Conference paper

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Production of chitosan oligosaccharides for inclusion in a plant biostimulant

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Abstract: The use of biostimulants to enhance crop productivity is beginning to be adopted into mainstream agricultural practice. There is an emerging consensus on the critical role that low-cost and scalable chitosan oligosaccharide production systems can play in meeting the demands of this "greener" approach in agriculture. The objective of our research was to produce chitosan oligosaccharides (CHOS) mixtures that can work as plant biostimulants using cost effective enzymes. Commercial chitosans with a consistent formulation and available in bulk were used in the study. Chitosans were characterized in terms of degree of N-acetylation (pH-metric titration) and molecular weight (Ubbelohde viscometer). The yield of the CHOS were determined along with their physicochemical characteristics. The biological activity of the different CHOS mixtures were evaluated for efficacy against a fungal pathogen (*F. oxysporum*) in the susceptible tomato cultivar 'Moneymaker'. The performance of some CHOS resulted in significant enhancements in a number of plant health indicators such as increased biomass, disease control and induction of ISR markers. Finally, the optimal CHOS preparation in terms of plant bioactivity was scaled up and validated by a preliminary field trial with the industrial tomato cultivar 'H9661'. The effectiveness of this treatment on crop productivity was consistent with the results observed in the lab and similar to other commercial plant biostimulants.

Keywords: agriculture; chitosan oligosaccharides; commercial; EUCHIS-12; field trial; ICCC-13; plant biostimulants; sustainable; tomato.

Introduction

Agriculture faces a challenge to find effective, sustainable and ecologically sound solutions for enhancement of crop productivity. A growing world population will require more food, while the prospect of global warming threatens to reduce agricultural productivity due to more demanding climatic conditions [1]. Plant biostimulants are currently playing an important role in making agriculture more efficient in terms of crop yield and environmental sustainability. The definition of plant biostimulants proposed by du Jardin is now gaining widespread acceptance [2]: "Plant biostimulants are substances and materials, with the exception of nutrients and pesticides, which, when applied to plant, seeds or growing substrates in specific formulations, have the capacity to modify physiological processes of plants in a way that provides potential benefits to growth, development and/or stress response".

According to several reports from the literature, water soluble chitosan oligosaccharides (CHOS) have been shown to induce various plant defense-related cellular responses [3] (Fig. 1). Moreover, these compounds

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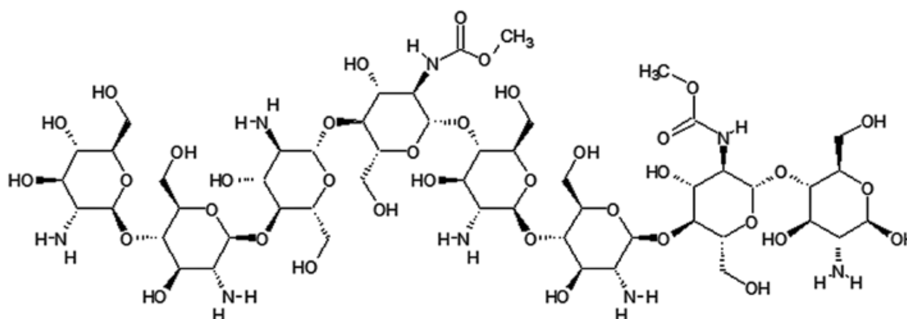

Fig. 1: Chemical structure of chitosan oligosaccharide (CHOS). Schematic representation of the octamer (DA: 25 %).

have been reported to be enhancers of plant photosynthesis [4] and inducers of flowering [5]. However, most of these research studies have investigated the effects and mode of action of CHOS in cell suspensions or isolated plant organs [3, 6, 7] and unfortunately there is a deficit of published information on how these compounds can improve crop performance in the field.

