Growth of Xylella fastidiosa at Different pH Levels of Xylem Sap

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Abstract Xylella fastidiosa is a xylem-limited bacterium that causes Pierce's Disease (PD), which leads to mortality in grapevines. There have been recordings of symptomatic grapevines recovering from PD over winter, which led to speculation that X. fastidiosa is sensitive to seasonal shifts in temperature or pH (pH is a function of seasonality). This research analyzes the affects pH has on the growth of X. fastidiosa. X. fastidiosa was grown in xylem sap (extracted with a Scholander pressure bomb) and adjusted to pH levels 4, 5, 6, and 7. Two test were conducted to determine the affects pH had on the growth of X. fastidiosa. The first test had xylem sap pooled together from 3 different European grape cultivars (*Vitis vinifera*); Cabernet Sauvignon, Pinot Noir, and Sylvaner. The second test only used xylem sap from Cabernet sauvignon. Xylem sap that was adjusted to pH of 6 had the highest population count, while at pH 4 the bacteria died after 24 hours. Bacteria grown at pH 5 and 7 had similar population levels at nearly every sample time. Although X. fastidiosa showed different growth patterns for the two different test, there was a consistency in the affects pH has on X. fastidiosa growth. The natural pH of the xylem sap (pH of 6) proved to be the optimum for X. fastidiosa growth for all test conducted. Knowing how pH affects X. fastidiosa can lead to better methods in combating it, such as knowing which season to prune off infected grape vines, when to apply insecticides, or when the optimal time would be to inoculate grapevines for research purposes.

Introduction

The main objective of the Research was to find the survivorship of X. fastidiosa in different pH levels of xylem sap. The secondary objective was to find the survivorship of X. fastidiosa grown in a 96 well plate. Because the composition of xylem sap is predominantly water it was expected that the X. fastidiosa bacteria will have higher populations and a faster growth rate at pH levels close to that of water, estimated to be around 6. We expected X. fastidiosa to grow best in the most simulated natural environment.

PD is caused by a bacterium known as Xylella fastidiosa (Wells et al. 1987) and lives in the xylem of grapevines (Hopkins 1989); the disease inhibits the water transport system by encumbrance of the xylem vessels causing the grapevine to dehydrate and die (Hewitt 1970, Goodwin and Meredith 1988, Newman et. al. 2003,). PD causes the grapevines to have symptoms of water stress, pre-mature aging, chlorosis of leaves, and eventually leads to early plant mortality (Hopkins 1989).

Vectors of PD, primarily sharpshooters (Homoptera: *Cicadellidae*) (Hill and Purcell 1997), readily pick up the disease with no latent time when feeding on a sick grapevine and transmit it to healthy grapevines (Almeida and Purcell 2003). Recent PD epidemics in Southern California have caused significant economic loss for the grape industry (Redack et al. 2004, Alemeida and Purcell 2003). Although there are no methods proven to cure PD there have been techniques developed to slow down the spread (Redack et al. 2004).

Research conducted at a UC study site in Northern California has shown that a significant amount of symptomatic PD grapevines recover over the winter season (Almeida 2003, Purcell 1980). There is strong evidence that shows that pH of xylem sap changes depending on the season (Alves et. al. 2003). Although unclear as to why X. fastidiosa have trouble surviving the winter season, there is speculation that the decrease in pH of xylem sap during the winter makes for an inhabitable living environment for the bacteria. Grapevines inoculated with X. fastidiosa in the late summer or early fall often recover by the following spring. Usually spring inoculations are able to persist past the winter months (Almeida and Purcell 2003) knowing how the bacteria react at different pH levels would be useful in choosing the best times (with regards to seasons) to inoculate grapevines for research purposes. Learning when X. fastidiosa is most critical relative to pH will also help grape growers combat PD, such as pruning before winter or application of pesticides targeting sharpshooters in the early spring. A possible new alternative

would be altering the pH of xylem sap by changing the concentration of abscisic acid (ABA) in the grapevine (Davies 2002). A new irrigation technique known as partial rootzone drying (PRD) changes the concentration of ABA in the xylem sap which causes a change in pH (Stoll et al. 2000).

