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In-Vitro Inhibition of *Pythium ultimum*, *Fusarium graminearum*, and *Rhizoctonia solani* by a Stabilized Lactoperoxidase System alone and in Combination with Synthetic Fungicides

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Abstract: Advances in enzyme stabilization and immobilization make the use of enzymes for industrial applications increasingly feasible. The lactoperoxidase (LPO) system is a naturally occurring enzyme system with known antimicrobial activity. Stabilized LPO and glucose oxidase (GOx) enzymes were combined with glucose, potassium iodide, and ammonium thiocyanate to create an anti-fungal formulation, which inhibited in-vitro growth of the plant pathogenic oomycete *Pythium ultimum*, and the plant pathogenic fungi *Fusarium graminearum* and *Rhizoctonia solani*. *Pythium ultimum* was more sensitive than *F. graminearum* and *R. solani*, and was killed at LPO and GOx concentrations of 20 nM and 26 nM, respectively. *Rhizoctonia solani* and *F. graminearum* were 70% to 80% inhibited by LPO and GOx concentrations of 242 nM and 315 nM, respectively. The enzyme system was tested for compatibility with five commercial fungicides as co-treatments. The majority of enzyme + fungicide co-treatments resulted in additive activity. Synergism ranging from 7% to 36% above the expected additive activity was observed when *P. ultimum* was exposed to the enzyme system combined with Daconil® (active ingredient (AI): chlorothalonil 29.6%, GardenTech, Lexington, KY, USA), tea tree oil, and mancozeb at select fungicide concentrations. Antagonism was observed when the enzyme system was combined with Tilt® (AI: propiconazole 41.8%, Syngenta, Basel, Switzerland) at one fungicide concentration, resulting in activity 24% below the expected additive activity at that concentration.

Keywords: *Pythium*; *Fusarium*; *Rhizoctonia*; enzyme stabilization; lactoperoxidase; glucose oxidase; fungicide

1. Introduction

Fungicides are critical for the control of many economically important plant diseases and have been an integral component of crop production for decades [1–3]. All fungicides impose a selection pressure on their target pathogen populations, and some fungicides are prone to the development of resistance. This is especially true for fungicides with a single target site, as mutations in the target gene can lead to the selection of a resistant sub-population in the pathogen population with fungicide applications [4–7]. Some widely used examples of such fungicides include succinate dehydrogenase inhibitors (SDHIs), quinone outside inhibitors (QoIs), de-methylation inhibitors (DMIs), and phenylamides (PAs). Fungicides with multi-site modes of action are less likely to select for resistance [8].

Managing fungicide resistance has been a priority since it began to appear in the 1960s and 1970s [8]. Concerns over the loss of fungicide efficacy have resulted in the development and implementation of various resistance management strategies. Such strategies include applying

mixtures of fungicides with different modes of action (often a site-specific with a multi-site), rotating fungicides with different modes of action, restricting the number of applications per season, maintaining the manufacturer's recommended dose, and implementing cultural control practices as part of an integrated pest management (IPM) program [8]. An important consideration regarding fungicide mixtures is the potential interaction between different fungicides, which may lead to antagonistic or synergistic activity against target pathogens [9–11]. Identifying such interactions is crucial for the effective and efficient deployment of new fungicide chemistries prior to their incorporation into an IPM program.

An element of IPM that has gained attention over the past several years is the use of biologically-based pest control solutions, known as biocontrols or biopesticides. Mechanisms of biocontrols and biopesticides include antibiosis, resource competition, and disease resistance-induction [12]. Regarding plant disease management, other than the use of resistant host varieties, biocontrols are considered as living organisms that suppress the activity or reduce populations of plant pathogens. Products that achieve the same outcome, but do not contain living organisms, and are fermented or extracted from natural sources, are considered biopesticides [12]. Enzymes are integral to the antagonistic activity of certain biocontrol microorganisms. Lytic enzymes, such as chitinases, glucanases, and proteases, are secreted by a number of microbes and are known to be suppressive to plant pathogenic fungi and bacteria [13–17]. However, deploying these enzymes directly for plant disease management has received little attention, probably in part due to the costs that are associated with obtaining commercially relevant quantities of enzyme, and subsequently stabilizing the enzymes for industrial use.

Advances in the field of biocatalysis, particularly in the areas of protein engineering enzyme immobilization and stabilization, are making the use of enzymes in agriculture increasingly feasible [18–20]. As pesticide resistance and government regulation continue to decrease the number of effective compounds that are available to farmers [8,21], enzymes represent a promising new frontier in crop protection. Enzyme immobilization can be achieved through several approaches, including adsorption of the proteins onto a carrier, protein encapsulation inside a carrier, or cross-linking. Enzyme immobilization increases enzyme stability and has been shown to increase enzyme activity in certain systems [18]. For example, immobilization of horseradish peroxidase by adsorption onto magnetic nanoparticles and the subsequent entrapment by self-assembly increased enzyme activity and reduced inhibition by the H_2O_2 substrate and reaction products when compared to the free enzyme [22,23]. Maximizing enzyme activity and stability will likely be paramount for the economic viability and, ultimately, the adoption of stabilized enzymes as alternatives to conventional synthetic pesticides by the agriculture industry.