To overcome this practical limitation, the aim of our research was to produce agriculturally-beneficial water soluble CHOS mixtures from commercial chitosans using a low-cost hydrolase. We evaluated the efficacy of CHOS applied by foliar spray on induced resistance markers, photosynthetic performance and yield in tomato plants (cv. Moneymaker) under controlled conditions. The optimal CHOS preparation in terms of plant bioactivity was scaled up and validated by a preliminary field trial with the industrial tomato cultivar 'H9661'. The results showed a similar effectiveness of this CHOS treatment compared to other commercial plant biostimulants.

Materials and methods

CHOS production

To perform plant assays, five CHOS with different physico-chemical properties were used. The CHOS were obtained by the enzymatic hydrolysis of chitosans with a consistent formulation and available in bulk from different industrial suppliers [50–200 cP; DA (%): 10–25] under the action of a commercial low-cost hydrolase. The scale-up of CHOS 1 production was reproduced in a 5000 L-batch reactor.

Chitosan and CHOS characterization

The viscometric average molecular weight (M_v) was obtained according to the Mark-Houwink equation [8]. The degree of N-acetylation (DA) was determined by pH-metric titration following the methodology "Titraton I" described by Czechowska-Biskup et al. [9].

Plant material

Tomato seeds (*Lycopersicon esculentum*, cv. Moneymaker) were purchased from Liscahane Nurseries, Tralee. Seeds were surface sterilized with sodium hypochlorite for 1 min before being thoroughly rinsed with distilled water. Seeds were set in plug trays using growth medium of compost: vermiculite: perlite (5:1:1). On day 19, seedlings were then transferred to 2 L pots (same growth medium as previous). The resultant plants were raised in a growth room at a temperature of $27/22 \pm 2\,°C$ (day/night; 16/8 h) under a light intensity of 120 $\mu mol \cdot m^{-2} \cdot s^{-1}$ in a complete randomized block design.

Treatment application

On day 28, tomato leaves were sprayed with the treatments. β-aminobutyric acid (BABA; Sigma-Aldrich) was dissolved in distilled water and applied at a rate of 0.1 $mg \cdot mL^{-1}$. CHOS treatments were also dissolved in distilled water and applied at a rate of 0.25 $mg \cdot mL^{-1}$. Control plants were sprayed with equal volume of distilled water.

Pathogen preparation and inoculation procedures

Race (1) *Fusarium oxysporum* f. sp. *lycopersici* was purchased from DSMZ (Germany). It was grown in a shaking incubator (150 rpm) at 27 °C for 7 days in sterile potato dextrose broth (PDB). The mycelium and conidia were both collected. Spores were separated from the mycelium by filtering the suspension through a fine cheesecloth. Spore density was determined using a Neubauer hemocytometer. Spore density was adjusted with sterilized water to $1 \cdot 10^6$ spores/mL per plant. Pathogen was applied to plants through a combination of dip/pour method 7 days after the foliar application of the treatments (on day 35).

Phenotypic assessment

Tomato plants were assessed on day 42 (21 days after treatment and 14 days after pathogen inoculation). Plants were assessed for biomass (fresh weight), disease severity and chlorophyll content. Disease severity was measured on a scale of 0–100 %. Plants with a score of 0 show no signs of disease, plants with a score of 33.3 % have vascular browning below the cotyledon or a reduced height, plants with a score of 66.6 % show vascular browning above the cotyledon and dead plants have a score of 100 %. Chlorophyll content (SPAD units) was measured with a Minolta SPAD 502 meter.

ISR enzyme assays

The induction of defence related enzymes (endochitinase and peroxidase/POD) were measured in fresh tomato leaves. Tomato leaves were harvested at various time intervals (0, 7, 14, 21 days) after each treatment, immediately frozen in liquid nitrogen, and stored at −80 °C until they were processed for enzyme extraction.