Initial pH measurements of xylem sap extracted with the Scholander pressure bomb had pH readings between 4.4 – 5.6 when expected to be between 6 and 7. However these pH readings were taken with pH paper and not a pH electrode. PH readings with an electrode were not possible at the time due to the difficulty in extraction of xylem sap and low amounts of xylem extracted.

This paper examines the affects pH has on the Temecula (TEM) strain of X. fastidiosa bacteria when grown in grapevine xylem sap. The TEM strain was obtained from a stock solution that was kept in storage at the Purcell lab. The TEM strain originated from the city of Temecula in Southern California where PD had eradicated many of the grape vineyards. Xylem was taken from European grape (*Vitis vinifera*) cultivars; Cabernet Sauvignon, Pinot Noir, and Sylvaner grapevines that were grown from cuttings of mature grapevines.   Cuttings were provided by UC Davis.

Methods

The experiment was conducted in the Hilgard lab and the Oxford Greenhouse tract at the University of California, Berkeley. Due to negative pressures that are present during plant transpiration (Schurr 1998) the most efficient method of xylem extraction was the Scholander pressure bomb (Scholander et al. 1965), which is considered to be an improved method of xylem sap extraction (Bextine and Miller 2004). For all test conducted the X. fastidiosa TEM isolate was used.

SCP creation 1.0g Na_2 Succinate, 1.5g K_2HPO_4, 1.0g KH_2PO_4, 1.0g Na_3 Citrate, dissolved in 1 liter of deionized water, then 2 ml of the solution was placed into test tubes which were autoclaved for 15 minutes at 140 degrees Celsius prior to use.

PWG creation   4g phytone peptone, 1g trypticase, 0.4g $MgSO_4$, 1.2g K_2HPO_4, 1.0g KH_2PO_4, 10ml of phenol red stock (0.2% in di H_2O) 10 ml hemin chloride (0.1% in 0.05 N NaOH) 8.0g Gelrite gum, all placed into 850 ml of deionized water and autoclaved for 15 minutes at 140 degrees Celsius. After solution cools to room temperature (25 degrees Celsius),

bovine serum albumin (BSA) (dissolved into 50 ml of deionized water) and L-glutamine (dissolved into 100 ml of deionized water) are filter sterilized with a .22 µm Whitman filter in the flow hood, and then mixed in with the autoclaved solution.) (Hill and Purcell 1995, Davis et al. 1981).

Stock creation   Petioles from PD symptomatic grapevines, which had been needle inoculated following the method of Hopkins and Adlerz (1988), were plucked off and weighed between 0.1 - 0.3 grams then put through the sterilization process described by Hill and Purcell (1995). First each petiole was submerged for two minutes in a solution of 70% ethanol alcohol and 30% autoclaved iodized water, then 2 minutes in 30% bleach and 70% autoclaved iodized water, and finally in a sterilized cup with 100 ml of autoclaved deiodized water, with the final step repeated twice (Hill and Purcell 1995). The petioles were diced on sterile Whitman #1 filter paper with a flame sterilized razor blade in a laminar flow hood and placed into 2 ml of autoclaved succinate-citrate-phosphate (SCP) buffer. The petioles were homogenized using a Polytron tissue grinder for 15 seconds. Twenty micro liters of the homogenized petioles were plated onto Modified Periwinkle-Gelrite (PWG) plates to allow for X. fastidiosa growth. The X. fastidiosa bacteria are able to form mature colonies after ten days, which are then scraped off with a flame sterilized loop and placed in a 1.5 ml eppendorph with 1 ml of PW broth (similar to PWG but without the Gelrite gum) and 0.2 ml of glycerol. It is referred to as the stock solution and stored in a -70 degree Celsius freezer (Lopes et al. 2005).

Bacteria into SCP Twenty micro liters of stock solution were streaked onto PWG plates, and grown for ten days (Souza et al. 2004). After the tenth day the drops are scraped off with a flame sterilized loop and placed into 2 ml of SCP. A spectrometer was used to determine the population density of X. fastidiosa in the SCP.

