STUDY DESIGNED FOR

The Institute for Sustainable Food Systems (ISFS)

By

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Title: Assessing *Bacillus subtilis* S-713, Cow's Milk, and Sodium Bicarbonate as a Control for Powdery Mildew in Field Cucurbit Crops.

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Abstract

Three suppression methods for powdery mildew in winter squash were tested to determine the most effective in open field conditions. A commercially available strain of *Bacillus subtilis*: strain 713 sold as Serenade Opti by Bayer, a solution of 25% cow's milk, and a 2% sodium bicarbonate solution ($NaHCO_3$) all demonstrated an ability to suppress the fungal pathogen within a confidence interval of 95%. The sodium bicarbonate solution was shown to be more effective than cow's milk, which in turn was more effective than the bio-control. All methods were more effective at suppressing powdery mildew than the untreated control.

Introduction

Managing powdery mildew in cucurbit crops caused by *Sphaerotheca fuliginea, Podosphaera fuliginea* and *Erysiphe cichoracearum* is a large challenge for growers in most areas of the world (McGrath and Thomas, 1996), including British Columbia (Pest Management Centre, 2006). The fungal infection occurs commonly both in field and greenhouse production and can drastically reduce yield in both size or number of fruit if improperly managed (McGrath, 1996b) by reducing vegetative growth, absorbing leaf nutrients, and causing defoliation (Zhang et al 2016) (Pest Management Centre, 2006). Yields are further reduced as fruits ripen improperly, or become susceptible to sunburn (McGrath, 1996b). The crop loss increases as the disease severity increases, with earlier infections resulting in the largest crop reduction (Nuñez-Palenius et al, 2009).

Powdery mildew appears as a white, talc-like powdery that begins as round spots on older leafs; it has the potential to quickly spread to cover the entire leaf surface and even stems if given the proper environmental conditions (McGrath, 1996b) (Pest Management Centre. 2006). These obligate parasites thrive best in temperatures ranging from 22 to 31 degrees celsius, when the relative humidity is over 80%, and low overall precipitation (Pest Management Centre, 2006).

As global climate change gives rise to increasing overall temperatures in British Columbia (Government of B.C., 2016), growers in the Fraser Valley have noticed increases in powdery mildew pressure over the past half decade (Scholefield and Dessureault, 2016). With average temperatures of 20C to 28C throughout British Columbia (Current Results, 2019a), relative humidity that can fluctuate between 34% and 99% in a single day (Vancouver Historical Relative Humidity, 2019) and an average of 1 day or 1 to 2 inches of rainfall (Current Results, 2019b) (Current Results, 2019c), August in B.C. often provides an optimal environment for powdery mildew spore growth and spread (Pest Management Centre, 2006).

Producers have relied heavily on fungicides such as benomyl and triadimefon to control the disease, however an over-reliance has given rise to concerns over their environmental impacts and increasing levels of fungicide tolerance in *S. fuliginea* (McGrath, 1996a) (McGrath 1996c). The common cultural practice of crop rotation has been shown to be ineffective at controlling powdery mildew, due to the ability of its spores to remain viable in the soil for multiple growing seasons (Gay et al, 1985).

Due to the aforementioned factors of climate change, fungicide tolerance, and ineffectualness of some traditional controls, growers both in Canada and abroad, at any scale, have a vested interest in finding economically viable ways to protect their cucurbit crops.

In this study, three alternate foliar spray applications were put into practice and assessed on their ability to control the disease, and protect yield, relative to one another. One is the commercially available, broad spectrum bio-fungicide Serenade Opti, produced by Bayer. It is strain QST 713 of *Bacillus subtilis*, a bacteria commonly used to suppress a wide range of fungal infections, including powdery and downy mildew, botrytis and sclerotinia, or early and late blight (Bayer, 2017). The naturally occuring bacteria has been demonstrated to produce multiple antifungal and antimicrobial compounds that inhibit the vital structures of powdery mildew, its hyphae and conidia (Romero et al, 2007). Many strains of *B. subtilis* have shown at least a partial ability to reduce disease presence and severity, in a variety of commercial crops (Romero et al, 2007) (Gilardi et al, 2012) (Kim et al, 2013).