Leaf samples (50 mg fresh weight) were thoroughly ground in liquid nitrogen and homogenized for 1 h with 0.5 mL of 100 mM ice-cold sodium acetate buffer (pH 5.0) containing 2 % (w/v) polyvinylpolypyrrolidone (PVPP), 1 mM PMSF and 1 mM EDTA for endochtinase, but using 50 mM sodium phosphate buffer (pH 7.5) containing 2 % (w/v) PVPP, 1 mM PMSF and 1 mM EDTA for POD. The homogenates were centrifuged (14 000 × *g* for 15 min at 4 °C) and the supernatant was decanted carefully and used to measure the enzymatic activities. All enzyme extract procedures were conducted at 4 °C.

Endochitinase activity was determined with a highly sensitive substrate that produces the fluorescent product 4-methylumbelliferone (4-MU) upon enzymatic hydrolysis of 4-MU-$(GlcNAc)_3$ (Sigma–Aldrich). Reaction mixtures, containing 20 µL enzyme extract, 160 µL 100 mM sodium acetate buffer (pH 5.0) and 40 µL 180 µM 4-MU-$(GlcNAc)_3$ substrate, were prepared in 96 wells microplates. The plates were then incubated in a shaking incubator at 37 °C and 180 rpm for 1 h. The reaction was stopped by the addition of 100 µL 0.2 M Na_2CO_3 and the release of free 4-MU was measured by fluorescence spectrophotometry with excitation and emission wavelengths of 360 nm and 460 nm. A calibration curve was constructed with 4-MU and units of activity were defined as nmol 4-MU released per min per milligram soluble protein.

POD activity was determined using guaiacol as the substrate in 96 well microplates. The reaction mixture (200 µL) contained 126.6 µL 100 mM sodium phosphate buffer (pH 7.5), 40 µL guaiacol 1 % (v/v) aqueous solution and 13.3 µL of the enzyme extract. The assay mixture was allowed to incubate for 5 min at 25 °C in the

microplate reader. The reaction was started by adding 20 µL of 1 % (v/v) hydrogen peroxide. The increase in absorbance at 470 nm was measured immediately for 3 min at 25 °C. POD activity was expressed as units of enzymatic activity (U). One U represents the amount of enzyme catalyzed for the formation of 1 nmol of tetra-guaiacol ($\varepsilon = 26\,600\ M^{-1}\cdot cm^{-1}$) per min per milligram soluble protein.

Protein concentration was determined by the Bradford method, using a protein-dye reagent (Bio-Rad) and BSA (bovine serum albumin) as a standard. The standard procedure for 96 well microplate was applied according to the manufacturer's manual.

Direct growth rate inhibition of *F. oxysporum* by CHOS

Aliquots (150 µL) of sterile PDB containing the required amount of CHOS for dose response were prepared in 96 well microplates. Ten µL of a spore suspension ($1\cdot 10^4$ spores/mL) of *Fusarium oxysporum* (race I) or distilled water (blank) was added and the plates were incubated at 25 °C and 200 rpm under agitation for 7 days. Growth rate was calculated after determining the absorbance at 405 nm according the equation described by Oliveira et al. [10].

Field trials

Open field trials were conducted in 2014 in Spain to evaluate the effect of the optimal CHOS preparation on performance of a well established industrial tomato variety cv. H9661. 2 other commercial plant biostimulants (BIO1 and BIO2), BABA and an untreated control were included in each field experiment. Treatments were applied four times at the same concentration used in the greenhouse experiment as a foliar spray. Two fungicide programs were carried out: one according to the standard grower practice and another one using 50 % of the standard rate. The fungicides used were penconazole and bupirimate and were applied as a foliar spray. Both fungicide conditions were arranged in a randomized complete block design with six replicates of 24 m^2/plot (3 m × 8 m) per each treatment and two rows of plants per plot. Plant density on the field was 16 666 plants/ha. Total marketable fruit yield (kg/ha) was recorded to evaluate crop performance.