Pre-test Bacteria into PW One ml from "bacteria into SCP" was mixed into 30 ml of PW media. Then 200 µl was placed into each well of a 96 well plate. The 96 well plate was wrapped in parafilm to prevent the media from evaporating and kept on a shaker at 120 rpm. Every 24 hours the population density of 8 randomly selected wells (never re-using the same well) was determined using the dilution methods of Hill and Purcell (1995).

Collection of xylem Xylem was collected using the Scholander pressure bomb (Scholander et al. 1965). Grapevines were grown for 7 months from cuttings in 1 gallon pots in Super Soil potting soil. Terminal ends of grape stems 15 cm long were placed into the pressure bomb, and

the external severed end had the layer of skin peeled off, then scraped with a razor to minimize phloem contamination with the xylem (Scholander et al. 1965, Bextine and Miller 2004). The pressure bomb was allowed to go up to 400 Lbs psi. Xylem was collected from the tip of the grape stem with a 100 µl pipette and placed into 1.5 ml eppendorfs which were stored in a -70 degrees Celsius freezer (Bextine and Miller 2004).

Test 1 X. fastidiosa grown in various xylem sap at different pH levels Thirty ml of xylem sap from 3 different European grape cultivars (*Vitis vinifera*); Cabernet Sauvignon, Pinot Noir, and Sylvaner were pooled together in a 50 ml beaker. Six ml of the xylem sap from the 50 ml beaker was placed into a 10 ml beaker and had the pH adjusted to 4.0. After pH adjustment the xylem sap was sterilized by placing it in a sterilized 0.13 mm syringe with a sterilized 0.22 µm filter cap, and discharged into a sterilized 10 ml beaker. Then 145 µl of "bacteria into SCP" is combined with the sterilized xylem sap and dispersed into the 96 well plate, at 200 µl per well. This process is repeated again for pH's 5, 6, and 7, with the 6 ml of xylem sap coming from the originally pooled xylem sap in the 50 mL beaker. The pH was lowered with Hydrochloride acid or raised with Sodium hydroxide. PW media with X. fastidiosa was used as the positive control. The negative control was SCP. (See Appendix Fig. 5).

Test 2 X. fastidiosa grown in Cabernet Sauvignon xylem sap same procedure as test 1 with the exception of only using Cabernet Sauvignon xylem sap.

Results

As we were unclear as to how well X. fastidiosa was able to grow in a 96 well plate, a preliminary test were conducted by growing different initial quantities of the bacteria in a 96 well plate with 200 micro liters of PW per well. The preliminary test showed that X. fastidiosa had a large initial die off the first 24 hours, Then began to increase between hours 24 – 48, peaking at the 96 hour, and died off again (Fig. 1). This information was useful in finding the X. fastidiosa growth pattern, and it demonstrated that X. fastidiosa was able to grow in small volumes of liquid (Fig 1).
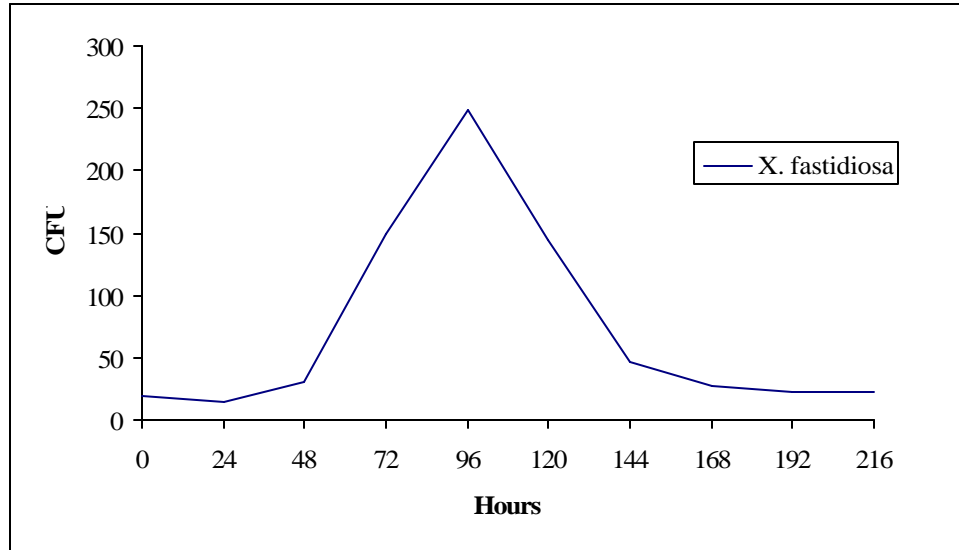
Figure 1 Average rate of growth for X. fastidiosa in a 96 well plate. Each well contains 200 microliters of PW. Colony formation units (CFU) are counted on PWG plates under a 30x microscope. One colony of X. fastidiosa is counted as 1 CFU (Pre-test).