Lactoperoxidase (LPO) is an enzyme with antimicrobial properties and occurs naturally in tears, saliva, and milk [24,25]. Lactoperoxidase is extracted from bovine milk and can be obtained relatively simply [26], making it an attractive option for experimentation as well as commercialization. LPO catalyzes the oxidation of thiocyanate and iodide ions to antimicrobial hypothiocyanite ($OSCN^-$) and hypoiodite (OI^-), respectively, in the presence of hydrogen peroxide (H_2O_2). The H_2O_2 can be provided by the activity of glucose oxidase (GOx), which is extracted from the fungus *Aspergillus niger*, on β-D-glucose in the presence of oxygen. These unstable compounds, in turn, oxidize sulfhydryl groups in the cell membranes of microbes, leading to the inhibition of glucose transport, glycolysis, respiration, and ultimately cell death [24]. The LPO system, which here refers to LPO, GOx, and substrates, is known to inhibit Gram positive and Gram negative bacteria, as well as fungi [27]. Furthermore, once $OSCN^-$ and OI^- are depleted, H_2O_2 accumulates through the activity of GOx which leads to additional oxidative stress on microbial cells [28].

The objectives of this study were to (i) test the ability of a stabilized LPO and GOx system to inhibit the growth of *P. ultimum*, *F. graminearum*, and *R. solani* in vitro; and, (ii) determine if the combined effect of stabilized LPO and GOx with five commercial fungicides resulted in synergistic, additive, or antagonistic activity when compared to each active ingredient alone.

2. Results

2.1. Optimizing the Stabilized Enzyme Formulation

P. ultimum, *F. graminearum*, and *R. solani* all showed sensitivity, to varying degrees, to the stabilized enzyme formulation in preliminary tests. As a result, an experiment was conducted to identify enzyme concentrations that would result in a measurable reduction in fungal growth without being completely inhibitive. *P. ultimum* showed greater sensitivity to the enzyme formulation than *F. graminearum* and *R. solani* in preliminary experiments so *P. ultimum* was tested at lower enzyme concentrations than *F. graminearum* and *R. solani* for formula optimization (Table 1). The three highest enzyme concentrations tested on *P. ultimum* resulted in 100% inhibition. The lowest concentration tested resulted in 31% inhibition (Table 1). All three of the enzyme concentrations that were tested on *F. graminearum* and *R. solani* resulted in reduced growth, and ranged from 74% to 57% growth reduction for *F. graminearum* and 72% to 63% growth reduction for *R. solani* (Table 1). Based on these results, the lowest concentration tested was chosen for *P. ultimum* (4.0 nM LPO and 5.2 nM GOx) and *F. graminearum* and *R. solani* (119.0 nM LPO and 154.7 nM GOx) fungicide experiments. The stabilized enzyme formulation was prepared as carboxymethyl cellulose (CMC) film disks at these concentrations for the fungicide synergy experiments.

Table 1. Enzyme concentrations used to optimize formulations for in vitro experiments.

Enzyme Concentrations (nM) [1]		**Reduction in Growth** [2]			
Lactoperoxidase	**Glucose Oxidase**	***P. ultimum***	**SD** [3]		
40	53	100%	0%		
32	42	100%	0%		
20	26	100%	0%		
4.0	5.3	31%	7%		
		F. graminearum	**SD** [3]	***R. solani***	**SD** [3]
323	420	74%	3%	72%	4%
242	315	77%	14%	72%	8%
119	155	57%	4%	63%	1%

[1] Amount of each enzyme (lactoperoxidase (LPO) and glucose oxidase (GOx)) in each 50 µL enzyme disk; [2] Reduction in growth relative to non-treated controls. Each value is the mean of two replicates; [3] Standard deviation.

To test whether these enzymes are phytotoxic, tomato seeds were coated with the stabilized enzyme formulations and planted. Germination and growth were observed for four weeks. After four weeks, plant height and root and shoot mass were measured (Table 2). There was no difference between the coated seeds and the uncoated control seeds in germination rate or in vigor, as measured by shoot height and weight and root weight.

Table 2. Plant vigor data for tomato seeds coated at different enzyme concentrations.

Enzyme Concentrations (nM) [1]		**Plant Vigor Data** [2]					
Lactoperoxidase	**Glucose Oxidase**	**Shoot Weight (g)**	**SD** [3]	**Root Weight (g)**	**SD** [3]	**Plant Height (cm)**	**SD** [3]
Control	Control	1.64	0.4	1.17 ab [4]	0.49	16	0.71
20	26	1.44	0.25	1.92 b	0.39	15.8	1
40	52	1.81	0.56	1.02 a	0.28	18	1.8
119	153	1.37	0.24	1.58 ab	0.56	16.3	1.7
162	208	1.47	0.29	1.60 ab	0.13	16.3	1.1
	Tukey's HSD	0.54		0.84		2.4	
	p value	0.132 (ns [5])		0.033		0.070 (ns [5])	

[1] Amount of each enzyme (LPO and GOx) in each 50 µL enzyme disk; [2] Plant growth data. Each value is the mean of five replicates; [3] Standard deviation; [4] Means followed by the same letter are not significantly different, Tukey's honest significant difference (HSD) ($p < 0.05$); [5] Not significant.

