Project Title: New products for the prevention and control of shoot trauma blight

Report Type: Final Project Report

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Budget 1

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Item	3/1/2023	3/1/2024
Salaries	$20,064.00	$20,867.00
Benefits	$9,000.00	$9,361.00
Wages		
Benefits		
RCA Room Rental		
Shipping		
Supplies	$10,328.00	$10,749.00
Travel	$250.00	$250.00
Plot Fees		
Miscellaneous		
Total	$39,642.00	$41,227.00

Footnotes: 4% inflation for year 2. [1]Salary 4.5 months; [2]Benefits rate 44.9%; [3]Lab and field supplies include the tested compounds, apple trees, substrate and fertilizers, culture media and reagents for qPCR.

Objectives:

1. *In vitro* screening for antibacterial activity, dosage, and potential additive and/or synergistic effects in different products (e.g., soluble chitosan derivatives, rhamnolipids, lauric acid, caprylic acid) or mixtures with already used antimicrobials.
2. Elaborate a methodology to screen materials for shoot blight prevention and control, mimicking natural infection conditions after wind and hail damage.
3. Evaluate *in planta* efficacy of products and/or product combinations in greenhouse and field conditions.

Significant Findings

- Chitosan lactate demonstrated the strongest in vitro inhibitory activity among all tested products.
- A reproducible wounding and inoculation method to mimic trauma blight symptoms in crabapple and apple was developed.
- Sodium caprylate exhibited significant phytotoxicity on apple trees when applied at its effective in vitro bactericidal concentration (0.4%). The maximum safe concentration of sodium caprylate for apple trees was determined to be 0.08%, based on dose-response testing assays with 'Fuji' seedlings.
- Among tested commercial chitosan-based products, TidalGrow SPECTRA used at concentrations above the recommended by the manufacturers, was the only one providing significant disease control and reducing disease severity in both greenhouse and field trials. The other commercial products, and also chitosan lactate alone, had reduced or no activity controlling shoot blight.
- Rhamnolipids also reduced symptom severity and provided effective disease control consistently among trials, particularly in the days immediately following infection.
- There were important differences in the efficacy of rhamnolipids and the chitosan formulation of TidalGrow SPECTRA between greenhouse and field trials, with fire blight symptoms developing faster and more aggressively in the field. Factors that could explain some of these differences are:
    - Greenhouse and field trials were performed with trees of different host cultivars, 'Pink Lady' and 'Gala', respectively, with differing susceptibilities to fire blight.
    - Greenhouse trials were performed during mid and late summer, with higher top temperatures recorded than during field trials, which were carried out at the beginning of summer.
    - The pH of rhamnolipids in field trials was adjusted to 5.5 instead of 4.0, in order to compare results with mixed treatments with Cueva adjusted to the same pH to avoid phytotoxicity issues with copper.
- None of the products tested in the field caused russeting
- Single spray applications of both rhamnolipids and chitosan from TidalGrow SPECTRA induced plant defense-related genes, with peak expression observed at 3-7 days post-spray. This suggests that their efficacy in greenhouse and field trials may be partially attributed to the activation of plant's natural defenses. These results indicate that treatment efficacy might be improved by spacing sprays more than 3 days apart.
- In vitro tests showed rhamnolipids synergistically enhanced antibiotic activity when co-applied with streptomycin and especially kasugamycin. This means that rhamnolipids could be used as co-adjuvants to increase antibiotic efficacy. However, apple seedling assays revealed incompatibilities between rhamnolipids and both Actigard and chitosan-based products, combined treatments with other plant defense elicitors are discommended.
- Chitosan formulation in TidalGrow SPECTRA enhanced the activity of other plant defense inducers like Actigard when combined. These combinations worked at lower product concentrations while maintaining or improving efficacy compared to individual treatments at full concentration.
- Overall, while rhamnolipids and chitosan in TidalGrow SPECTRA provided consistent protection against shoot blight and/or significantly reduced symptom severity in both greenhouse and field trials. Although the observed protection could be considered intermediate-low, the trials were performed under very high infection pressure. Their consistent activity suggests these treatments may be viable eco-friendly alternatives to other agrochemicals that can be integrated into existing management strategies, particularly under lower infection pressure conditions.

Results and Discussion

1. Most of the assayed materials possess bactericidal activity against *E. amylovora*

As part of **Objective 1**, we tested the in vitro antimicrobial activity of different products with potential use in the orchard against shoot blight. Of all the tested products, chitosan lactate demonstrated the strongest in vitro activity against *E. amylovora*. It significantly reduced *E. amylovora* populations by 50% and 95% at very low concentrations of 0.03% and 0.05%, respectively (**Figure 1C**). The next most effective products were sodium caprylate, with IC_{50} and IC_{95} values of 0.17% and 0.45%, respectively (**Figure 1A**), and rhamnolipids (**Figure 1B**). Rhamnolipids exhibited a biphasic growth inhibition curve, with two distinct concentrations (IC_{50} at 0.10% and IC_{50} at 0.40%) causing a 50% reduction in populations during each phase.

Chitosan oligosaccharides derived from mushrooms and crabs showed some bactericidal activity but did not completely inhibit *E. amylovora* growth under the test conditions, indicating low effectiveness against the pathogen (**Figure 1D** and **1E**, respectively).

Raw chitosan could not be included in this assay due to incompatibilities with the experimental conditions. Chitosan solutions are highly viscous and require acidic conditions for solubilization. Sodium lactate was soluble but precipitated during incubation, making it impossible to measure bacterial growth (**Table 1**).