Tests were conducted on how well and how long X. fastidiosa was able to grow in xylem sap. It was expected that the growth patterns would remain the same. However it was unclear as to how long the bacteria would survive and react to the different pH levels. Test 1 used xylem sap that was pooled together from 3 grape cultivars; Cabernet Sauvignon, Sylvaner, and Pinot Noir in order to fill the 96 well plate. The same pattern of growth was seen as when X. fastidiosa was grown in PW, except in a more condensed time scale (Fig. 2).
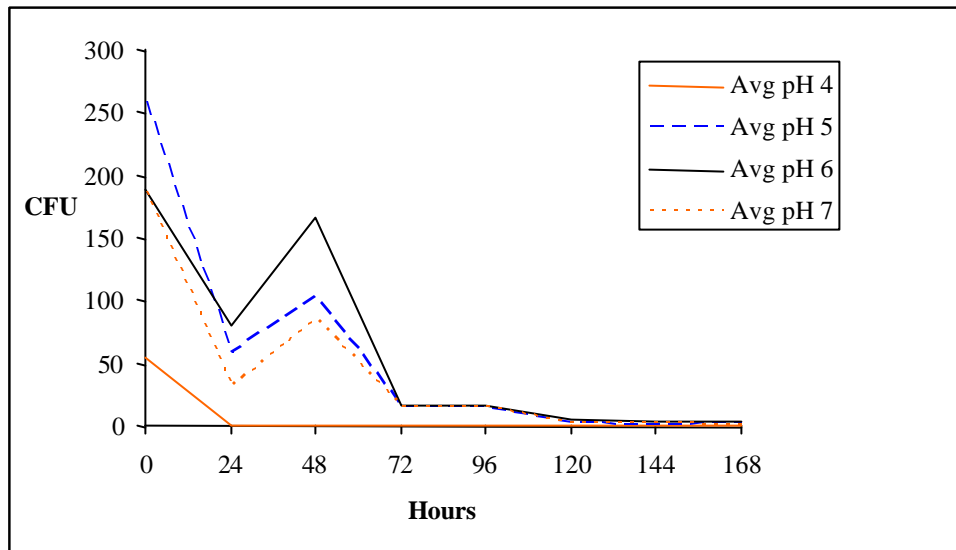

Figure 2 X. fastidiosa grown at different pH levels in various xylem saps; Cabernet Sauvignon, Pinot Noir, and Sylvaner (Test 1).