Statistics

At least five physico-chemical determinations (molecular weight and DA) were performed per chitosan sample. Hydrolysis experiments were repeated at least three times. Growing, inoculation and sampling of plants were done in three independent experiments with at least six plants per treatment. For phenotypic assessment, a minimum of six plants were evaluated for each experiment and treatment. For enzyme assays, 2 extractions of each treatment were performed and the assay was repeated three times. For fungal growth experiments, two independent experiments with five replicates were carried out by treatement. Standard deviation (SD) was calculated and its range is shown in the figures. Standard analysis of variance (ANOVA) were conducted using the SigmaPlot software program (version 12.0) to compare the data between each treatment and control.

Results and discussion

CHOS production and characterization

In an attempt to develop an efficient process for the production of CHOS on a large scale, we studied the hydrolysis of five different commercials chitosans. Initially, we confirmed that the dynamic viscosity of

chitosan aqueous solutions decreased suddenly after adding an unexpensive commercial enzyme under the optimal temperature and pH values. Despite the fact that chitosans with different DA (11.6–26.1 %) and M_v (33.3–97.4 kDa) were used as the substrate of enzymatic depolymerization, the yield of five water soluble CHOS generated ranged from 55 % to 70 %. Physicochemical characteristics of CHOS products showed a similar low molecular weight (1–5 kDa) and significant decreases with respect to the DA value (10.1–19.3 %).

Assessment of induced resistance in tomato plants

To compare the ability to induce protection in tomato plants against the soilborne pathogen *F. oxysporum*, plant assays were performed in the susceptible tomato variety 'Moneymaker'. Foliar spray of all CHOS tested resulted in 25–30 % decreases in disease severity values relative to the untreated control. The higher values of tomato plant protection were correlated with increases in plant fresh weight (Fig. 2a). However, only CHOS 1 treatment showed significant differences with the untreated control. The phenotypical assessment illustrated the remarkable induced resistance of this treatment (Fig. 2b). Interestingly, despite BABA having been shown to control a large variety of plant diseases by inducing resistance in the plants [11], this treatment showed a lower protection and plant biomass than all CHOS used in this experiment.

Measurements of chlorophyll concentration indicated a negative effect of the fungal pathogen. However, it is interesting to note that there was a clear linear correlation of the SPAD values with the disease severity (Fig. 3). Former authors reported that root pathogens can affect several physiological processes, such as water absorption or gas exchange, resulting in the damage of the photosynthetic system of the plant [12]. The results of this work reinforced the potential of water soluble CHOS 1 to become a useful product to induce resistance and retain leaf greenness in tomato plants.

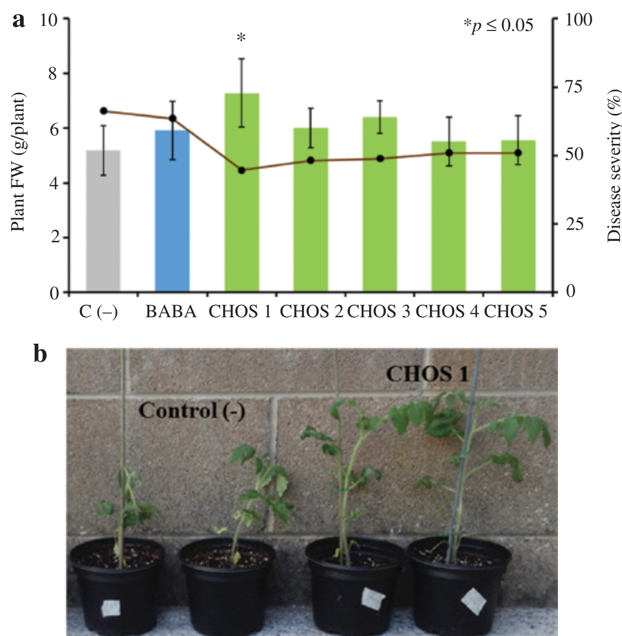

Fig. 2: Effect of CHOS treatments on fitness of tomato plants (cv. Moneymaker) that were exposed to *F. oxysporum* (I). (a) Fresh weight (FW) and disease severity values of 42-day-old-tomato plants (21 days after treatment and 14 days after pathogen inoculation) were represented as bar and line chart respectively. All data were analysed for significant differences by one-way ANOVA using Holm-Sidak test at $p \leq 0.05$ against the control. (b) Final condition of untreated and CHOS 1-treated infected tomato plants.

