS of fungal growth under the lab condition. After 5 dpi, the concentration at 0.05% completely inhibited the growth of *Phytophthora cactorum*. The concentration at 0.01% allowed minimal pathogen growth (**A**,**B**). Bars represent the standard deviation of three replicates and significant changes are marked: *** $p < 0.001$ (Student's *t*-test).

3.2. Evaluation of Fungal Growth and PR Gene Expression upon Curative and Preventive CUSTOS Treatments

Next, we tested the effects of CUSTOS on the two devastating ascomycete pathogens *Alternaria dauci* and *Botrytis cinerea* during carrot plant infection. Fungal conidia (10^8 and 10^3 conidia ml^{-1}, respectively) were sprayed in a volume of 10 mL per plant. At 7 dpi, carrot leaves and petioles showed heavy symptoms of gray mold caused by *Botrytis cinerea*. Gradual fungal growth was observed in plants inoculated with *Alternaria dauci* conidial solutions, taking up to six weeks for the total infection of all parts of the plants. In contrast, the untreated plants were strongly infected and infection symptoms were visible in virtually every part of the plants (Figure S1). To estimate the level of infection on both CUSTOS treated and untreated plant material, we quantified the expression of *Alternaria dauci Glyceraldehyde-3-phosphate dehydrogenase* (*Adgpd*) and *Botrytis cinerea β-tubulin* genes (*Bc1*) by the qPCR and normalized it with the carrot *ubiquitin* gene. Both preventive and

curative treatments of carrot plants with CUSTOS strongly reduced *Alternaria dauci* and *Botrytis cinerea* infections (Figure 4 and Figure S2).

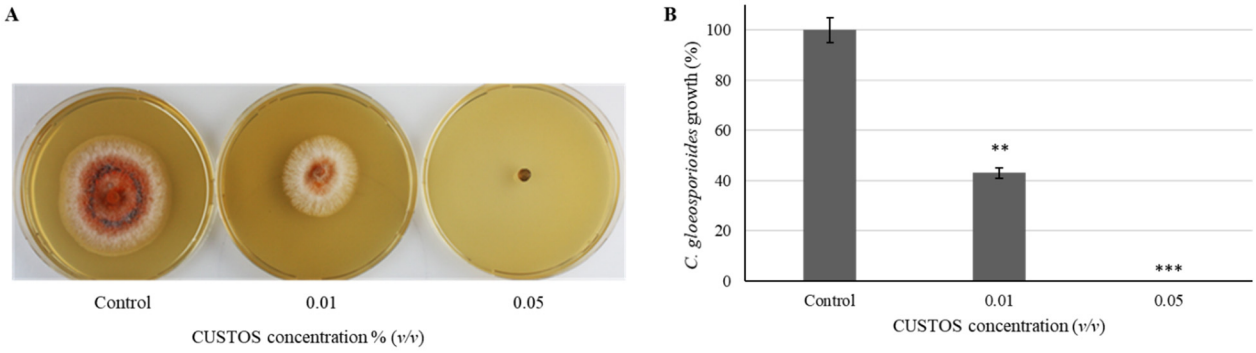

Figure 2. Inhibition by CUSTOS of fungal growth under lab conditions. After 5 dpi, the concentration at 0.05% completely inhibited the growth of *Colletotrichum gloeosporioides*, while less growth was observed at 0.01%. (**A**,**B**). Bars represent standard deviation of three replicates and significant changes are marked: ** $p < 0.01$; *** $p < 0.001$ (Student's *t*-test).