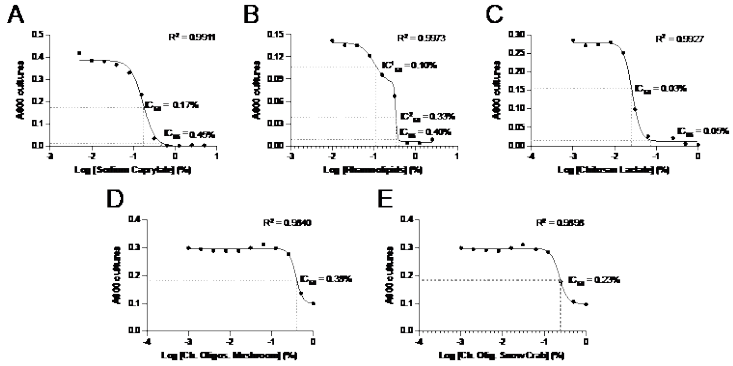

Figure 1. Effect of sodium caprylate (A), rhamnolipids (B), chitosan lactate (C), chitosan oligosaccharides from mushroom (D), and chitosan oligosaccharides from snow crab (E) on *E. amylovora* growth. Each chart shows the IC_{50} and IC_{95} values obtained in triplicate assays. Curves were adjusted by non-linear regression after log-transformation of the assayed compound concentrations. The R^2 values indicate the goodness of fit of the curves to the obtained data.

Table 1. Performance of different compounds inhibiting *E. amylovora* growth.

Compound	Concentration Range	IC50	IC95
Sodium Caprylate	0 - 5 %	0.17%	0.45%
Sodium Laurate [a]	0 - 5 %	ND	ND
Rhamnolipids [b]	0 - 5 %	0.10% 0.33%	0.40%
Chitosan [c]	0-1 %	NA	NA
Chitosan Lactate	0 - 1 %	0.03%	0.05%
Chitosan Oligos (Crab) [d]	0 - 1 %	0.38%	NA
Chitosan Oligos (Mushroom) [d]	0 - 1 %	0.23%	NA

[a] Although sodium laurate was soluble in water and culture medium, after overnight incubation, the salt precipitated on the bottom of the wells, making it impossible to quantify *E. amylovora* growth. ND, not determined.

[b] The effect of rhamnolipids on *E. amylovora* growth fitted a biphasic curve with two significant decreases of bacterial growth, defined by the first and second IC_{50} values (0.10 % and 0.33%). In this case, the IC_{95} value was calculated manually, interpolating the value directly from the chart.

[c] Due to low solubility without extremely altering the pH under the assayed conditions, this product was removed from these assays. NA, not applicable.

[d] The assayed concentrations did not completely inhibited *E. amylovora* growth, and higher concentrations were difficult to dissolve. NA, not applicable.

In summary, all the tested substances demonstrated antimicrobial properties that could be useful for managing fire blight in the field. A key aspect of this assay was its design to determine whether the compound itself, rather than just its pH, had inhibitory effects on *E. amylovora*. The test was conducted under controlled pH conditions in a medium that supports pathogen growth. This is significant because many agricultural products are prepared at low pH levels before application, and this acidity may also contribute to killing pathogen cells in the field. Notably, the activity of caprylic acid, rhamnolipids, and chitosan has been reported to persist or even increase at low pH levels, such as pH 4.0 or 5.0.

2. Optimization of treatment conditions using apple seedlings and crabapples.

Key findings from 'Fuji' seedling assays revealed:

1. Caprylic acid showed significant phytotoxicity at 0.4% (concentration selected based on in vitro assays). All treated plantlets developed extensive necrotic lesions across their leaves within 24 hours of initial application. Follow-up experiments confirmed this effect, showing necrotic spots on leaf surfaces even at 2-fold dilutions (0.4% to 0.16%). Concentrations of 0.08% and lower did not cause phytotoxicity under experimental conditions, leading us to adopt this concentration for subsequent potted plant experiments.
2. Some plants treated with Armour-Zen 15% (8 qt/50 gal) and chitosan oligosaccharides from mushroom (1%) showed isolated necrotic spots on some leaves in one out of three/six plants at the end of the experiment. As a precautionary measure, we reduced these product concentrations in subsequent assays (**Table 2**).
3. Commercial chitosan-based products contained acetic acid with pH ranges from 3.77 to 5.22. Control tests using water buffered to pH 3.2-3.7 (with glacial acetic acid and K_2HPO_4) showed no effect on *E. amylovora* infections compared to distilled water controls, ruling out potential bactericidal effects of acetic acid alone.
4. Plantlets treated with Armour-Zen 15% and inoculated with *E. amylovora* developed significantly larger necrotic lesions than untreated controls at 10 days post-inoculation.
5. Plants treated with rhamnolipids and caprylic acid showed smaller necrotic lesions compared to untreated controls sprayed with water or water buffered to pH 3.2, although these differences were not statistically significant (data not shown).

3. Development and optimization of an inoculation method mimicking trauma blight conditions

As part of **Objective 2**, we evaluated two methods to replicate trauma blight symptoms: (1) cutting with sterile scissors followed by spray inoculation, and (2) spray inoculation followed by wounding with scissors. The first method simulates plant tissue damage from strong winds or hail, where *E. amylovora* cells subsequently reach wounds through biological vectors (e.g., insects) or physical vectors (e.g., wind-driven rain). The second method represents a less common scenario where *E. amylovora* is present on plant surfaces before wind or hail damage creates entry points into plant tissues. Generally, *E. amylovora* is considered a poor colonizer of leaves and other plant organs besides flowers. Abundance of *E. amylovora* cells on other plant surfaces than flowers are more likely in heavily damaged orchards with abundant ooze, necrotic lesions, or infected flowers.