2.2. Sensitivity of P. ultimum, F. graminearum, and R. solani to the Stabilized Enzyme Formulation Slone and in Combination with Fungicides In Vitro

A total of 28 pathogen X fungicide X concentration combinations were tested using enzyme film disks and a filter paper diffusion disk method. Among those, six were significantly synergistic, 21 were additive, and one was significantly antagonistic. Synergism was observed with Daconil® (active ingredient (AI): chlorothalonil 29.6%, GardenTech, Lexington, KY, USA), tea tree oil, and mancozeb when tested on *P. ultimum* and ranged from 7% to 36% above the expected combined effect. The effects of Stratego®YLD (AI: prothioconazole 10.8% + trifloxystrobin 32.3%, Bayer, Leverkusen, Germany) on *F. graminearum* were additive when combined with the stabilized enzyme formulation. Tilt® (AI: propiconazole 41.8%, Syngenta, Basel, Switzerland) when combined with stabilized enzymes resulted in additive activity against *R. solani*. The one instance of significant antagonism occurred when Tilt® was tested on *F. graminearum* and resulted in an observed combined effect that was 24% lower than the expected combined effect.

Daconil® combined with the stabilized enzyme formulation produced the most consistent synergistic effect among the groups tested. Three out of four combinations resulted in statistically significant synergistic activity against *P. ultimum,* ranging from 13% to 20% above the expected combined effect (Table 3). The fourth concentration resulted in an additive effect. The combined effects were significantly greater than the effects of the enzyme formulation alone and each of the four fungicide concentrations alone. The effect of the enzyme formulation alone was also significantly greater than the fungicides alone (Table 3).

Table 3. Inhibition of *Pythium ultimum* by the stabilized lactoperoxidase (LPO) formulation alone and in combination with Daconil® using fungicide-impregnated filter paper disks.

% Reduction in Growth [1]							
Daconil® Dose (μg)	**(+) Enzyme [2]**	**SD**	**(−) Enzyme**	**SD**	**Tukey's HSD**	**Combined Effect (Observed−Expected) [3]**	**Combination Result**
128	87 a [4]	0.8	21 e	0.5	7.5	+20	synergistic
12.8	75 b	0.3	16 e	0.8		+13	synergistic
6.4	77 b	2.3	14 ef	0.3		+17	synergistic
1.3	59 c	1.6	8 f	0.0		+5	additive
0	46 d	0.5	NA	NA			

[1] Reduction in growth relative to non-treated controls. Each value is the mean of two replicates; [2] Enzyme concentration for *P. ultimum* was 4.0 nM LPO and 5.2 nM GOx and for *F. graminearum* and *R. solani* was 119.0 nM LPO and 154.7 nM GOx; [3] Difference between observed effect of fungicide (+) enzyme formulation and expected additive effect. Expected value calculated by adding the effect of fungicide alone and the effect of the enzyme formulation alone for each fungicide concentration; [4] Means followed by the same letter are not significantly different, Tukey's HSD ($p < 0.05$); NA indicates data not available, fungi grown as controls without fungicides or enzymes are the base for which % reduction in growth is measured against.

Stratego®YLD, combined with the stabilized enzyme formulation, resulted in an additive effect against *F. graminearum* at all four of the fungicide concentrations tested (Table 4). The combined effects were significantly greater than the effects of the fungicide alone at the three highest fungicide concentrations tested. The effect of the stabilized enzyme formulation alone was not significantly different from the combined effects nor the fungicides alone (Table 4).

Table 4. Inhibition of *Fusarium graminearum* by the stabilized LPO formulation alone and in combination with Stratego®YLD using fungicide-impregnated filter paper disks.

	% Reduction in Growth [1]						
Stratego®YLD Dose (µg)	**(+) Enzyme [2]**	**SD**	**(–) Enzyme**	**SD**	**Tukey's HSD**	**Combined Effect (Observed–Expected) [3]**	**Combination Result**
20	97 a [4]	0.2	32 bc	26.5	58.3	+10	additive
10	96 a	1.5	32 bc	28.7		+9	additive
2.5	94 a	0.3	30 bc	7.8		+9	additive
0.25	82 ab	3.2	19 c	2.7		+8	additive
0	55 abc	17.2	NA	NA			

[1] Reduction in growth relative to non-treated controls. Each value is the mean of two replicates; [2] Enzyme concentration for *P. ultimum* was 4.0 nM LPO and 5.2 nM GOx and for *F. graminearum* and *R. solani* was 119.0 nM LPO and 154.7 nM GOx; [3] Difference between observed effect of fungicide (+) enzyme formulation and expected additive effect. Expected value calculated by adding the effect of fungicide alone and the effect of the enzyme formulation alone for each fungicide concentration; [4] Means followed by the same letter are not significantly different, Tukey's HSD ($p < 0.05$); NA indicates data not available, fungi grown as controls without fungicides or enzymes are the base for which % reduction in growth is measured against.