The second method compared is a sodium bicarbonate solution, in the form of publicly available baking soda mixed with water. While baking soda alone has not been approved as a pesticide for production in Canada, both sodium and potassium bicarbonate have been shown to inhibit the growth of powdery mildew when applied as a foliar spray, both preventatively and as a post-infection control (Zaki et al, 2011). Sodium bicarbonate ($NaHCO_3$) is known as a bio-rational material, ei a material with a low toxicity to plants, that do not cause phytotoxic responses, the mechanics of disease suppression may or may not be known, depending on the type of bio-rational material (Zaki et al, 2011). With $NaHCO_3$ the exact mode of action for its antifungal properties isn't fully understood, however there is some belief that it works by compromising the cell wall membranes of spores (Kuepper and Thomas, 2001). There are several concerns with using $NaHCO_3$ in an agricultural setting; the solution may potentially run-off the leaves once applied, both potentially decreasing its effectiveness and altering the pH of the soil (Kuepper and Thomas, 2001); over application can also potentially lead to sun damage of the foliage (Kuepper and Thomas, 2001), further reducing yield. These issues stem from over application of $NaHCO_3$, which this study attempted to avoid by using the same concentration used in Zaki et al, 2011, of 2% $NaHCO_3$, which they showed to be the most effective without causing foliar damage. Run-off was minimal, as the environmental conditions that increase the likelihood of run-off (ei high daily precipitation) are generally not the prevailing conditions in the lower mainland throughout August (Current Results, 2019b).

The final method is a solution of cows milk (with 2% milk fat) and water. Also unregistered for use in Canada, but a 1999 greenhouse study by Wagner Bettiol found that high concentrations of milk were more effective at controlling *Sphaerotheca fuliginea* than the conventional fungicides fenarimol or benomyl (at concentrations of 0.1 g/l). A range of concentrations were evaluated, at 5, 10, 20, 30, 40, and 50%, both once and twice a week. Applications of 10% or higher twice a week was shown to control the disease as effectively or better than the fungicides tested (Bettiol, 1999). For the once a week applications, milk concentrations of 20 to 50% were required to control *S. fuliginea* as effectively as the conventional fungicides (Bettiol, 1999). Mold was found growing on leaves when using concentrations of 30% or higher, but it did not appear to impact overall plant health or yield (Bettiol, 1999). In order to keep applications uniform between treatments, every solution was applied once per week, and therefore the solution was required to be above 20%; to prevent mis-evaluating powdery mildew levels due to the presence of mold, this study looked to avoid a milk solution concentration of 30% or over. These factors led to the decision to use a 25% milk solution for the course of this study. While the mode of action that allows milk to discourage powdery mildew isn't fully understood, electron microscope evaluation after milk foliar application showed significant damage to powdery mildew hyphae and conidiophores, and a higher abundance of yeast based microorganisms that coated the conidia (Medeiros et al, 2012).

Methods and Materials

Varieties

The study was conducted on 3 varieties of cucurbit; Red Kuri pumpkin, Galeux D'eysines pumpkin, and Styrian pumpkin, chosen for their similar space requirements, time to maturity of 90 to 95 days (KCB Samen, 2019) (Baker Creek Rare Seeds, n.d.) (Sherck Seeds, n.d.), and marketability. A variety of pumpkins were chosen to broaden the applicability of the study beyond a single cultivar.

Spacing & block design / set up

The study was organized as a randomized complete block design (RCBD), with each block consisting of the three treatments plus control (four total: Serenade Opti, $NaHCO_3$ solution, milk solution, and control), with each treatment plot containing one of each variety (three total); Red Kuri, Galeux D'eysines, and Styrian. Each plant within the individual treatments were spaced at 1.5m sq, with 3m spacing between treatments. Treatments were further separated from one another by a buffer of buckwheat, in an attempt to avoid cross contamination of sprays via wind drifting. There were a total of five replicates, for a total of 60 individual plants (20 of each variety, 15 for each treatment including the control). The replicates were located at KPU's teaching farm at the Garden City Lands in Richmond, British Columbia. The plants were watered via drip irrigation to avoid an additional disease vector.

Planting / transplanting seedlings

The cucurbits were initially direct seeded into the field June 20th, 2019; however the initial seedlings were destroyed by birds approximately a week after emergence. A second planting of 30 seedlings of each variety was started in the KPU geodesic dome greenhouse on July 10th, in 2x2 inch cell trays with standard potting mix. These were kept in the dome for an extended period to allow them to progress to the point where they'd be less desirable to birds. They were hardened off from July 24th to 31st, when the strongest 20 seedlings of each variety were chosen to be transplanted into the field. Reflectors made of pie plates on strings were placed within the plots to further discourage wayward fowl. A buckwheat buffer between treatment plots was direct seeded on August 5th to prevent cross contamination of foliar sprays via wind.