Trend lines at all time intervals showed that xylem sap with a pH of 6 had the best growth, while pH's 5 and 7 had no significant difference (Fig 3). There was no growth on xylem sap that was altered to a pH of 4.
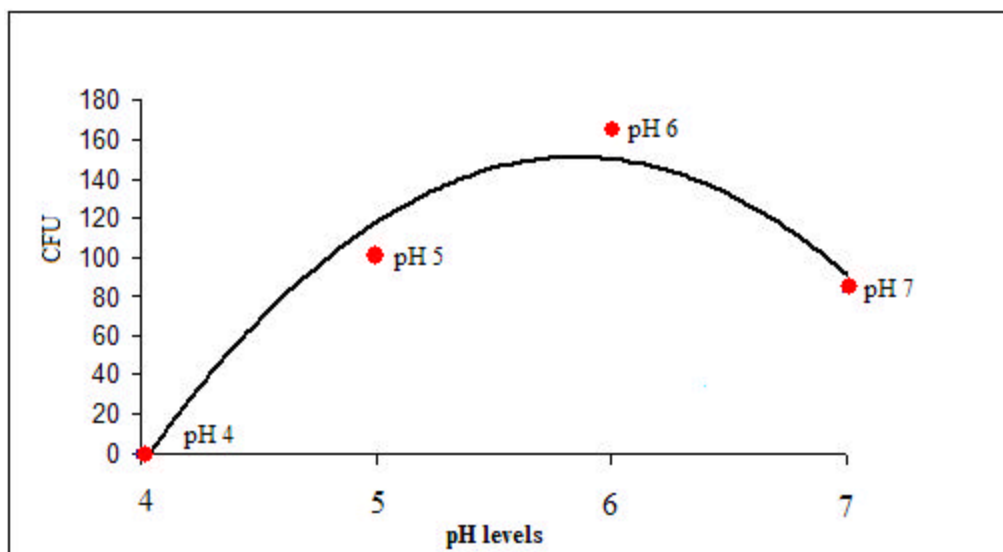

Figure 3 Trend line of X. fastidiosa growth at the 48th hour

The pH of xylem sap for both test 1 and test 2 changed between the initial pH recording (time=0) and the final pH recording (time=168 for test 1 and time=96 for test 2). For both test xylem sap that was altered to pH levels 4, 5, and 6 had an average increase of 0.3 in the pH, while xylem sap adjusted to pH 7 only had an increase of 0.01 in pH (Table 1).

Table 1 Acidic and Basic additives to the various xylem sap and pH measurements

Test 1

Targeted pH	HCL added	NaOH added	Initial pH t=0	Final pH t=168	Change in pH
4	10μL	3μL	4.12	4.4	0.28
5	11μL	5μL	5.1	5.5	0.4
6	0	0	6.13	6.44	0.31
7	0	6μL	7.13	7.14	0.01

Test 2

Targeted pH	HCL added	NaOH added	Initial pH t=0	Final pH t=96	Change in pH
4	4μL	0	4.14	4.4	0.26
5	3μL	0	5.15	5.52	0.37
6	0	0	6.11	6.61	0.5
7	4μL	6μL	7.01	7	0.01