Tilt®, combined with the stabilized enzyme formulation, resulted in a statistically significant antagonistic effect against *F. graminearum* at the third lowest fungicide concentration tested; while the remaining three concentrations were additive (Table 5). The combined effects were significantly greater than the effects of the corresponding fungicide concentration alone at the three lowest concentrations; but was not significantly different at the highest concentration. The effect of the stabilized enzyme formulation alone was significantly lower than the combined effects at the two highest fungicide concentrations and the highest fungicide concentration alone; but was not significantly different from the other combinations (Table 5). The Tilt® results for *R. solani* were similar to those for *F. graminearum*. The combined effects were additive at all four of the concentrations tested. The combined effect was significantly greater than the corresponding fungicide concentration alone at the lowest concentration tested. Combined effects versus fungicides alone were not significantly different at the three highest concentrations. Combined effects at all four of the fungicide concentrations were significantly greater than the enzyme formulation alone. The effect of the highest fungicide concentration alone was significantly greater than the enzyme formulation alone; and the three lowest fungicide concentrations alone were not significantly different from the enzyme formulation alone.

Table 5. Inhibition of *Fusarium graminearum* and *Rhizoctonia solani* by the stabilized LPO formulation alone and in combination with Tilt® using fungicide-impregnated filter paper disks.

	% Reduction in *F. graminearum* Growth [1]						
Tilt® Dose (µg)	**(+) Enzyme [2]**	**SD**	**(–) Enzyme**	**SD**	**Tukey's HSD**	**Combined Effect (Observed–Expected) [3]**	**Combination Result**
10	92 a [4]	3.2	71 abc	1.0	21.7	−8	additive
5	91 a	1.5	50 cd	4.1		−9	additive
2.5	76 ab	0.2	46 d	0.8		−24	antagonistic
0.25	81 ab	3.4	34 d	9.4		−19	additive
0	69 bc	12.8	NA	NA			
	% Reduction in *R. solani* Growth [1]						
Tilt® Dose (µg)	**(+) Enzyme**	**SD**	**(–) Enzyme**	**SD**	**Tukey's HSD**	**Combined Effect (Observed–Expected) [2]**	**Combination Result**
10	98 a [3]	3.0	93 a	7.8	34.3	−2	additive
5	92 a	11.0	67 abc	7.6		−8	additive

2.5	80 ab	16.2	50 bcd	2.3	−7	additive
0.25	51 bc	9.6	16 d	3.5	−1	additive
0	36 cd	7.1	NA	NA		

[1] Reduction in growth relative to non-treated controls. Each value is the mean of two replicates; [2] Enzyme concentration for *P. ultimum* was 4.0 nM LPO and 5.2 nM GOx and for *F. graminearum* and *R. solani* was 119.0 nM LPO and 154.7 nM GOx; [3] Difference between observed effect of fungicide (+) enzyme formulation and expected additive effect. Expected value calculated by adding the effect of fungicide alone and the effect of the enzyme formulation alone for each fungicide concentration; [4] Means followed by the same letter are not significantly different, Tukey's HSD ($p < 0.05$); NA indicates data not available, fungi grown as controls without fungicides or enzymes are the base for which % reduction in growth is measured against.

Tea tree oil, combined with the stabilized enzyme formulation, resulted in a statistically significant synergistic effect at the two lowest fungicide concentrations, and was additive at the two highest concentrations (Table 6). The combined effects were significantly greater than the effects of the enzyme formulation alone and three of the four fungicide concentrations alone. The highest fungicide concentration alone produced a significantly greater effect than enzyme formulation alone, and was not significantly different from any of the four combined effects (Table 6).

Table 6. Inhibition of *Pythium ultimum* by the stabilized LPO formulation alone and in combination with tea tree oil using fungicide-impregnated filter paper disks.

	% Reduction in Growth [1]						
Tea Tree Oil Dose	**(+) Enzyme [2]**	**SD**	**(–) Enzyme**	**SD**	**Tukey's HSD**	**Combined Effect (Observed–Expected) [3]**	**Combination Result**
30%	100 a [4]	0.0	91 a	12.1	23.4	0	additive
20%	100 a	0.0	66 b	9.4		+7	additive
15%	100 a	0.0	45 bc	2.1		+28	synergistic
10%	94 a	6.6	31 c	1.6		+36	synergistic
0%	27 c	4.7	NA	NA			

[1] Reduction in growth relative to non-treated controls. Each value is the mean of two replicates; [2] Enzyme concentration for *P. ultimum* was 4.0 nM LPO and 5.2 nM GOx and for *F. graminearum* and *R. solani* was 119.0 nM LPO and 154.7 nM GOx; [3] Difference between observed effect of fungicide (+) enzyme formulation and expected additive effect. Expected value calculated by adding the effect of fungicide alone and the effect of the enzyme formulation alone for each fungicide concentration; [4] Means followed by the same letter are not significantly different, Tukey's HSD ($p < 0.05$); NA indicates data not available, fungi grown as controls without fungicides or enzymes are the base for which % reduction in growth is measured against.