Both methods were highly reproducible, reaching infection rates of 100% regardless of the pathogen concentration used. This suggests that even at 10^6 CFU/mL, these conditions could match a scenario of high infection pressure. The only factor significantly affecting symptom severity after 7 days was pathogen concentration, accounting for 36.4% of the observed variability ($P = 0.0016$). Multiple comparison tests revealed that *E. amylovora* concentrations had a significant effect when cuts were performed before spray inoculation ($P = 0.0185$) but not when the pathogen was sprayed before performing the cuts ($P = 0.0568$) (**Figure 2A**). Based on these results, and considering the first scenario seems more plausible, we selected the strategy of making cuts before spray inoculation as the preferred method for reproducing trauma blight symptoms in apples.

For the inoculations in this experiment, we made two cuts at the base of the leaf toward the midrib and one transversal cut to the leaf tip (**Figure 2B**). While all cuts showed signs of infection, the ones at the base of the leaf exhibited highly reproducible symptom severities, unlike those on the leaf tip (**Figure 2B**). Therefore, for the greenhouse assays with potted 'Pink Lady' trees and field trials with 'Gala' trees (see below), we only made cuts at the base of the leaves before *E. amylovora* spray inoculation.

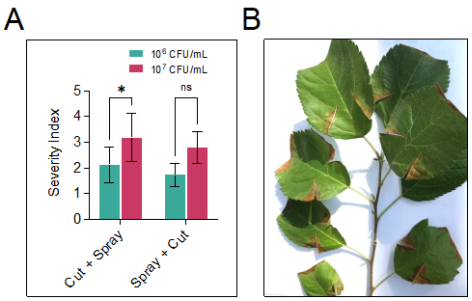

Figure 2. Optimization of an inoculation method to recreate trauma blight-like symptoms using white crabapple plants. Two inoculation methods, cutting leaves plus spray inoculation with *E. amylovora* (Cut + Spray), and spray inoculation followed by cutting with sterile scissors (Spray + Cut), were used. Results were average values of 5-8 replicate leaves per shoot in two different shoots. The asterisk indicates significant differences associated with inoculum concentration when sprayed after cutting ($P \leq 0.05$) (A). Cut positions and representative fire blight symptoms 7 days after inoculation (B).

3. Evaluation of treatments for trauma blight control in greenhouse trials

Based on results from **Sections 1, 2**, and **3**, we optimized treatments with different compounds as well as an inoculation method mimicking trauma blight conditions (**Table 2**) for testing on two-year-old 'Pink Lady' apple trees. The treatments included Actigard as a plant defense elicitor control, along with copper (Cueva), streptomycin (FireWall), and oxytetracycline (FireLine) as bactericide controls. Disease control efficacy was calculated as the percentage reduction in disease incidence in inoculated wounds compared to the untreated control. Treatments performed during the first greenhouse trial in 2023 are summarized in **Table 2**, with results reported in **Figure 3**.

In this first trial, among the 10 treatments tested, only FireWall, TidalGrow SPECTRA, and FireLine provided significant fire blight disease control throughout the experiment, with median efficacy values of 76.8%, 63.9%, and 56.6%, respectively, at 33 days post-inoculation ($P \leq 0.0117$) (**Figure 3A**). Rhamnolipids showed relatively good disease control of 46.7% at 11 dpi ($P = 0.0120$) but declined to 12.4% by the end of the experiment ($P = 0.0021$). Other treatments, such as Cueva and ChitoAg-80, provided similarly low but statistically significant disease control at 33 dpi, with values of 12.4% and 13%, respectively ($P \leq 0.0482$) (**Figure 3A**). However, these values, based on infection

incidence, do not fully capture the activity of the tested products. **Figure 3B** shows that although Actigard, Cueva, and rhamnolipids provided low disease control efficacy percentages, they reduced lesion size by 30-60% compared to untreated controls during the same period ($P \leq 0.0356$). Conversely, the other commercial chitosan-based compounds, Armour-Zen 15% and ChitoAg-80, performed poorly in reducing disease symptoms (**Figure 3B**) and controlling the disease, particularly Armour-Zen 15% (**Figure 3A**).

Table 2. Spray treatments applied on 2-year-old potted 'Pink Lady' trees in the 2023 greenhouse trial.

Treatment	No. Trees	Active compound	Application concentration	% Active compound	Application Pattern [d]
Actigard 50 WG	5	Acibenzolar-S-methyl (50%)	2 oz/100 ga	0.007	A
FireWall 50 WP	4	Streptomycin sulfate (65.8%)	8 oz/100 ga	0.039	B
Fire Line 45 WP	4	Oxytetracycline hydrochloride (48.8%)	9 oz/100 ga	0.033	B
Cueva	5	Copper octanoate (10%)	4 qt/100 ga	0.100	B
Armour-Zen 15%	4	Chitosan (15%)	4 qt/100 ga [a]	0.150	A
ChitoAg-80	5	Chitosan (4%)	2 qt/100 ga	0.020	A
TidalGrow SPECTRA	4	Chitosan (5.75%)	22 fl oz/ 2 ga	0.494	A
Rhamnolipids [b]	5	Rhamnolipids (90%)	0.40%	0.36	A
Sodium caprylate [b]	5	Sodium caprylate (98%)	0.08% [c]	0.076	A
Chitosan Oligos. [b]	5	98.2% Deacetylated chitin (mushroom)	0.20%	0.200	A
Untreated control	5	NA	NA	NA	A

[a] Because of potential phytotoxicity at the highest recommended concentration, we used a lower one.

[b] Products adjusted to pH 4.0 with glacial acetic acid.

[c] Due to high phytotoxicity, we used a concentration 5 times lower than the original one (0.4%), which showed no phytotoxicity in apple plantlets.