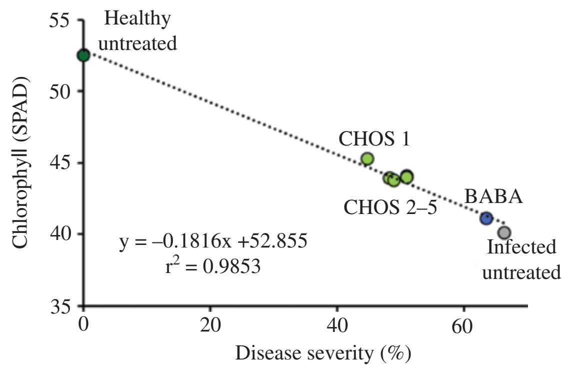

Fig. 3: Effect of CHOS treatments on chlorophyll levels and disease severity of 42-day-old-tomato plants (cv. Moneymaker) that have been exposed to *F. oxysporum* (I). Values were determined 21 days after treatment and 14 days after pathogen inoculation.

Induction of ISR enzymes in tomato plants

Further biochemical analysis was conducted to elucidate the induction of defence responses triggered in response to foliar spray of CHOS and BABA. All treatments applied by foliar spray to the infected plants caused significant increases of endochitinase and POD activities relative to the untreated control. CHOS 1 and CHOS 3 induced the highest endochitinase activity levels after 21 days of foliar application (Fig. 4). However, all CHOS used induced 2–3 times higher levels of POD activity than the control plants at the end of the experimental time course (Fig. 5).

As shown in Fig. 6, CHOS 1 treatment differentially activated endochitinase and POD enzymes before pathogen infection. This behavior could be related to a transient defence response mobilized after plant treatment with elicitors. This priming effect could be the reason why the endochitinase and POD activity levels of CHOS 1 treated plants were significantly higher than untreated plants after pathogen infection. The results of this study also demonstrate that the resistance against *F. oxysporum* found in tomato plants induced with CHOS 1 was significantly related to endochitinase and POD activation. Previous studies have reported that the induction of resistance by plant elicitors elevate the level of some defence compounds and sensitize the plants to rapidly produce some compounds promptly after infection and thereby, provide protection against disease [13].

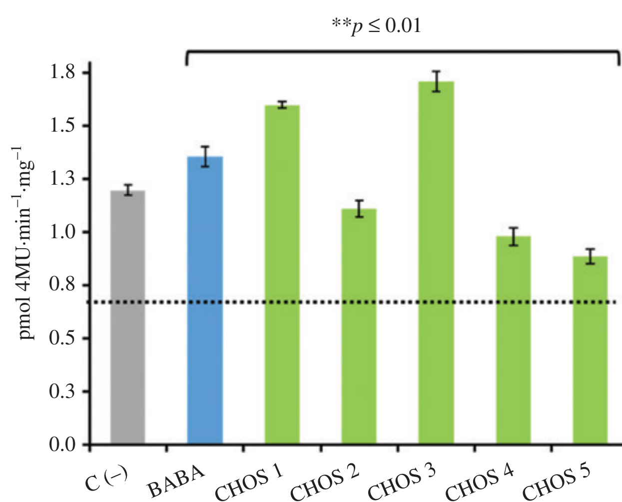

Fig. 4: Endochitinase activity in 42-day-old-tomato leaves (cv. Moneymaker) on a time course after elicitation with CHOS and BABA and infection with *F. oxysporum* (I). An uninfected untreated sample was evaluated as baseline control (----). Values were determined 21 days after treatment and 14 days after pathogen inoculation). All data were analysed for significant differences by one-way ANOVA using Holm-Sidak test against the control.