Mancozeb was tested using fungicide-amended media due to the insensitivity of *P. ultimum* and *F. graminearum* to mancozeb-impregnated filter paper disks. Mancozeb, combined with the stabilized enzyme formulation, resulted in a statistically significant synergistic effect on *P. ultimum* at the lowest fungicide concentration, and was additive at the three highest concentrations (Table 7). The combined effects were significantly greater than the effects of the enzyme formulation alone, and all four of the fungicide concentrations alone. The effect of the enzyme formulation alone was significantly greater than the three lowest fungicide concentrations alone, but was not significantly different from the highest concentration alone (Table 7). Combined activity against *F. graminearum* was additive at all four of the fungicide concentrations (Table 7). The combined effects were significantly greater than the corresponding fungicide concentrations alone at the three highest concentrations. The effect of the enzyme formulation alone was not significantly different from any of the combined effects nor the fungicides alone (Table 7).

Table 7. Inhibition of *Pythium ultimum* and *Fusarium graminearum* by the stabilized LPO formulation alone and in combination with mancozeb using fungicide-amended media.

Mancozeb Concentration (mg/L)	% Reduction in *P. ultimum* Growth [1] (+) Enzyme [2]	SD	(–) Enzyme	SD	Tukey's HSD	Combined Effect (Observed–Expected) [3]	Combination Result
10	53 a [4]	0.3	23 d	1.7	6.04	+4	additive
5	45 b	0.5	16 e	0.4		+3	additive
2	34 c	1.3	10 e	0.8		−2	additive
0.5	35 c	3.7	2 f	0.5		+7	synergistic
0	26 d	0.9	NA	NA			
Mancozeb Concentration (mg/L)	**% Reduction in *F. graminearum* Growth [1] (+) Enzyme [2]**	**SD**	**(–) Enzyme**	**SD**	**Tukey's HSD**	**Combined Effect (Observed–Expected) [3]**	**Combination Result**
10	58 a [4]	5.7	26 bcd	6.0	32.4	+3	additive
5	53 ab	15.6	17 cd	5.4		+7	additive
2	51 ab	5.3	1 d	5.1		+21	additive
0.5	39 abc	10.9	6 cd	3.8		+4	additive
0	29 abcd	8.9	NA	NA			

[1] Reduction in growth relative to non-treated controls. Each value is the mean of two replicates; [2] Enzyme concentration for *P. ultimum* was 4.0 nM LPO and 5.2 nM GOx and for *F. graminearum* and *R. solani* was 119.0 nM LPO and 154.7 nM GOx; [3] Difference between observed effect of fungicide (+) enzyme formulation and expected additive effect. Expected value calculated by adding the effect of fungicide alone and the effect of the enzyme formulation alone for each fungicide concentration; [4] Means followed by the same letter are not significantly different, Tukey's HSD ($p < 0.05$); NA indicates data not available, fungi grown as controls without fungicides or enzymes are the base for which % reduction in growth is measured against.

3. Discussion

The stabilized LPO system inhibited the in vitro growth of plant pathogenic *P. ultimum*, *F. graminearum*, and *R. solani*. *Pythium ultimum* was more sensitive to the LPO system than *F. graminearum* and *R. solani*, requiring approximately 30 times less enzyme to achieve comparable levels of inhibition. The difference in sensitivity between fungi and oomycetes was not surprising, given that different organisms are known to vary in their sensitivity to the LPO system [27]. To date, most of the studies involving the LPO system have focused on human bacterial pathogens, such as *Staphylococcus* spp., *Pseudomonas aeruginosa*, *Escherichia coli*, *Streptococcus* spp., and yeast like *Candida albicans* [27–29]. A few studies have demonstrated activity against spores of filamentous fungi, such as *Aspergillus fumigatus*, *Aspergillus niger*, *Mucor rouxii*, and *Byssochlamys fulva* [30,31]. To our knowledge, this is the first report of activity against the vegetative state of filamentous fungi and oomycetes, which may have important implications for crop disease management.

The LPO system is complex, and the concentration of each enzyme and substrate in the system ultimately affects its antimicrobial activity, making it difficult to directly compare results across studies. Bosch et al. demonstrated that LPO at a concentration of 387 nM in an LPO system similar to the one used here was sufficient to kill 1,000,000 CFU of *C. albicans*, *E. coli*, *Staphylococcus aureus*, and *P. aeruginosa* when exposed to the LPO system in solution for at least two hours [27]. We found that a slightly lower concentration of LPO (323 nM) was sufficient to inhibit, but not kill, the fungi *R. solani* and *F. graminearum*, and a much lower concentration (20 nM) was sufficient to completely kill the oomycete *P. ultimum*. Although the LPO concentrations that were used in the Bosch et al. study were relatively similar to the highest LPO concentration that was included in this study, the assay conditions were very different [27]. The majority of studies on the antimicrobial activity of the LPO system conducted to date, including the Bosch et al. study, have involved bacteria and yeasts that were exposed to a non-stabilized LPO system in a liquid suspension [27]. The effect of fungicides on filamentous fungal growth is typically assayed by exposing the fungus to the active ingredient in a

petri dish containing solid growth media and measuring the relative reduction in mycelial growth. The disparity in assay conditions, along with differences in LPO system parameters and the use of free versus stabilized enzymes further contributes to the challenge of comparing the antimicrobial activity of the LPO system across studies. Utilizing a standardized set of assay conditions for further study of the LPO system on filamentous fungi would be helpful for building on existing work and establishing a body of knowledge on this system.