X. fastidiosa that was grown in Cabernet Sauvignon xylem sap (test 2) did not follow the expected growth patterns that were seen in the pre-test and test 1. The bacteria in xylem sap at pH of 4 died off after 24 hours as expected. Xylem sap adjusted to pH levels of 5 and 7 had a sharp decline in population during the first 24 hours as expected. However at the 48th hour bacteria in pH of 5 died off. Between the 24th and 96th hour X. fastidiosa in pH of 7 had a steady decline. The population level of X. fastidiosa in xylem at pH of 6 remained constant for the first 24 hours and then had a large die off between the 24th hour and 48th hour. The population stabilized between the 48th and 72nd hour, with a decline in population levels after the 72nd hour to the 96th hour. Supprisingly the control also did not follow the expected growth pattern as seen in the pre test or test 1. Test 2 only showed death with no resurgence in bacterial populations (Fig. 4).
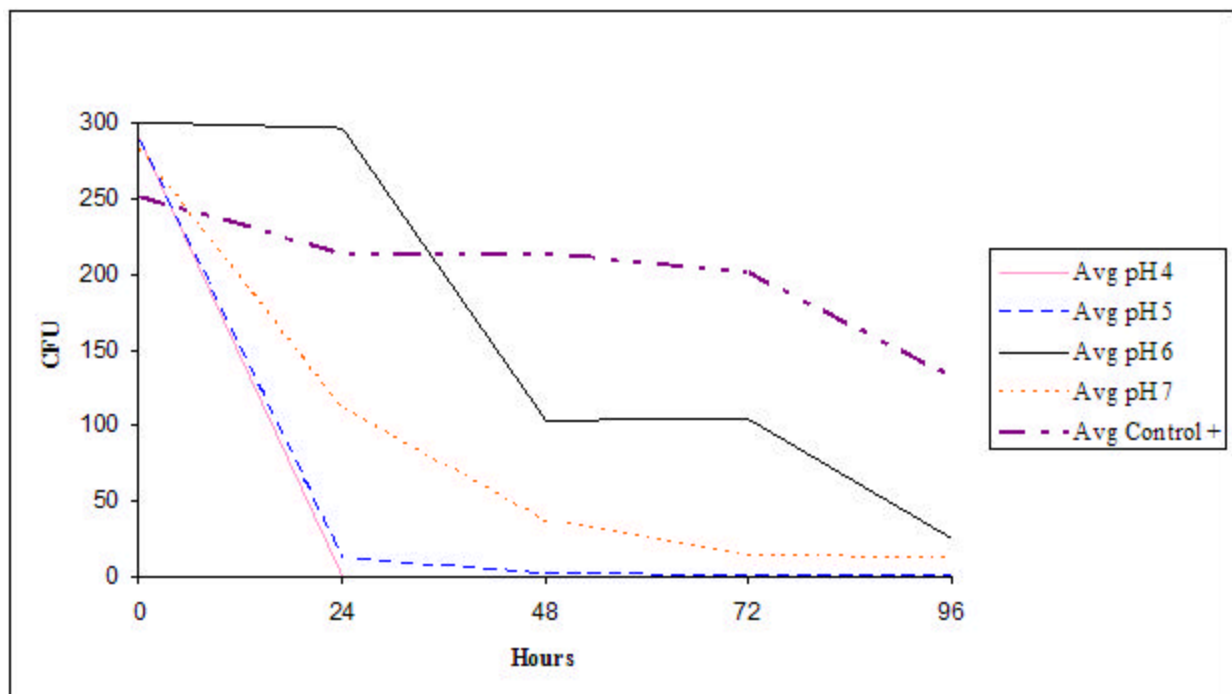

Figure 4 Growth of X. fastidiosa in various pH levels of Cabernet Sauvignon Xylem sap (Test 2)

At all time intervals pH 6 showed to have the highest populations. At the 96th hour pHs 6 and 7 reached critically low levels and were not expected to resurge based on our pre-test (Fig. 1) and test 1 (Fig. 2). To account for inaccurate counts of bacteria due to biofilm formation in the 96 well plate, 3 randomly selected wells from each pH level were rinsed out 3 times with SCP

and then refilled with 200 ml of SCP and sonicated for 10 minutes (Feil 2001). There were no significant amounts of bacteria found after sonification. Xylem adjusted to pH levels 4, 5, and 7 had zero population counts, while pH 6 had some bacterial growth. However the amount of bacteria grown was insignificant and did not change the results or statistics.

Statistics Only data from test 2 was statistically analyzed. In order to analyze my data effectively I used two statistical methods. In each case I tested the null hypothesis, that the mean number of CFU's are the same at each pH level. For there to be significant evidence to reject the null hypothesis, meaning that the means differ, a p-value of less than 0.05 is required, as this shows that the probability of the means actually being the same is less than 5%.

First, I applied the analysis of variance test to my data to compare the mean CFU's at each time interval for pH5, pH6 and pH7. The p-values obtained from these tests showed that there is significant evidence that the mean CFU's for each pH are different at every time interval, excluding the initial time (t=0). I then applied the t-test to compare each pair of pH values (pH5 vs. pH6, pH5 vs. pH7, and pH6 vs. pH7) at each time interval. Again, the p-values obtained show strong evidence that there is a difference between the mean CFU's when comparing two pH levels, excluding t=96 when comparing pH6 and pH7 and t=0, for all comparisons.

The reasoning for there being an insignificant p-value for t=96 in the comparison of pH6 and pH7 is that the CFU's were expected to dramatically die off by this time, at these pH's. Therefore this implication in similarity of the mean CFU's for pH6 and pH7 at t=96 actually supports my previous assumptions about the population characteristics at these pH's and time.