[d] **A**, for products with recognized or potential plant-defense inducers: treatment application on 7-29-23, 8.01.23; tree wounding and inoculation on 8.03.23; treatment application 8.04.23 (9-11h post-inoculation) and 8.07.23. **B**, for products with bactericidal activity only: treatment application 8.02.23; tree wounding and inoculation on 8.03.23; treatment application on 8.04.23 (9-11h post-inoculation) and 8.07.23.

Comparisons among the three commercial chitosan-based products indicated that high chitosan concentrations might be important for significant protection against fire blight, although other factors probably contribute to chitosan efficacy. Treatments with TidalGrow SPECTRA, Armour-Zen 15% and ChitoAg-80 were designed to achieve different final chitosan concentrations of around 0.5%, 0.15% and 0.02%, respectively (**Table 2**). The most effective protection was observed with TidalGrow SPECTRA prepared with the highest chitosan concentration. Interestingly, despite its lower chitosan concentration, ChitoAg-80 provided better disease control than Armour-Zen 15%, which contained 7.5 times more chitosan. Similarly, symptom severity was slightly reduced in plants treated with ChitoAg-80 compared to those treated with Armour-Zen 15% (**Figure 3B**). These findings suggest that additional ingredients within the commertial product composition may contribute to enhance ChitoAg-80 activity at lower doses or decrease Armour-Zen 15% efficacy despite its higher chitosan dosage.

For treatments with sodium caprylate, none of the trees sprayed at a 0.08% concentration exhibited signs of phytotoxicity on the leaves. While the analysis did not show statistically significant differences compared to the control, it is notable that, except for one tree, symptom severity was generally reduced relative to the control.

Based on these results, we designed the treatments for the 2024 greenhouse trial, which included Actigard (2 oz/100 gal), FireWall 50 WP (8 oz/100 gal), and Cueva (4 qt/100 gal) as controls for plant defense induction and bactericides, respectively. In this trial, we reduced the number of treatments, focusing only on those that showed better results in the previous trial, specifically rhamnolipids (0.4%) and TidalGrow SPECTRA (1100 fl oz/100 gal). For comparison, we also

included a treatment with pure chitosan lactate derived from snow crab, prepared at a 0.2% concentration (**Table 3**).

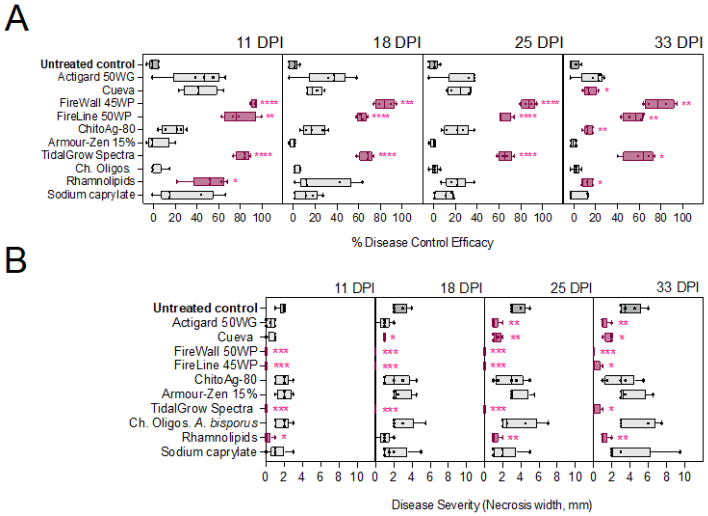

Figure 3. Efficacy of different products in trauma blight control and symptom severity reduction throughout time in potted 'Pink Lady' trees, in a first greenhouse trial in 2023. The **%** disease control was calculated as the % disease incidence reduction with respect to the % disease in the untreated control, multiplied by 100. Disease severity was measured as the longest distance between the cut and the necrotic lessions in the inoculated leaf. Asterisks show statistically significant differences between the indicated treatment and the untreated control ($\alpha = 0.05$). Treatments providing significant disease control and/or symptom reduction are highlighted in pink.

Table 3. Spray treatments applied on potted 'Pink Lady' trees in the 2024 greenhouse trial.

Treatment	No. Trees	Active compound	Application concentration	% Active compound	Application Pattern[b]
Actigard 50 WG	5	Acibenzolar-S-methyl (50%)	2 oz/100 ga	0.007	A
FireWall 50 WP	4	Streptomycin sulfate (65.8%)	8 oz/100 ga	0.039	B
Cueva	4	Copper octanoate (10%)	4 qt/100 ga	0.1	B
TidalGrow SPECTRA	6	Chitosan (5.75%)	1100 fl oz/ 100 ga	0.494	A
Rhamnolipids [a]	7	Rhamnolipids (90%)	0.40%	0.36	A
Chitosan lactate. [a]	6	Deacetylated chitin (snow crab)	0.20%	0.2	A
Untreated control	9	NA	NA	NA	A

[a] Products adjusted to pH 4.0 with glacial acetic acid.

[b] **A**, for products with recognized or potential plant-defense inducers: treatment application on 6.20.24, 6.23.24; tree wounding and inoculation on 6.25.24; treatment application 6.26.24 (9-11h post-inoculation) and 6.29.24. **B**, for products with bactericidal activity only: treatment application 6.24.24; tree wounding and inoculation on 6.25.24; treatment application on 6.26.24 (9-11h post-inoculation) and 6.29.24.