When the stabilized LPO formulation was combined with five commercial fungicides, the effects ranged from synergistic to antagonistic and were dependent on the pathogen X fungicide X concentration combination. This is in agreement with previous studies, which found that synergistic enhancement of fungicidal activity is dependent on the sensitivities of the target species, or strains within a species, to each individual component of the mixture [9]. For example, Samoucha and Cohen (1986) found that a mixture of the systemic fungicide metalaxyl and any of five different contact fungicides resulted in the synergistically enhanced control of potato late blight caused by either a metalaxyl-sensitive or metalaxyl-resistant isolate of *Phytophthora infestans* [32]. However, the synergistic activity was much greater against the metalaxyl-resistant isolate when compared to that of the metalaxyl-sensitive isolate. Similar results were observed with mixtures of metalaxyl and mancozeb against metalaxyl-sensitive and -resistant isolates of the cucumber downy mildew pathogen *Pseudoperonospora cubensis* [33].

The goal of the fungicide portion of this study was to characterize the interactions of select fungicides in combination with the stabilized LPO system with regard to two species of fungi and one oomycete. Fungicides were chosen based on whether they were labeled for use against each individual species, which is why each fungicide was not tested against each species. However, mancozeb was tested on both *P. ultimum* and *F. graminearum*, and Tilt® was tested on both *F. graminearum* and *R. solani*, allowing for inter-species comparisons of LPO-fungicide interactions. Although a pattern of synergy was observed with both of the species that were tested with mancozeb, the relatively large experimental errors between *F. graminearum* replicates made it difficult to detect statistically significant synergy in that experiment. Significant synergy was observed with *P. ultimum* at the lowest mancozeb concentration tested. In the Tilt® experiments, a pattern of antagonism was observed with both of the species tested, but once again the large experimental errors between replicates allowed for the detection of only one case of statistically significant antagonism with *F. graminearum* at the second to lowest Tilt® dose that was tested. The variance was generally greater in experiments involving *F. graminearum* and *R. solani* when compared to *P. ultimum*.

Fungicides are critical for crop disease management. Some fungicides, however, have been shown to have phytotoxic effects at high concentrations that are required to effectively control disease, and the number of fungicides that are available to farmers is declining due to increasing government regulation and loss of efficacy due to resistance [8,21,34–36]. The introduction of novel fungicide chemistries that meet government regulatory standards, and the protection of existing chemistries against the threat of obsolescence due to resistance will likely be crucial for the assurance of food security in the future. Our results show that the stabilized LPO system may be a viable tool for controlling crop diseases that are caused by fungi and oomycetes, and that it does not affect the germination or vigor of tomato seedlings under nutrient rich conditions. Future studies of the LPO system as a potential seed treatment should evaluate its efficacy in greenhouse and field trials, under low nutrient conditions, and other environmental stresses. Furthermore, the stabilized LPO system was shown to be compatible with several commercial fungicides, and its broad-spectrum activity makes it a good candidate for co-formulations with the goal of controlling multiple diseases in a single application or reducing the probability of selecting for resistance in treated pathogen populations. Further research is required to examine the mechanism of diffusion into the complex soil environment by the stabilized LPO system. The stabilized LPO system may also meet organic certification standards because it utilizes naturally derived enzymes, making it a potentially valuable tool for organic and conventional crop protection.

4. Materials and Methods

4.1. Isolates

Isolates of *Pythium ultimum* (isolate Geneva16), *Fusarium graminearum* (isolate GZ014NNY98), and *Rhizoctonia solani* (isolate AC1-A1) were all originally collected in New York State and were obtained from collaborators in the Section of Plant Pathology and Plant-Microbe Biology at Cornell University. *F. graminearum* and *R. solani* were maintained on potato dextrose agar (PDA) (Sigma-Aldrich, St. Louis, MO, USA) and *P. ultimum* was maintained on cornmeal agar (CMA) (Sigma-Aldrich).

4.2. Fungicides, Enzymes and Reagents

Commercial fungicide formulations that were used in the study were Daconil® (active ingredient (AI): chlorothalonil 29.6%, GardenTech, Lexington, KY, USA), Tilt® (AI: propiconazole 41.8%, Syngenta, Basel, Switzerland), Stratego YLD® (AI: prothioconazole 10.8% + trifloxystrobin 32.3%, Bayer, Leverkusen, Germany), mancozeb flowable with zinc (AI: mancozeb 37%, Bonide, Oriskany, NY, USA), and tea tree oil (AI: tea tree oil 100%, Mason Natural, Miami Lakes, FL, USA). Potassium iodide (KI), ammonium thiocyanate (NH_4SCN), carboxymethyl cellulose (CMC), glucose, lactoperoxidase (LPO, ≥150 U/mg), and glucose oxidase (GOx, 177 U/mg) were obtained from Sigma-Aldrich.