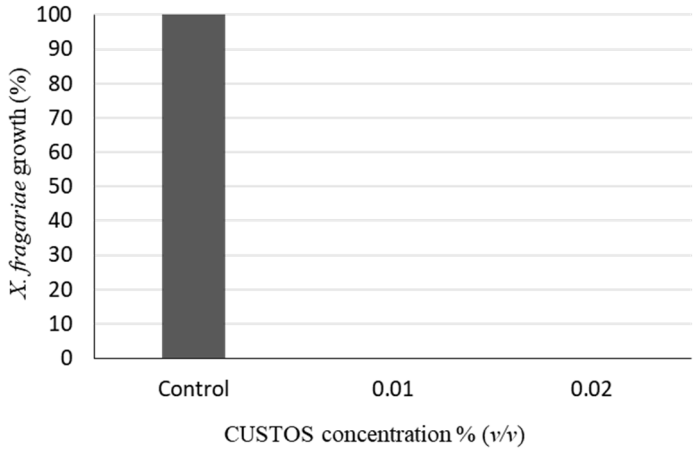

Figure 3. Inhibition by CUSTOS of bacterial growth under the lab condition. All the CUSTOS concentrations tested (0.01% and 0.02%) completely inhibited the growth of *Xanthomonas fragariae*. The bacterial activity was measured by optical activity (OD600) 48 h post-treatment. The experiment was repeated twice. In both cases, no bacteria were grown at 0.01 and 0.02% (*v*/*v*).

Next, we analyzed the expression levels of the genes *Dau c1.01, Dau c1.02*, and *Dau c1.03*, which all belong to the *PR 10* gene family of carrot, in treated vs. untreated samples. The carrot plants were inoculated with *Alternaria dauci* and *Botrytis cinerea*, respectively, and treated with CUSTOS vs. the mock. We found that in all the treated carrot plants the expression of *PR* 10 family genes was significantly reduced compared with the untreated plants (Figure 5), corroborating that the development of the fungi is greatly reduced after the CUSTOS treatment.

3.3. Pre-Harvest CUSTOS Application on a Strawberry Prevented Post-Harvest Pathogenic Growth

To evaluate the effect of CUSTOS in extending the shelf life of strawberries, nine field fruits from each row were harvested and stored in the fridge in an uncovered small box and observed daily for the presence of fungal growth. We found that pre-harvest CUSTOS application in a strawberry field prevents post-harvest pathogenic growth of fruits compared with control samples showing visible fungal presence at 17 days post-harvest (Figure S3).

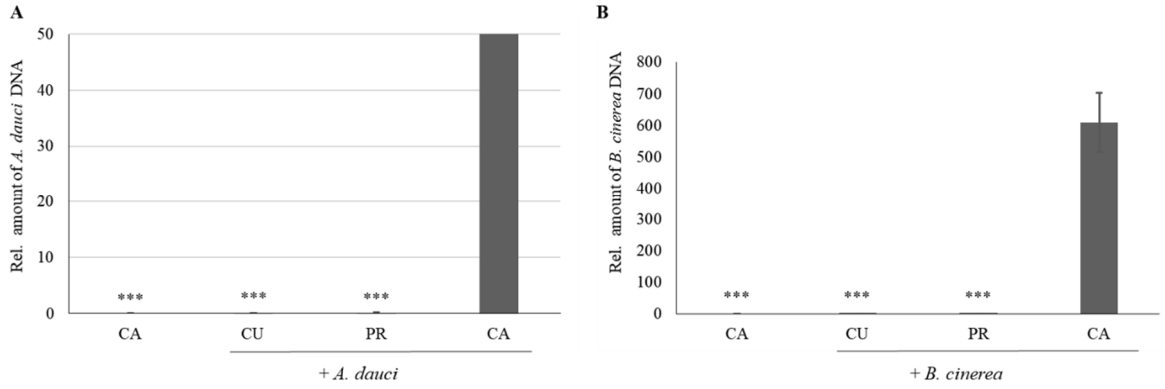

Figure 4. Quantification of fungal DNA in carrot sprayed with fungal conidia. The relative amount of DNA was quantified by qPCR. (**A**) The relative amount of DNA (*Adgpd* gene) of *Alternaria dauci* in carrot plants normalized with the carrot *ubiquitin* gene. (**B**) The relative amount of DNA (*β-tubulin* gene) of *Botrytis cinerea* in carrot plants normalized with carrot *ubiquitin* gene. Bars represent the standard deviation of three replicates and significant changes are marked: *** $p < 0.001$ (Student-test). PR = preventive; CU = curative; CA = control non-treated.