In the 2024 trial, all tested products, including pure chitosan lactate, initially demonstrated strong trauma blight control, with median control efficacy percentages ranging from 56.1% to 92.6% (**Figure 3**). However, none of the products prevented wound infections completely, and the

differences compared to untreated controls diminished over time. By the end of the experiment (36 dpi), only TidalGrow SPECTRA (12.5%) and FireWall (15%) maintained statistically significant control against shoot blight infections ($P \leq 0.0081$), although at low levels (**Figure 3A**). Similar to the 2023 greenhouse trial, while most tested products could not prevent infections (**Figure 3A**), many significantly reduced symptom severity ($P \leq 0.0067$) (**Figure 3B**). Chitosan lactate was the only tested product that showed no clear effects on symptom severity, with treated trees developing lesions slightly smaller in size but statistically undistinguishable from to those of untreated controls (**Figure 3B**). These results together with the ones from 2023 trial indicate that chitosan derivatives like chitosan lactate and chitosan oligosaccharides alone are not good options for shoot blight control. Even among the chitosan-based commercial products, only the chitosan formulation in TidalGrow SPECTRA showed significant differences from the controls among trials.

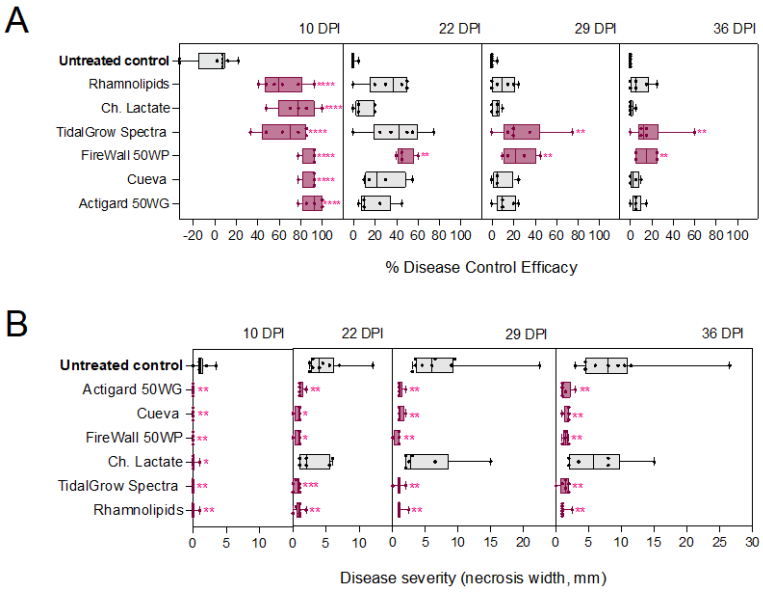

Figure 4. Efficacy of different products in trauma blight control and symptom severity reduction throughout time in potted 'Pink Lady' trees, in a second greenhouse trial carried out in summer of 2024. Asterisks show statistically significant differences between the indicated treatment and the untreated control ($\alpha = 0.05$). Treatments providing significant disease control and/or symptom reduction are highlighted in pink.

In summary, analysis of both disease control and severity measurements from the 2023 and 2024 greenhouse trials showed that TidalGrow SPECTRA consistently reduced disease incidence and severity across both trials. Its performance often matched that of conventional chemical treatments, suggesting its potential as a sustainable disease management option. Rhamnolipids demonstrated consistent efficacy in controlling shoot infections, particularly during the initial days following shoot inoculations, despite showing reduced efficacy at later time points. This product effectively reduced symptom severity in both trials, achieving results comparable to Actigard and even copper or antibiotic treatments. Both TidalGrow SPECTRA and rhamnolipids maintained consistent performance patterns across trials, although with different efficacies (**Figures 2 and 3**). These results

were achieved under high infection pressure conditions (with 100% wound infection in control trees by trial end). This suggests that these products may demonstrate even better performance under less infection-conducive conditions.

4. Rhamnolipid and chitosan performance assessment under field conditions

Based on results from the 2023, we designed treatments for a field trial using 'Gala' apple trees, at WSU Columbia View Research Farm in Wenatchee, WA. While we standardized conditions in greenhouse trials by pruning trees and applying treatments when new shoots reached 5-10 inches in length, field assays were timed using petal fall as a reference point. Products with potential plant defense-activating properties were applied days before and after *E. amylovora* inoculation, following the greenhouse trial protocol (**Table 4**). For field applications, we modified the pH of rhamnolipids to 5.5 instead of 4.0. This pH more closely aligns with standard agrochemical buffering practices and prevents copper phytotoxicity when used in combined treatments with copper (Cueva) (**Table 4**).

Table 4. Spray treatments applied on 'Gala' trees in a field trial carried out in spring of 2024.

Treatment	Rate per 100 gallons water	pH	Surfactant	Timing*
Untreated, Inoculated Check	na	na	na	PF, PF+3, PF+6, PF+9
Firewall 50WP	8 oz	5.6	Regulaid @16 oz	PF+6
Cueva	4 qt		na	PF, PF+6
Rhamnolipids	0.4 %	5.5	na	PF, PF+3, PF+6, PF+9
Cueva + Rhamnolipids	3 qt 0.4 %	5.5	na	PF, PF+6
Chitosan (TidalGrow SPECTRA)	1100 fl oz	na	na	PF, PF+3, PF+6, PF+9

*PF, petal fall; PF+3, petal fall + 3 days; PF+6, petal fall + 6 days (morning after evening inoculation); PF+9, petal fall + 9 days.

The results from the field trial are summarized in **Figure 4**. Disease symptoms developed and progressed more rapidly and aggressively in field trials compared to greenhouse trials. These differences may be attributed to both cultivar variation ('Gala' versus 'Pink Lady') and environmental conditions. The spring to early summer field conditions proved more conducive to disease development than the greenhouse environment, where higher peak temperatures were recorded during mid to late summer trials.