4.3. Optimizing Enzyme Formulation for In-Vitro Assays, and Enzyme Stabilization

The concentration of LPO and GOx in the dry enzyme film disks was optimized prior to beginning fungicide interaction experiments to identify enzyme concentrations that resulted in a measurable reduction in growth for each species without being completely inhibitive. LPO and GOx were always included in a 1:1.3 M LPO:GOx ratio, and the final enzyme concentration that is reported is the amount of each enzyme (LPO and GOx) in each 50 µL enzyme disk. *P. ultimum* was tested at four LPO and GOx concentrations, and *F. graminearum* and *R. solani* were tested at three LPO and GOx concentrations (Table 1). Optimization tests were done in duplicates. Dry enzyme film disks were made by combining 3 µL KI (1M), 5 µL NH_4SCN (1M), 175 µL 4% carboxymethyl cellulose (CMC), varying volumes of stabilized LPO + GOx, and 3 µL blue food dye brought up to a final volume of 1 mL with distilled deionized water (DDI H_2O). Enzyme stabilization was performed, as previously described [22,37]. Briefly, LPO (125 µg/mL, pH 7.4) and GOx (330 µg/mL, pH 7.4) were mixed to achieve a 1:1.3 M LPO:GOx ratio and stored on ice. Magnetite nanoparticles (NP) (1.277 mg/mL, pH 3, approximately 5 mL stock) were ultrasonicated at 40% amplitude for 1 min, cooled to ambient temperature (approximately 21 °C) in a water bath, and pipette mixed with the LPO:GOx enzyme suspension in a 1:1 enzyme:NP ratio. Dry substrate film disks were made by combining 30 µL KI (1M), 50 µL NH_4SCN (1M), 350 µL 4% CMC, 500 µL glucose (1M), and 3 µL red food dye brought up to a final volume of 1 mL with DDI H_2O. Dyes were included to differentiate enzyme disks from the substrate disks and were not biologically active or structural components of the disks. Each solution was pipette mixed several times and vortexed briefly. Solutions were dispensed in 50 µL aliquots onto parafilm and were dried at an ambient temperature in a vacuum oven containing desiccant at −50 kPa. The diameter of the dried enzyme and substrate film disks is approximately 5 mm. After approximately 2 h, dry enzyme and substrate disks were stored in the dark at 4 °C until use.

To test the sensitivity of each species to the enzyme formulation, one substrate disk was removed from the parafilm sheet and was placed on the center of a petri dish containing CMA (*P. ultimum*) or PDA (*F. graminearum* and *R. solani*). Next, one enzyme disk was removed from the parafilm sheet and was placed on top of the substrate disk. Finally, one culture plug was placed on the disks mycelia-side down. *P. ultimum* was tested using 7 mm-diameter culture plugs, and *F. graminearum* and *R. solani* were tested using 4 mm-diameter culture plugs. Once non-treated control colonies had grown to nearly the edge of the plate (two days for *P. ultimum*, four days for *R. solani*, five days for *F.*

graminearum) two perpendicular colony diameter measurements were taken for *P. ultimum* and *R. solani*. Colonies of *F. graminearum* were non-circular, therefore photos of each plate were taken and colony areas were measured using the public domain image processing program ImageJ (available at https://imagej.nih.gov/ij/).

4.4. Evaluating Phytotoxicity of Stabilized Enzyme Formulation

Tomato seeds (*Solanum lycopersicum*, 'Celebrity F1' Lot #53815, Johnny's Selected Seeds, Winslow, ME, USA) were coated with the stabilized enzyme formulation as follows: For each seed, a dry substrate disc was placed on parafilm. A single tomato seed was placed on top of the dry substrate disc, and then covered with a dry enzyme disc. A 10 µL drop of sterile DDI H_2O was added to the stack to adhere the dry enzyme and substrate discs to the seed. The coated seeds were allowed to dry for 1 hour. Following the drying time, the coated seeds were rolled between gloved fingers to ensure good contact between the seed and the substrate and enzyme discs. Five seeds were coated with four concentrations of the stabilized enzyme formulation, plus five seeds coated with just inert components of the coating and five seeds uncoated as controls. Coated seeds were planted in pre-moistened Miracle-Gro potting mix (Scotts Miracle-Gro Company, Marysville, OH, USA) and were placed under t5 high-output grow lights (Hydroplanet, Wheat Ridge, CO, USA). Pots were watered with DDI H_2O until seedlings emerged, then watered with label rate Miracle-Gro fertilizer (Scotts Miracle-Gro Company, Marysville, OH, USA). After four weeks, plant height was measured and seedlings were harvested. Soil was washed from the roots with water, and then root and shoots were separated and weighed.