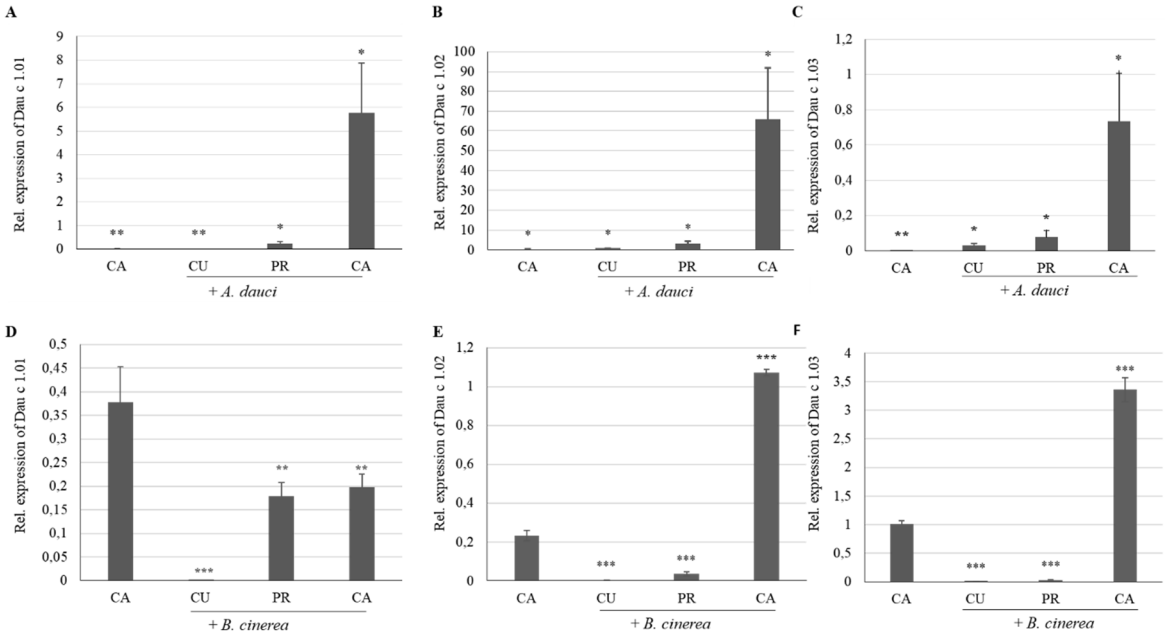

Figure 5. Quantification of *PR* 10 gene expression in carrot sprayed with fungal conidia. The relative expression of three different carrot *PR* 10 genes (syn. *Dau c 1.01; Dau c 1.02* and *Dau c 1.03*) was quantified with qPCR and normalized with carrot *ubiquitin*. (**A**–**C**) Plants sprayed with *Alternaria dauci* conidia. (**D**–**F**) Plants sprayed with *Botrytis cinerea* conidia. Bars represent standard deviation of three replicates and significant changes are marked: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ (Student's *t*-test. PR = preventive; CU = curative; CA = control non-treated.

4. Discussion

Plant extracts are favorable alternatives for fungicides to control phytopathogenic fungi in the presence of bioactive chemicals such as flavonoids, phenols, tannins, and alkaloids. These secondary metabolites are more adaptable, associated with fewer environmental consequences than chemical pesticides, and more acceptable to consumers. Bioactive compounds are part of an innate response elicited by most living organisms and are produced ubiquitously in plant roots, seeds, flowers, stems, and leaves. The bioactive ingredients of CUSTOS (http://d2bioinc.com// accessed on 22 March 2022) are proprietary;

however, previous research on Allium-based compounds indicated that the thiosulphinate allicin and its derivatives account for the antibacterial activity of garlic [28]. Garlic is a well-known plant with anti-microbial activity. Out of 345 plant extracts tested on *Botrytis cinerea*, garlic extract had one of the strongest inhibition reactions [29]. Biologically active compounds derived from garlic, which are known for their broad spectrum of antifungal activity [30–32], have demonstrated that the antimicrobial activity of the garlic (*Allium sativum*) hydro-alcoholic extract against biodeteriogenic fungal and bacterial strains is associated with the presence of allicin and thiosulfinic compounds identified by UHPLC. Allicin disrupts the sulfhydryl protein groups, as well as pools of other thiol stress constituents, such as glutathione [33,34]. Due to the universality of allicin target sites, we predicted that the activity of CUSTOS would be durable, with no anticipated development of microbial resistance. This study tested the antimicrobial properties of CUSTOS on the growth of plant pathogenic microorganisms such as fungi, oomycetes, and bacteria in vitro, using both curative and preventive applications.