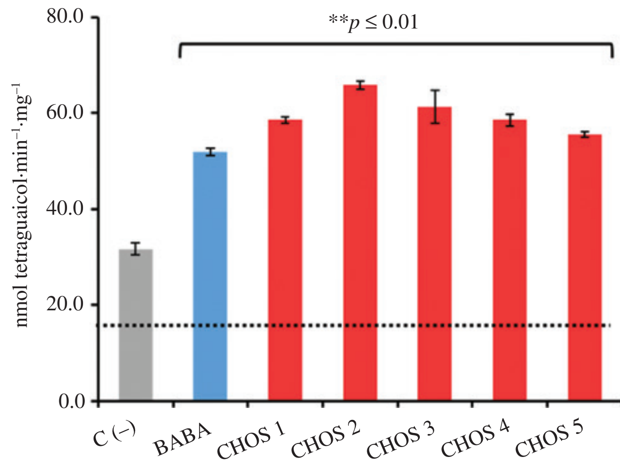

Fig. 5: POD activity in 42-day-old-tomato leaves (cv. Moneymaker) on a time course after elicitation with CHOS and BABA and infection with *F. oxysporum* (I). An uninfected untreated sample was evaluated as baseline control (----). Values were determined 21 days after treatment and 14 days after pathogen inoculation. All data were analysed for significant differences by one-way ANOVA using Holm-Sidak test against the control.

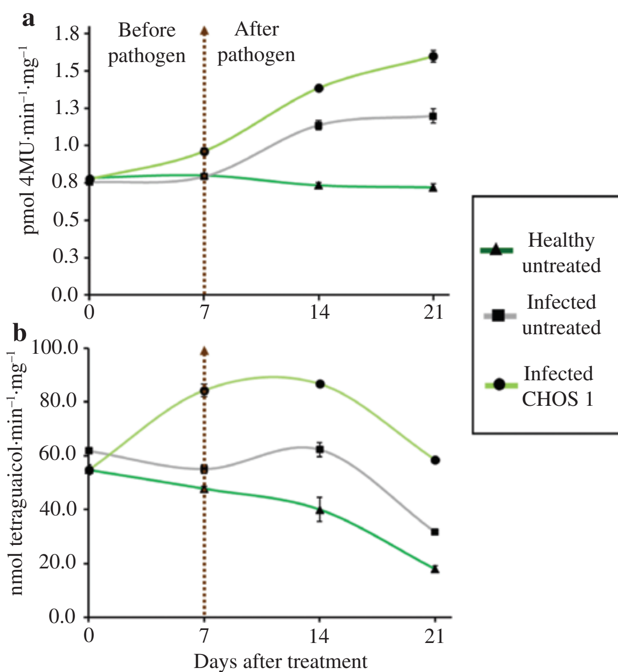

Fig. 6: Endochitinase (a) and POD (b) activity in tomato leaves (cv. Moneymaker) on a time course after elicitation with water and CHOS 1 and infection with *F. oxysporum* (I). An uninfected untreated sample was evaluated as baseline control.

Examples of these defence mediators include POD which catalyzes the production of antimicrobial quinones [14] and endochitinase which is involved in degradation of major cell wall components of many pathogens [15].

Direct growth rate inhibition of *F. oxysporum* by CHOS

Table 1 shows the direct effect of the CHOS on the growth of *F. oxysporum* (I). It was observed that the growth rate was not inhibited significantly by any CHOS at concentration ≤ 1 mg·mL^{-1} after seven days of incubation

Table 1: Changes in growth *F. oxysporum* (race I) on day seven in PDB with CHOS.