Application methods and timing

All 3 solutions were applied with the hand-pressurized spray equipment on hand at KPU, before any sign of powdery mildew was observed. Treatments were applied weekly for a total of 6 times, starting August 14th, August 22nd, August 29th, September 5th, September 10th, and September 19th. The slight variation in time between treatments was due to scheduling and transit complications; 7 day intervals were desired, and on average this was achieved (7.1 day intervals on average). While the concentrations of the solutions were kept consistent throughout the study, the total volume used increased as plant biomass increased to allow for full foliar coverage; 2 liters per treatment on August 14th, 3 liters on August 22nd, 4 liters on August 29th, and 6 liters September 5th and 10th. The sprayer was decontaminated between treatment applications, using a diluted bleach rinse, followed by two pure water rinses, as recommended by AgPhd (AgPhd, 2013).

Spraying occurred in the afternoon, between 1pm and 5pm. This timing was not ideal, but was required due to scheduling conflicts, travel time, and preceding observations / data collection.

Formulas / solution concentrations

Bayer's Serenade Opti is used on cucurbit crops for suppressing powdery mildew at a rate of 1.7 to 3.3 kg per hectare, or 0.7 to 1.3 kg per acre, increasing the concentration as disease severity increases (Bayer, 2017). Due to the smaller scale of the study, this was converted to a per plant application rate using the following logic: Penn State suggests that an 8 lb pumpkin be planted at a rate of 2800 plants per acre, or at the widests spacing, 1600 plants per acre (Penn State, 2005). The expected average weight of the varieties chosen is approximately 10 to 15 lbs (KCB Samen, 2019.) (Baker Creek Rare Seeds, n.d.) (Sherck Seeds, n.d.), with Red Kuri being the exception, with fruits weighing around 4.5 - 6 lbs. The spacing was based around the larger varieties, and maximized to prevent cross-contamination, so the spray was applied as if there were 1600 plants per acre. With an estimate of approximately 1600 plants per acre, the rate of application of Serenade Opti per plant was estimated to be at 0.0008 kg, or 0.8 grams. Proper mixing procedures for Serenade Opti involve half filling the applicator with water, adding the desired amount of powdered formula, agitating, and filling with the remaining water needed (Bayer, 2017). Agitation continued throughout the spraying process to insure even distribution of Serenade Opti. Only the amount being used immediately was prepared.

As recommended by Zaki et al, the concentration of the $NaHCO_3$ solution was 2%, mixed with water, which should control powdery mildew while not damaging the plants through photosensitivity or altered soil pH (Zaki et al, 2011). The milk solution was 25% milk (containing 2% milk fat), 75% water, in order to prevent mold forming while still adequately suppressing powdery mildew through a once per week application, as per Bettiol, 1999.

Disease monitoring and data collection

Anand et al, 2009 describes a simple method for monitoring disease progress, which was done on the day of each foliar spray application, before the spray was applied. The intensity of the disease is described per leaf on a 0 to 5 scale, where 0= no infection, 1= 0.1% to 10%, 2= 10.1% to 15%, 3= 15.1% to 25%, 4= 25.1% to 50%, 5= over 50%. The percentages listed refer to area infected per individual leaf. These figures are used to calculate the Percent Disease Index (PDI), using the formula "(Sum of numerical ratings / Total number of leaves observed) X (100 / Maximum disease grade in score chart[5])" (Anand et al, 2009).

A score from 0 - 5 was collected at a rate of 3 to 6 randomly selected leaves per plant; 3 leaves on August 14th and 22nd, then 6 leaves the remaining 4 weeks, once the plants had reached sufficient size. These scores were used to calculate the PDI per leaf, and at the end of the growing season the mean of the weekly PDI per treatment plot was calculated, along with the total yield per treatment being measured, providing a mean PDI score and a total yield for each plot in each of the four treatments (three methods plus control).

Yield was measured at the end of the season by weighing all fruit, and taking the mean weight of each plot. Squash was harvested on October 10th, after frost had begun to kill off the plants. Minor frost damage occurred on the squash, but not to the point of compromising the yield data.