4.1. In Vitro Inhibition Assays

The inhibitory activity of CUSTOS was initially evaluated by determining the concentration needed to inhibit microbial colony growth on an agar medium. Both *Colletotrichum gloeosporioides* and *Phytophthora cactorum* growth was inhibited at the concentration of 0.01% (Figures 2 and 3). This result is consistent with those of [24] who have demonstrated the inhibitory activity of CUSTOS (former name: VEG'IYS) on various fungi and oomycetes such as *Alternaria dauci* and *Botrytis cinerea*. A reduction in growth rates of a group of soil-borne pathogens of cereals was observed at the sub-inhibitory concentrations of CUSTOS. It was concluded that the inhibition range might depend on both the pathogen and treatment [35]. Additionally, we could show a strong anti-bacterial activity of CUSTOS on the bacterium, *Xanthomonas fragariae* (Figure 3), which is associated with strawberry plants in vitro at 0.01%. Furthermore, a minimum CUSTOS concentration should be determined as below 0.01%.

4.2. The Curative and Preventive Effects of CUSTOS on Carrot Plants

To evaluate the effects of CUSTOS against pathogens on carrot plants, curative and preventive methods were used. The results confirmed that CUSTOS prevents the growth of the necrotrophic fungus, *Botrytis cinerea*. Recently, several genes and signaling factors have been identified that play roles in pathogenesis, particularly in appressorium formation and penetration, including the NOX complex, MAPK cascades, heterotrimeric G proteins, histidine kinases, and the cyclic adenosine monophosphate (cAMP) signaling pathways [36,37] review article). In the present study, differential expression levels of the *PR 10* gene were analyzed by RT-qPCR after the CUSTOS treatment on infected carrots. Both in treated and untreated plants, the expression level of *PR-10* correlated with the magnitude of infection.

4.3. Pre-Harvest Application of CUSTOS in a Strawberry Field

Strawberry fruits are popular worldwide due to their taste, aroma, soft texture, nutritional, and curative values. The fruit has high anthocyanin, phenol, and vitamin C contents and these compounds are imperative for an effective free radical scavenging activity [38]. However, strawberry fruits are highly susceptible to fungal pathogens. In the past, chemical fungicides were used to improve preservation and provide longer shelf life. Today, WHO regulations prevent the use of chemicals as preservatives and warn about their effects on public health and the environment [25]. The strawberry contains 90 percent water, and the high levels of respiration make it vulnerable to microbial contamination and transpiration, which result in a short shelf life [38]. Some studies suggest increasing the strawberry shelf life by applying ascorbic acid [39], salicylic acid, radiation, and various plant extracts [40]. All these methods have their pros and cons. This study analyzed the effectiveness of CUSTOS in extending the shelf life of strawberry fruits. These results demonstrated that even 17 days post-harvest, 75 percent of fruits treated with CUSTOS were still not infected by

fungi. The control strawberry exhibited a massive loss of the bright red color, juicy texture, and sweetness of the fruits due to the presence of pathogen(s). This was in contrast to fruits treated with different concentrations (0.1 and 0.2%) of CUSTOS, in which no pathogen presence could be ascertained but some had a slight loss of the bright red color and the succulence of the fruits. Most interestingly, in fruits harvested from the row sprayed with 0.5% CUSTOS, the tissue integrity was maintained (there was no trace of pathogen growth; the bright red color, juicy texture, and sweetness was intact, as observed in the graphical result shown) and this concentration did not affect the taste and the sweetness of the fruits.

CUSTOS inhibited the growth of *Botrytis cinerea* and *Alternaria dauci* on carrot plants applied both curatively and preventively. The level of infection or the damage inflicted by the pathogens on the inoculated carrot plants was rated by visual observation of the presence of fungal growth, which was supported by molecular analysis through qPCR. The data generated from molecular quantification of fungal growth in plant samples corroborated with the physical rating of infection levels. These results suggest that these plant extracts (i.e., CUSTOS) have a prospective potential for plant protection and can be used to keep up with a constantly growing global demand for food supplies.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ijpb13020006/s1, Figure S1: Carrot plants showing infection growth after being sprayed with A. dauci and B. cinerea conidia solution; Figure S2: The effect of CUSTOS against B. cinerea and A. dauci on carrot plants; Figure S3: Experiments with strawberry fruits.