CHOS	Changes in normalized growth rate (%)[a]			
	0 mg·mL^{-1}	0.25 mg·mL^{-1}	0.50 mg·mL^{-1}	1 mg·mL^{-1}
1	100.0 ± 4.1[b]	105.5 ± 0.6	106.3 ± 4.2	105.5 ± 0.6
2	100.0 ± 2.2	100.8 ± 1.9	103.9 ± 2.9	99.5 ± 6.7
3	100.0 ± 0.9	104.1 ± 0.8	100.9 ± 6.5	101.4 ± 3.2
4	100.0 ± 3.0	99.5 ± 4.2	103.9 ± 2.0	102.1 ± 3.8
5	100.0 ± 2.5	100.1 ± 2.3	99.3 ± 5.1	99.9 ± 3.7

[a]Growth rate ($A_{405\ day\ 7}$–$A_{405\ day\ 0}$)/164 normalized to control sample (0 mg·mL^{-1}).
[b]Values did not significantly differ according to Holm-Sidak test ($p \leq 0.05$).

at 25 °C in PDB. Therefore, the results lead to the conclusion, that CHOS produced in this study did not show any direct antifungal activity against the pathogen used in the plant experiments.

Field trials

From the results presented above, it is possible to conclude that CHOS 1 is a water-soluble and non-toxic substance which can elicit significant defence responses in tomato plants. Therefore, this CHOS product was selected to be scaled up and validated by a preliminary field trial study. The CHOS 1 production process was scaled up in a 5000 L batch reactor, achieving a 91 % yield with respect to the lab production and similar physicochemical properties (data not shown).

Tomato field trials were carried out with the industrial tomato cultivar ‘H9661’, a popular variety that carries the gene of resistance to some diseases such as Fusarium or Verticillium wilt. Tomato plants were grown in a near optimal environment with little biotic or abiotic stress events recorded during the growing period.

It is evident from the data presented in Table 2 that no significant treatment effects were observed with the 100 % fungicide regime. The CHOS 1 treatment provided a 7 % increase in marketable yield although this was not statistically significant versus the untreated control at a $p \leq 0.1$. A non-significant negative effect, with a 9 % decrease in productivity, was obtained for treatment BIO 1. However, use of both commercial biostimulants and CHOS 1 treatment for the 50 % fungicide program outperformed the untreated control with a 19–22 % significant increase in marketable yield.

The biostimulants tested in this trial enhanced yield even in the absence of significant biotic stress under field conditions however future trials will require a more focussed setup to specifically assess benefits to reducing Fusarium wilt in the field. This tomato trial did provide useful data on the fungicide reduction benefits of these biostimulants and data on the effectiveness of CHOS 1 treatment compared to other commercial plant biostimulants.

Table 2: Tomato yield after treatment with biostimulants and different fungicide regimes.

Treatment	Marketable yield (kg/ha)	
	100% Fungicide	50% Fungicide
Untreated	66 305 ± 3777	62 726 ± 2592
BABA	65 896 ± 6341	63 299 ± 11 166
BIO 1	60 413 ± 912	74 384 ± 9855 (*)[a]
BIO 2	67 542 ± 9562	76 775 ± 3338 (*)
CHOS 1	70 927 ± 9926	74 186 ± 9855 (*)

[a]The asterisks indicate that the mean value is significantly different from that of the untreated control at $p \leq 0.1$ based on Holm-Sidak’s test.

Conclusion

In brief, this study provided the basis for commercial development of a platform of CHOS products to solve crop productivity challenges. The data generated provides a compelling case for the benefits of effective CHOS treatments in sustainable agricultural practices. Additional research to optimise processing conditions for CHOS production will be required in order to further enhance their efficacy. Further investigation of the CHOS composition and its impact on plant signaling pathways will also offer opportunity for enhanced efficacy which will deliver more yield for growers.

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