0.5% Chitosan (TidalGrow SPECTRA) and 0.4% rhamnolipids demonstrated low but statistically significant efficacy in controlling shoot infections in the field ($P \leq 0.0282$). TidalGrow SPECTRA achieved 32.3% and 8.5% control at 7 and 12 dpi respectively, while rhamnolipids showed 17.3% and 12.0% control at the same time points (**Figure 4A**). As observed in greenhouse trials, control plants exhibited 90-100% wound infection by the experiment's end, indicating very high infection pressure. This extreme disease pressure may explain why even FireWall, the most effective treatment, provided low protection (median of 16.1%) against shoot blight by the end of the experiment (**Figure 4A**). Neither Cueva alone nor its combination with rhamnolipids provided any protection against shoot infections, even during early post-inoculation periods.

Interestingly, treatments with rhamnolipids, but not chitosan (TidalGrow SPECTRA), significantly reduced symptom severity, with lesions being 92.5% smaller than those in untreated control trees at

the final experimental time point (23 dpi) (**Figure 4B**). However, the combination of rhamnolipids with copper provided less protection than rhamnolipids alone. These findings suggest a potential negative interaction between copper and rhamnolipids, which may reduce the latter's efficacy in controlling infections.

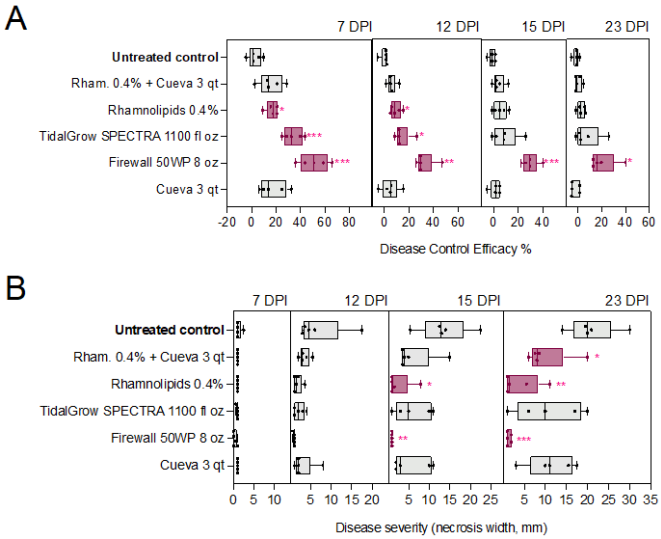

Figure 5. Efficacy of different products in trauma blight control and symptom severity reduction throughout time in a field trial on 'Gala' trees, carried out in 2024. Asterisks show statistically significant differences between the indicated treatment and the untreated control ($\alpha = 0.05$). Treatments providing significant disease control and/or symptom reduction are highlighted in pink.

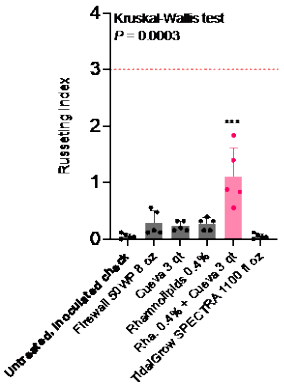

Figure 6. Russeting marks in fruit after treatments. Each dot in the graph is an average value of 25 fruit per tree (5 trees/treatment), which were monitored for the presence of russeting marks, and recorded based on a standard russeting index ranging from 0 to 5, where values above 3 (dotted line in the graph) are considered detrimental for the fruit marketability. Asterisks denote statistically significant differences with the untreated control.

Although the combination of Cueva + rhamnolipids increased the russeting marks with respect to the control ($P = 0.0003$), none of the applied treatments induced significant fruit russeting above the threshold of 3 (**Figure 6**), demonstrating that both rhamnolipids and chitosan are bioactive compounds that can be safely used without compromising fruit marketability. While these treatments

did not completely prevent infections under high infection pressure conditions, our results indicate that both rhamnolipids and the chitosan formulation from TidalGrow SPECTRA show promise as sustainable alternatives to conventional chemical treatments. These products could be valuable components of integrated pest management strategies, particularly for controlling shoot blight under low to intermediate infection pressure conditions.

5. A single spray treatment with rhamnolipids or chitosan (TidalGrow SPECTRA) induces plant defense-related genes

Results from greenhouse and field trials suggested that rhamnolipids and chitosan (TidalGrow SPECTRA) may induce plant defense responses. To verify this hypothesis, we monitored the expression of key defense-related genes: pathogenesis related proteins 1 and 2 (*PR1*, *PR2*), which are associated with salicylic acid-mediated defense responses and systemic acquired resistance; Plant Defensin 1 (*PDF1*), a marker for jasmonic acid and ethylene-mediated defenses; and phenylalanine ammonia-lyase (*PAL*), a key enzyme in the phenylpropanoid pathway involved in both basal and induced resistance.

The analysis revealed that rhamnolipid treatments significantly affected the expression of all four analyzed genes ($P \leq 0.0476$). *PR1*, *PR2*, and *PAL* reached peak expression 7 days after spray application, while *PDF1* showed maximum expression levels 3 days after spray (**Figure 7A**). Similarly, TidalGrow SPECTRA treatments significantly increased expression levels of *PR1*, *PR2*, and *PAL* ($P \leq 0.0314$) (**Figure 7B**). These results support our observations from plant assays, indicating that while chitosan and rhamnolipids possess in vitro bactericidal activity, their efficacy in greenhouse and field trials can be partially attributed to the activation of multiple plant defense pathways.