Author Contributions: O.O. conducted the experiments, analyzed all data, drafted the figures, and wrote the manuscript; J.I. designed the study, analyzed all data, drafted the figures, and wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

Funding: Open Access funding is enabled and organized by Project DEAL.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Acknowledgments: We would like to thank Ute Micknass for the cultivation of carrot plants, and Udo Schnepp and Volker Weisel for the CUSTOS application in field experiments. We also thank Maximilian Reul (https://wetterauer-fruechtchen.de/ (accessed on 22 March 2022)) for providing the materials for field experiments.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Osusky, M.; Zhou, G.; Osuska, L.; Hancock, R.E.; Kay, W.W.; Misra, S. Transgenic plants expressing cationic peptide chimeras exhibit broad-spectrum resistance to phytopathogens. *Nat. Biotechnol.* **2000**, *18*, 1162–1166. [CrossRef] [PubMed]
2. Cook, J. Towards cropping systems that enhance productivity and sustainability. *Proc. Natl. Acad. Sci. USA* **2006**, *103*, 18389–18394. [CrossRef] [PubMed]
3. Alam, M.G.M.; Allinson, G.; Stagnatti, F.; Tanaka, A.; Westbrooke, M. Arsenic contamination in Bangladesh groundwater: A major environmental and social disaster. *Int. J. Environ. Health Res.* **2002**, *12*, 236–253. [CrossRef] [PubMed]
4. Sharma, M.; Schmid, M.; Rothballer, M.; Hause, G.; Zuccaro, A.; Imani, J.; Kämpfer, P.; Domann, E.; Schäfer, P.; Hartmann, A.; et al. Detection and identification of mycorrhiza helper bacteria intimately associated with representatives of the order Sebacinales. *Cell. Microbiol.* **2008**, *10*, 2235–2246. [CrossRef] [PubMed]
5. Dixon, R.A. Natural products and plant disease resistance. *Nature* **2001**, *411*, 843–847. [CrossRef] [PubMed]
6. Burt, S.A.; Reinders, R.D. Antibacterial activity of selected plant essential oils against Escherichia coli O157:H7. *Lett. Appl. Microbiol.* **2003**, *36*, 162–167. [CrossRef]
7. Kumar, J.; Ramlal, A.; Mallick, D.; Mishra, V. An Overview of Some Biopesticides and Their Importance in Plant Protection for Commercial Acceptance. *Plants* **2021**, *10*, 1185. [CrossRef]
8. Carlos, E.S.; Jesus, A.B.C.; Guadalupe, R.S.; Carmen, O.S. Biologically Active and Antimicrobial Peptides from Plants. *Biomedical. Res. Int.* **2015**, *10*, 21–29. [CrossRef]
9. Gutierrez, J.; Rodriguez, G.; Barry-Ryan, C.; Bourke, P. Efficacy of plant essential oils against foodborne pathogens and spoilage bacteria associated with ready-to-eat vegetables: Antimicrobial and sensory screening. *J. Food Prot.* **2008**, *71*, 1846–1854. [CrossRef]