4.5. Sensitivity of P. ultimum, R. solani, and F. graminearum to Fungicides Alone and in Combination with the Stabilized Enzyme Formulation In-Vitro

Commercial fungicides formulated as aqueous suspensions were thoroughly mixed to ensure homogeneity and diluted in sterile DDI H_2O. Fungicide dilutions are shown in Table 8. Whatman® qualitative grade 1 filter paper (FP) (Sigma-Aldrich) was cut into 7 mm disks using a 3-hole punch, and disks were autoclaved prior to use. FP disks were impregnated with 5 µL of each of four fungicide dilutions per fungicide tested resulting in a known mass of active ingredient (AI) per FP disk except for tea tree oil, which does not include mass of AI per unit volume on the label. Control FP disks were impregnated with 5 µL sterile DDI H_2O. Fungicide X enzyme disk interactions were tested by placing one substrate disk on the center of each petri dish containing PDA (*F. graminearum* and *R. solani*) or CMA (*P. ultimum*), followed by one enzyme disk, as previously described. Next, one fungicide-impregnated FP disk was applied on top of the enzyme and substrate disks. Finally, one culture plug was place on top of the FP disk mycelia-side down. Final enzyme concentrations in enzyme disks were 4 nM for *P. ultimum* and 119 nM for *F. graminearum* and *R. solani* based on results from preliminary enzyme formula optimization experiments. Plugs of *P. ultimum* measured 7 mm in diameter and plugs of *F. graminearum* and *R. solani* measured 4 mm in diameter. Preliminary testing revealed that *P. ultimum* is more sensitive to the enzyme treatment than *F. graminearum* and *R. solani*, so enzyme concentrations and plug sizes were chosen to achieve a measurable growth reduction, without being completely inhibitive, in enzyme-only treatments as compared to non-treated controls. Each experiment included the same fungicide dilution series plated without substrate and enzyme disks, as well as a substrate + enzyme disk-only treatment and a non-treated control. All of the plates, including controls, contained a FP disk, and each treatment was replicated once. Plates were left on the bench at ambient temperature for 2, 4, or 5 days for *P. ultimum*, *R. solani*, and *F. graminearum*, respectively, by which time control colonies had nearly grown to the plate edge. Two perpendicular colony diameter measurements were recorded for *P. ultimum* and *R. solani*. Colonies of *F. graminearum* measured using the public domain image processing program ImageJ.

Table 8. Fungicides and dilutions used in fungicide interaction experiments.

Trade Name	Active Ingredient (AI)	% AI in Commercial Formulation	FRAC Group	Mass AI per FP Disk (μg)			
Daconil®	Chlorothalonil	29.6%	Chloronitriles	128	12.8	6.4	1.3
Tilt®	Propiconazole	41.8%	DMI	10	5	2.5	0.25
Stratego® YLD	Prothioconazole + trifloxystrobin	prothioconazole 10.8% trifloxystrobin 32.3%	DMI + QoI	20	10	2.5	0.25
Tea tree oil	Tea tree oil	67%	Plant extract	4.0	2.7	2.0	1.3
				Concentration in amended media (mg/L)			
Mancozeb	mancozeb	37%	Dithiocarbamate	10	5	2	0.5

P. ultimum and *F. graminearum* were insensitive to mancozeb in impregnated-FP tests, so an additional experiment was done using mancozeb-amended media. CMA and PDA (for *P. ultimum* and *F. graminearum*, respectively) were amended with mancozeb to achieve final concentrations of the fungicide formulation of 10 mg/L, 5 mg/L, 2 mg/L, 0.5 mg/L, and 0 mg/L (controls). The same experimental design used in the impregnated-FP tests was used for amended-media tests, except FP was not included in any of the treatments or controls.

4.6. Statistical Analysis and Determination of Synergism, Additivity, and Antagonism

Reduction in growth was calculated using the following formula (1):

$$((\text{diameter control colony} - \text{diameter test colony})/\text{diameter control colony}) \times 100 \quad (1)$$

Experiments involving *F. graminearum* used colony area rather than colony diameter in the formula due to non-circular colony morphologies. Statistical analyses were done using R (version 3.3.0, R Foundation for Statistical Computing, Vienna, Austria). Each fungicide-by-pathogen combination was analyzed separately. Analysis of variance was done using the agricolae package [38] and treatment means were separated using the Tukey-Kramer honest significant difference (HSD) test ($p < 0.05$).

The effects of fungicide combined with stabilized enzyme formulation were characterized as synergistic, additive, or antagonistic based on the methods described by Couch et al. [9]. Briefly, an additive effect is observed when the level of disease control imparted by the co-operative action of two or more fungicides is equal to the control, as predicted by the response of each fungicide applied by itself. Synergism is observed when the disease control imparted by the joint application of two or more fungicides is greater than the control that would be predicted by an appropriate reference, in this case, the additive effect. Similarly, antagonism is observed when the disease control imparted by the joint application of two or more fungicides is less than the control predicted by an appropriate reference, again, in this case, the additive effect. Theoretical additive values (i.e., expected combined effects) were calculated by adding the observed effect of each fungicide alone with the observed effect of the enzyme formulation alone. Tukey's HSD value at $p < 0.05$ was then applied to the theoretical additive value to determine whether actual combined effects were synergistic, additive, or antagonistic. For example, if the theoretical additive value was 50% and the Tukey's HSD value was 10%, then the observed combined effect would be considered synergistic at >60%, additive between 40% and 60%, and antagonistic at <40%. If the theoretical additive value was >100%, then it was considered 100% (complete growth inhibition) for the purposes of this calculation.

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Conflicts of Interest: The authors of this manuscript were all working for Zymtronix Catalytic Systems, Inc. at the time of this study. Materials provided to us are acknowledged. Zymtronix is pursuing the development and commercialization of stabilized biocidal enzymes for agricultural applications based on the results of this study.

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