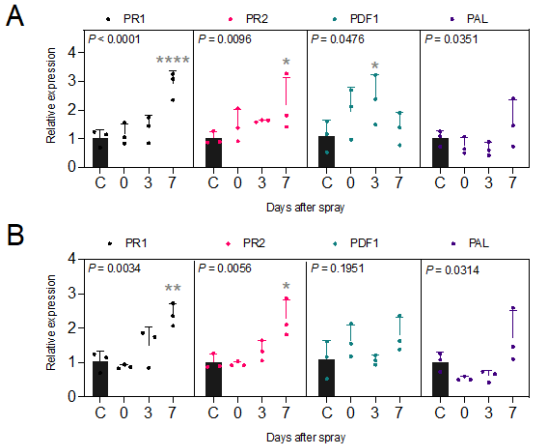

Figure 7. Relative expression of *PR1, PR2, PDF1* and *PAL* genes in potted 'Pink Lady' trees after one spray treatment with 0.4% rhamnolipids (A) and TidalGrow SPECTRA at 1100 fl oz/100 ga (0.49% chitosan) (B). Each dot corresponds to a pooled sample of three different trees. Columns show average relative expression values of 3 pooled samples (i.e., 9 trees) and the error bars indicate the SD. Untreated trees were used to normalize the relative expression of target genes across different samples, and the elongation factor 1 alpha gene (*EF1α*) was used as endogenous control. The *P* values indicate the significance of differences between the compared groups (untreated control trees, C, and sprayed trees at times 0, 3 and 7 days post-spray), assessed by one-way ANOVA analysis ($\alpha = 0.05$). Asterisks denote significant differences between control trees and sprayed trees at the specified time point, assessed by post hoc multiple comparisons tests.

6. **Characterization of product interactions with antibiotics and plant defense inducers**

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As part of **Objective 1**, we also explored interactions between products. We conducted an in vitro assay to evaluate interactions between rhamnolipids (Rha) and three antibiotics commonly used against *E. amylovora* in the field: streptomycin (Sm), kasugamycin (Kg), and oxytetracycline (OTc). This assay relied on turbidimetric measurements, which prevented testing the effects of TidalGrow SPECTRA due to its dark color, and turned the culture medium black. Accordingly, this experiment was performed only with rhamnolipids.

In this type of assay, if the product interactions are additive, the mixture components will contribute to the mixture IC_{50} proportionally to their individual IC_{50}. This implies that the observed mixture IC_{50} will be close to the average of the IC_{50} of each product alone, known as the estimated IC_{50}. To assess the type of product interaction, we use the FIC value (FIC = Observed IC_{50}/Estimated IC_{50}). In cases of additive interactions, the observed mixture IC_{50} aligns with the average IC_{50}, resulting in an FIC value of 1. Lower FIC values than 1 indicate that the mixture performs better than expected, considering the IC_{50} of the products acting separately (indicating a synergistic interaction).

For all tested compounds, the concentrations required to reduce *E. amylovora* populations by 50% were higher when used alone compared to when combined (**Figure 8**). Combinations of rhamnolipids with streptomycin and especially, kasugamycin, demonstrated strong synergistic interactions (FIC values of 0.4 and 0.29, respectively) (**Figure 8A,B**). This means that mixtures of antibiotics with rhamnolipids enhanced the bactericidal activity above the values expected in an additive model. In fact, mixtures of Rha+Sm and Rha+Kg at concentrations equal to the IC_{50} reduced *E. amylovora* populations below 95%, in comparison to the around 50% reduction observed with the products applied separately (**Figure 8A,B**). Combinations of rhamnolipids with oxytetracycline resulted in FIC values close to 1 (**Figure 8C**), meaning that rhamnolipids had no clear effect enhancing this antibiotic activity against *E. amylovora*. Overall, our findings suggest that rhamnolipids could serve as effective coadjuvants, enhancing the action of antibiotics in the field in a cost-effective manner.

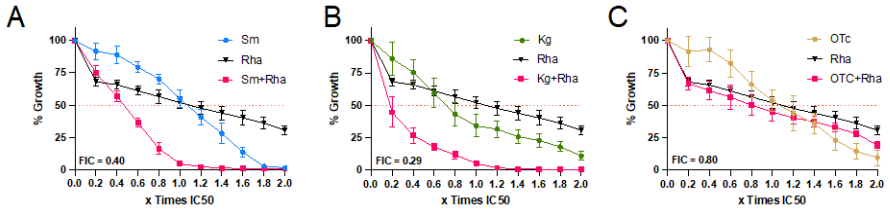


Figure 8. Interaction between rhamnolipids and antibiotics in inhibiting *E. amylovora* growth. Graphs show the percentage of *E. amylovora* growth inhibition by streptomycin (Sm), rhamnolipids (Rha) and their combination (Sm+Rha) (A), kasugamycin (Kg), Rha and their combination (Kg+Rha) (B), and oxytetracycline (OTc), Rha, and their combination (OTC+Rha) (C), at concentrations ranging from 0 to 2× their IC_{50}. The IC_{50} represents the concentration at which 50% growth inhibition is observed. Synergistic effects are indicated when the concentration of the products in the mixture required to reduce growth below 50% is lower than the IC_{50} of the individual products. The dotted red line marks the 50% growth inhibition threshold. Error bars represent the SD from three replicates in two independent assays.