10. Gurjar, M.S.; Ali, S.; Akhtar, M.; Singh, K.S. Efficacy of plant extracts in plant disease management. *Agric. Sci.* **2012**, *3*, 425–433. [CrossRef]
11. Amadioha, A.C. Evaluation of some plant leaf extracts against *Colletotrichum lindemuthianum* in cowpea. *Acta Phytopathol. Entomol. Hung.* **2003**, *38*, 259–265. [CrossRef]
12. Opara, E.U.; Wokocha, R.C. Efficacy of some plant extracts on the in vitro and in vivo control of *Xanthomonas campestris* pv. *vesicatoria*. *Agric. J.* **2008**, *3*, 163–170.
13. Dong, Y.; Zhao, M.; Zhao, T.; Feng, M.; Chen, H.; Zhuang, M.; Lin, L. Bioactive profiles, antioxidant activities, nitrite scavenging capacities and protective effects on H_2O_2-injured PC12 cells of *Glycyrrhiza glabra* L. leaf and root extracts. *Molecules* **2014**, *19*, 9101–9113. [CrossRef] [PubMed]
14. Shirazi, M.H.; Ranjbar, R.; Eshraghi, S.; Sadeghi, G.; Jonaidi, N.; Bazzaz, N.; Izadi, M.; Sadeghifard, N. An evaluation of antibacterial activity of *Glycyrrhiza glabra* extract on the growth of *Salmonella*, *Shigella* and ETEC *E. coli*. *J. Biol. Sci.* **2007**, *7*, 827–829. [CrossRef]
15. Hassan, S.M.; Byrd, J.A.; Cartwright, A.L.; Bailey, C.A. Hemolytic and antimicrobial activities differ among saponin-rich extracts from guar, quillaja, yucca, and soybean. *Appl. Biochem. Biotechnol.* **2010**, *162*, 1008–1017. [CrossRef]
16. Shenge, K.C.; Mabagala, R.B.; Mortensen, C.N. Identification and characterization of strains of *Xanthomonas campestris* pv. *vesicatoria* from Tanzania by biological system and sensitivity to antibiotics. *Afr. J. Biotechnol.* **2007**, *6*, 015–022.
17. Mbega, E.R.; Mortensen, C.N.; Mabagala, R.B.; Wulff, E.G. The effect of plant extracts as seed treatments to control bacterial leaf spot of tomato in Tanzania. *J. Gen. Plant Pathol.* **2012**, *78*, 277–286. [CrossRef]
18. Srivasata, S.; Singh, V.P.; Kumar, R.; Srivasata, M.; Sinha, A.; Simon, S. In vitro evaluation of Carbendazim 50 % WP, antagonists and botanicals against *Fusarium oxysporum* f. sp. *psidii* associated with rhizosphere soil of guava. *Asian J. Plant Pathol.* **2011**, *5*, 46–53.
19. Das, K.; Tiwari, R.K.; Shrivastava, D.K. Techniques for evaluation of medicinal plant products as antimicrobial agent: Current methods and future trends. *J. Med. Plant Res.* **2010**, *4*, 104–111.
20. Leontiev, R.; Hohaus, N.; Jacob, C.; Gruhlke, M.C.H.; Slusarenko, A.J. A Comparison of the Antibacterial and Antifungal Activities of Thiosulfinate Analogues of Allicin. *Sci. Rep.* **2018**, *8*, 6763. [CrossRef]
21. Koch, E.; Slusarenko, A. Arabidopsis is susceptible to infection by a Downy mildew fungus. *Plant Cell* **1990**, *2*, 437–445. [PubMed]
22. Slusarenko, A.J.; Patel, A.; Portz, D. Control of plant diseases by natural products: Allicin from garlic as a case study. *Eur. J. Plant Pathol.* **2008**, *121*, 313–322. [CrossRef]
23. Samadi, S.; Ghasemnezhad, A.; Naz, S.; Imani, J. An approved protocol for *Cynara scolymus* and *Momordica charantia* seed surface sterilization for pre-explant in-vitro culture using Allium extract. *J. Biol. Nat.* **2015**, *4*, 47–55.
24. Okuhara, P.; Kumar, N.; Hohenwarter, L.; Micknass, U.; Kogel, K.H.; Imani, J.; Graham, D.; Doty, S.L. Inhibition of fungal and bacterial plant pathogens by CUSTOS®, an Allium-BASED antimicrobial formulation. *J. Biol. Nat.* **2017**, *8*, 40–51.
25. Samadi, S.; Ghasemnezhad, A.; Imani, J. Extending shelf life of strawberry using some pre-storage treatments. *Acta Hortic.* **2017**, *1156*, 643–652. [CrossRef]