Statistical analysis

The final mean PDI scores and final mean yield weights were analyzed and compared using the JAMOVI interface of the statistical program R. All data was found not to be normally distributed using the Shapiro-Wilk test, so a square root transformation was used to achieve normality. The mean PDI scores were compared between treatments using a mixed model linear progression, where the independent variable was the square root of the mean PDI, and the factors being the date and treatment type (Serenade Opti, Milk, $NaHCO_3$, or Control). Tukey's HSD test was used for post-hoc analysis to determine which treatment was the most effective. For visualization purposes, the square root PDI score was converted back to PDI expressed as a percent once the analysis was done, which is displayed in the results section below.

Mean yield data was analyzed using analysis of variance (ANOVA), with the dependent variable being the square root of mean yield weight, and replicate and treatment type as the fixed factors.

For all statistical analysis, a confidence interval of 95% was used.

Results

The disease progress was slower than anticipated, with zero occurrences observed the first week in all treatment plots, and remaining under 50% for all treatments including the control for the first 4 weeks of observations. The mean PDI for every treatment besides the control stayed beneath 50% for the duration of the study (barely, in the case of Serenade Opti).

At the end of the season, it was shown within a 95% C.I. that the baking soda 2% solution was the most effective with a mean PDI of 30.7%, followed by the cow's milk solution with a mean PDI of 37.7%, and with the effectiveness of Serenade Opti falling behind both with a mean PDI of 49.4%. The untreated control performed the worst with a mean PDI of 67.7% at the end of the season.

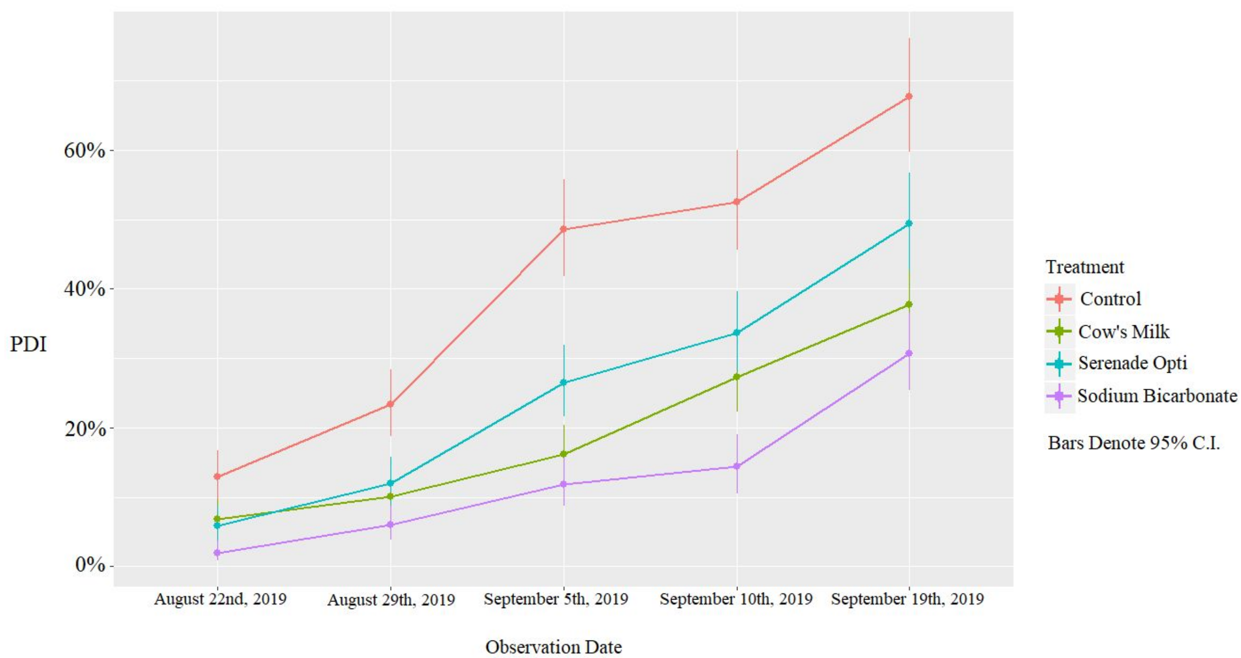

Fig 1. Linear Mixed Model Visual Plot

The preceding graph does not include August 14th, when all observed leaf scores were zero (ei no disease presence).