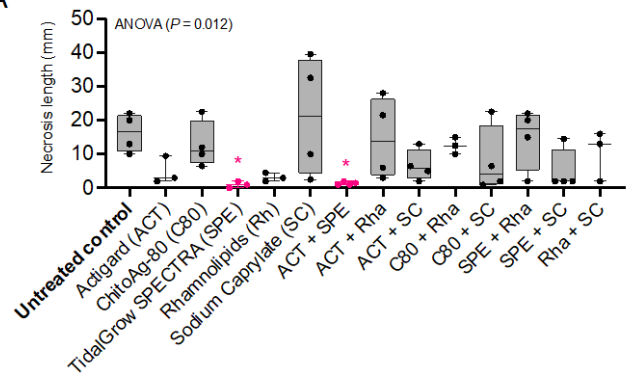
In a separate assay using 'Gala' seedlings, we tested plant defense inducers alone and in combination with rhamnolipids or chitosan-based products to evaluate potential enhancement of their activity. All products were tested at half the concentrations used in greenhouse trials to better detect positive interactions between product combinations. The tested treatments included Actigard (1 oz), rhamnolipids (0.2%), and the two chitosan-based products that performed best in greenhouse trials: ChitoAg-80 (1 qt) and TidalGrow SPECTRA (550 fl oz). Treatments and inoculations followed the greenhouse trial protocol, with *E. amylovora* sprayed after cutting five leaves twice at the base (10 wounds/plant). For comparison, treatments with sodium caprylate (0.04%) were also included. Each treatment was applied to 4-5 seedlings. Results showed that treatments significantly affected the

extension of necrotic lesions (ANOVA, $P = 0.012$). Multiple comparison tests indicated that TidalGrow SPECTRA (550 fl oz) and its combination with Actigard (1 oz) effectively reduced symptom severity (**Figure 9A**).

We additionally employed multivariable logistic regression analysis to determine whether the presence of rhamnolipids or chitosan in combined treatments had positive or negative effects on necrotic lesion extension. The analysis revealed that rhamnolipids in mixed treatments had a detrimental effect on the activity of other plant defense inducers, enhancing disease development (**Figure 9B**). The odds ratio for mixtures containing rhamnolipids was 3.656, indicating that the likelihood of observing necrotic lesions larger than those in control trees increased 3.7-fold when rhamnolipids were present in the mixture ($P < 0.0001$). Conversely, the presence of TidalGrow SPECTRA in mixtures reduced the probability of observing necrotic lesions larger than those in control plants by almost 50% ($P = 0.028$) (**Figure 9B**).

While further studies are needed to draw robust conclusions about the interactions of rhamnolipids and chitosan with other products, overall, our results suggest that rhamnolipids could serve as effective coadjuvants to enhance the efficacy of antibiotics in the field. Meanwhile, TidalGrow SPECTRA may positively impact the control of shoot blight infections and/or symptom development when combined with Actigard or other chitosan-based products. These findings also indicate that such effects and activity peaks might be achieved while reducing the concentrations of all active compounds in the mixture.

A



B

Variable	Odds ratio[a]	95% CI (profile likelihood)	*P* value[b]
ACT in MIX	0.646	0.3447 to 1.148	0.152
Rha in MIX	3.656	1.951 to 7.139	**<0.0001 (****)**
SPE in MIX	0.5085	0.2692 to 0.9071	**0.0281(*)**
Ch in MIX	0.6046	0.2767 to 1.273	0.1946
SC in MIX	1.064	0.5823 to 1.941	0.8403

[a] Values above 1 indicate increase of the likelihood to observe worsen symptoms. Values below 1 indicate the opposite.

[b] *P* values below 0.05 indicate statistically significant effects

Figure 9. Interaction between Actigard (ACT), rhamnolipids (Rh), ChitoAg-80 (C80), and TidalGrow SPECTRA (SPE) and their effects on fire blight symptom development. Effect of treatments, applied alone or in combination, on fire blight symptom severity. Asterisks indicate significant protection with respect to the untreated control (A). Multivariable logistic regression analysis showing the effect of the presence of specific plant defense elicitors in mixtures with other plant defense elicitors, on the extension of necrotic lesions (B).

Executive summary

Project title: New products for the prevention and control of shoot trauma blight

Keywords: Fire blight, rhamnolipids, chitosan, plant defense elicitors, sustainable disease management

Abstract

Extensive work on the prevention of blossom infection in Washington has been critical to reduce the impact of fire blight, which can cause severe infections in warm wet springs. However, few tools are available to prevent shoot blight infections, especially after hail and wind damage (trauma blight). This study investigated the potential of sustainable products, including chitosan-based formulations and rhamnolipids, for managing shoot blight. Initial in vitro screening revealed that all tested products had bactericidal activity against *Erwinia amylovora*, suggesting their potential to reduce pathogen populations on plant surfaces. Among these, two treatments showed promise: the chitosan formulation in TidalGrow SPECTRA, applied at 1100 fl oz/100 ga, and rhamnolipids applied at 0.4%. These products consistently affected disease development across two greenhouse trials and one field trial. While the level of shoot blight control varied from moderate to low depending on the trial, it is important to note that these results were obtained under extremely high infection pressure conditions. Under these challenging conditions, treatments with chitosan from TidalGrow SPECTRA and rhamnolipids provided protection levels and/or disease severity reduction comparable to conventional treatments such as copper (Cueva), Actigard, and antibiotics (FireWall and FireLine). This suggests these products might serve as potential alternatives to less environmentally friendly agrochemicals commonly used in fire blight management. Molecular analyses indicated that both chitosan from TidalGrow SPECTRA and rhamnolipids activated plant defense-related genes, including those associated with salicylic acid and jasmonic acid pathways. This suggests a dual mode of action: direct antimicrobial activity and stimulation of the plant's natural defenses. Additional in vitro studies revealed that rhamnolipids strongly enhanced the activity of streptomycin and kasugamycin, although they showed negative interactions with certain plant defense inducers, including Actigard. Chitosan in TidalGrow SPECTRA enhanced the activity of other chitosan-based products and Actigard when mixed together. Further research is required to refine application protocols, optimize product combinations, and evaluate long-term impacts on tree health and fruit quality. While the studied products show promise for reducing reliance on conventional chemicals, more extensive field trials are necessary before making definitive recommendations for commercial use.