26. Imani, J.; Kumar, N.; Sand, B.; Kogel, K.H. VEG'LYS, a product from allium, has a strong potential for plant disease control. In Proceedings of the ADIVK Aktuell, 26th General Assembly of German In Vitro Culture Working Group (ADVIK), Lünen, Germany, 2013; Available online: http://www.adivk.de/index.php?id=4 (accessed on 22 March 2022).
27. Schmittgen, T.D.; Livak, K.J. Analyzing real-time PCR data by the comparative C_T method. *Nat. Protoc.* **2008**, *3*, 1101–1108. [CrossRef]
28. Hughes, B.G.; Lawson, L.D. Antimicrobial effects of *Allium sativum* L. (garlic), *Allium ampeloprasum* L. (elephant garlic), and *Allium cepa* L. (onion), garlic compounds and commercial garlic supplement products. *Phytother. Res.* **1991**, *5*, 154–158. [CrossRef]
29. Wilson, C.L.; Solar, J.M.; El Ghaouth, S.; Wisniewski, M.E. Rapid Evaluation of Plant Extracts and Essential Oils for Antifungal Activity Against *Botrytis cinerea*. *Plant Dis.* **1997**, *81*, 204–210. [CrossRef]
30. Negri, M.; Salci, T.P.; Shinobu-Mesquita, C.S.; Capoci, I.R.; Svidzinski, T.I.; Kioshima, E.S. Early state research on antifungal natural products. *Molecules* **2014**, *19*, 2925–2956. [CrossRef]
31. Khan, S.; Imran, M.; Imran, M.; Pindari, N. Antimicrobial activity of various ethanolic plant extracts against pathogenic multi drug resistan *Candida* spp. *Bioinformation* **2017**, *13*, 67. [CrossRef]
32. Corbu, V.M.; Gheorghe, I.; Marinas, I.C.; Geană, E.I.; Moza, M.I.; Csutak, O.; Chifiriuc, M.C. Demonstration of Allium sativum Extract Inhibitory Effect on Biodeteriogenic Microbial Strain Growth, Biofilm Development, and Enzymatic and Organic Acid Production. *Molecules* **2021**, *26*, 7195. [CrossRef] [PubMed]
33. Miron, T.; Rabinkov, A.; Mirelman, D.; Wilchek, M.; Weiner, L. The mode of action of allicin: Its ready permeability through phospholipid membranes may contribute to its biological activity. *Biochim. Biophys. Acta* **2000**, *1463*, 20–30. [CrossRef]
34. Müller, A.; Eller, J.; Albrecht, F.; Prochnow, P.; Kuhlmann, K.; Bandow, J.E.; Slusarenko, A.J.; Leichert, L.I.O. Allicin induces thiol stress in bacteria through S-allylmercapto modification of protein cysteines. *J. Biol. Chem.* **2016**, *291*, 11477–11490. [CrossRef] [PubMed]
35. Okuhara, P.A.; Arroyo-Garcia, R.; Shen, K.A.; Mazier, M.; Meyers, B.C.; Ochoa, O.E.; Kim, S.; Yang, C.H.; Michelmore, R.W. A transgenic mutant of *Lactuca sativa* (lettuce) with a T-DNA tightly linked to loss of downy mildew resistance. *Mol. Plant-Microbe Interact.* **1997**, *10*, 970–977. [CrossRef] [PubMed]

36. Castillo, L.; Plaza, V.; Larrondo, L.F.; Canessa, P. Recent advances in the study of the plant pathogenic fungus *Botrytis cinerea* and its interaction with the Environment. *Curr. Protein Pept. Sci.* **2017**, *18*, 976–989. [CrossRef]
37. Kumar, J.; Ramlal, A.; Kumar, K.; Rani, A.; Mishra, V. Signaling Pathways and Downstream Effectors of Host Innate Immunity in Plants. *Int. J. Mol. Sci.* **2021**, *22*, 9022. [CrossRef]
38. Terefe, N.S.; Matihaies, K.; Simons, L.; Versteeg, C. Combined high pressure-mild temperature processing for optimal retention of physical and nutritional quality of strawberries. *Innov. Food Sci. Emerg. Technol.* **2009**, *10*, 297–307. [CrossRef]
39. Wright, K.P.; Kader, A.A. Effect of slicing and controlled-atmosphere storage on the ascorbate content and quality of strawberries and persimmons. *Postharvest Biol. Technol.* **1997**, *10*, 39–48. [CrossRef]
40. Boyraz, N.; Ozcan, M. Antifungal effect of some spice hydrosols. *Fitoterapia* **2005**, *76*, 661–665. [CrossRef]