Post Hoc Comparisons - Treatment

Comparison									
Treatment		Treatment	Difference	SE	t	df	p	$p_{bonferroni}$	p_{holm}
Control	-	Milk	1.937	0.156	12.45	75.0	< .001	< .001	< .001
Control	-	Sodium Bi	2.843	0.158	18.04	75.1	< .001	< .001	< .001
Control	-	Serenade	1.400	0.156	9.00	75.0	< .001	< .001	< .001
Milk	-	Sodium Bi	0.906	0.158	5.75	75.1	< .001	< .001	< .001
Milk	-	Serenade	−0.538	0.156	−3.46	75.0	< .001	0.005	< .001
Serenade	-	Sodium Bi	1.444	0.158	9.16	75.1	< .001	< .001	< .001

Fig 2. PDI Post-Hoc Tukey's HSD test results

Tukey's HSD test demonstrates that all differences between treatment types were statistically significant within a 95% confidence interval ($p < 0.05$).

Yield data was obtained on October 10th, with the mean weight of each treatment plot being compared through an ANOVA. No statistically significant effect of treatment was found on overall yield; a significant effect was found due to variety, but this is due to the Red Kuri cultivar being bred to produce smaller fruits than Styrian and Galeux D'eysines, and was therefore not included.

ANOVA

ANOVA

	Sum of Squares	df	Mean Square	F	p
Rep	0.3110	4	0.0778	0.1813	0.947
Treatment	0.0517	3	0.0172	0.0402	0.989
Rep ✻ Treatment	2.6939	12	0.2245	0.5234	0.887
Residuals	17.1554	40	0.4289		

[3]

Fig 3. Mean Yield ANOVA results

Conclusion and discussion

It was determined within a 95% C.I. that a 2% sodium bicarbonate solution is more effective at suppressing powdery mildew in the selected field winter squash cultivars than a 25% cow's milk solution with 2% milk fat, which in turn was more effective than the commercially available biofungicide Serenade Opti. All methods tested were found to be more effective than the untreated control at suppressing powdery mildew, and reducing the percent disease index.

However this disease suppression effect did not have a statistically significant effect on overall yield, even when compared to the untreated control. There are several potential reasons for this result, although it is unclear whether any or all of these reasons were the real culprit.

The late transplanting date, due to the original direct seeding being eaten by birds, resulted in a shortened growing season, preventing many of the squash from reaching full maturity. All three varieties had a collection of both mature and immature fruits which likely added variability to the yield data that was unrelated to treatment type. In an effort to allow full maturity to be reached, harvest was delayed as long as possible, until frost damage was observed in the squash, however the delay was not adequate to allow full maturity to be reached in many cases.

Another potential reason for the lack of yield variation between treatments was leaf burn observed in both the baking soda and Serenade Opti treatments, which likely resulted in reduced photosynthesis and therefore reduced yield. The Zaki et al 2011 study that determined 2% was the optimal concentration for a baking soda solution took place in a greenhouse, which may indicate that the optimal concentration for field production is less than 2%; however, the time of day that all the foliar applications were sprayed was sub-optimal, occurring shortly after noon when the sun is the most prominent (starting between 1pm and 3pm, finishing between 3pm and 5pm). This timing may have led to a higher level of leaf burn in both baking soda and Serenade Opti treatments, than if the foliar treatments had been applied either early in the AM, or later in the evening, when the incoming solar radiation is less intense. Greater care should be taken with spray timing if any follow up research is to be conducted.

While the results of this study are encouraging, there are several major changes and additions that would be beneficial to include in any follow up study, to further increase the reliability of the data, to explore the potential to protect yield, and to broaden the applicability to commercial farmers who may currently rely heavily on traditional synthetic fungicides.

These recommendations include using digital imaging and analyzing software to more accurately record and analyze the disease progress, rather than visual observation, to procure PDI scores; to design a larger scale study that covers acres to more closely mimic commercial field production, selecting commonly grown varieties, and using a mechanical sprayer to insure even and desired application rates; and to compare the treatments studied here with traditional synthetic fungicides and canola oil based fungicides to determine whether baking soda, cow's milk, *B. subtilis*, and canola oil based fungicides could replace the commonly used synthetics.

Finally, while the results for both unregistered controls (household baking soda and store bought cow's milk) are encouraging, it is worth noting that the volume of milk used was not insignificant, ranging from half a liter to 2 liters depending on the total volume of solution used. This amount would scale up dramatically, leading to a huge potential cost for farmers, meaning that unless a commercial product derived from cow's milk is invented, registered, and produced at a reasonable cost, any benefit of using cow's milk is quickly outweighed by the massive monetary investment. Serenade Opti, while showing the least amount of disease suppression, was used in very small amounts, which would cause far less economic strain on farmers than the cow’s milk alternative.

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