At the initial time (t=0) I did not expect any difference in the mean CFU's for each pH level because I wanted the same amount of bacteria to be present at the beginning of the time intervals for each pH. This is so it is a lot easier to compare the results and scaling of the data is not needed. The consistent resulting high p-values support the similarities in pH's as each p-value for the initial time is much greater than 0.05 regardless of the statistical test being used. Therefore there is no evidence that the mean CFU's differ between pH5, pH6 and pH7 at time zero (Table 2 in Appendix).

Discussion

Test 1 and 2 showed a sharp decline in population levels for the first 24 hours probably do to the bacteria re-adjusting to a new medium in which they live. The bacteria were grown on PWG

before they were placed into xylem sap. They could have also been affected by the difference in temperature change (Purcell 1980). The bacteria were grown for 10 days on a PWG plate in an incubator set at 25 degrees Celsius, but were put into thawed xylem then placed on a shaker at room temperature. There was inconsistent temperature, as temperature fluxed between night and day, typically between 20 and 25 degrees Celsius. Test 1 and 2 consistently demonstrated that pH 6 had the highest population counts with pH 5 and 7 having the second and third highest population counts. The bacteria were grown on the same 96 well plate, so I can assume they experienced the same external environmental affects such as temperature, and shakes on the shaker. The only variables would be the starting bacteria at the 24th hour, nutrients left in the xylem sap, and the different pH's. Assuming the nutrients are the same and normalizing the bacterial count, we can see the affect pH has on the growth of the bacteria (Fig. 6 in Appendix). pH 6 had the highest growth while pH's 5 and 7 were similar and had the next highest growth. So we can conclude that the optimal pH level for X. fastidiosa is a pH of 6, which also happens to be the natural ph level of the xylem sap.

Although the aim for the pH's was to be 4, 5, 6, and 7, they were a little off because small quantities of HCL or NaOH would drastically change the overall pH of the xylem sap (Table 1). The recorded difference in pH change between the initial pH's measured (at time=0) and the final pH's measured (at time = 168 for test 1 and time = 96 for test 2) could have resulted because of the addition of 145 µl of SCP (which has a pH of 7) that was added to the xylem sap after the pH change. For sterilization purposes it was not possible to alter the pH after the addition of SCP. This would also explain why pHs 4, 5, and 6 had an increase in pH of an average of 0.33, but ph of 7 only had an increases of 0.01 (Table 1).

For test 2 the unexpected difference in patterns could be because the Cabernet Sauvignon xylem sap was extracted mid winter, while xylem used for test 1 was extracted late summer (Purcell 1980). Difference in seasonality could have affected the concentrations of nutrients in the xylem sap leading to a difference in growth patterns. However xylem sap was extracted from greenhouse grapevines, where seasonality is not expected to affect the growth of the grape vine (Almeida and Purcell 2003). Also population levels are only taken every 24 hours so there could be unnoticed activity in the 96 well plate.

One final factor that could account for this anomaly could be the PWG plates that the bacteria were grown on. Plates are prepared by student workers who work in the lab part time,

gaining experience on lab techniques. There is a strong possibility that PWG plates made by student workers are incorrectly made or inconsistent with other PWG plates made by other student workers.

A trend line for any hour of bacteria sampled, which would eliminate all of the confounding factors, shows pH 6 having the highest population with pH 5 and 7 having relatively the same population counts and significantly lower populations then pH 6. A pH of 4 kills the bacteria. Xylem sap at a pH of 6, which is the natural pH, would be the optimum pH for X. fastidiosa bacteria and would promote and support a stable population size of X. fastidiosa. It was also found that X. fastidiosa was able to survive in a 96 well plate for a significant amount of time

Acknowledgements

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Appendix

Table 2 List of P-values for the T-test and ANOVA for test 2

T-test

Time	pH 5 vs. pH 6	pH 6 vs. pH 7	pH 5 vs. pH 7
0	0.78	0.64	0.82
24	0.00	0.02	0.00
48	0.00	0.01	0.00
72	0.00	0.01	0.01
96	0.02	0.17	0.01

ANOVA P-values

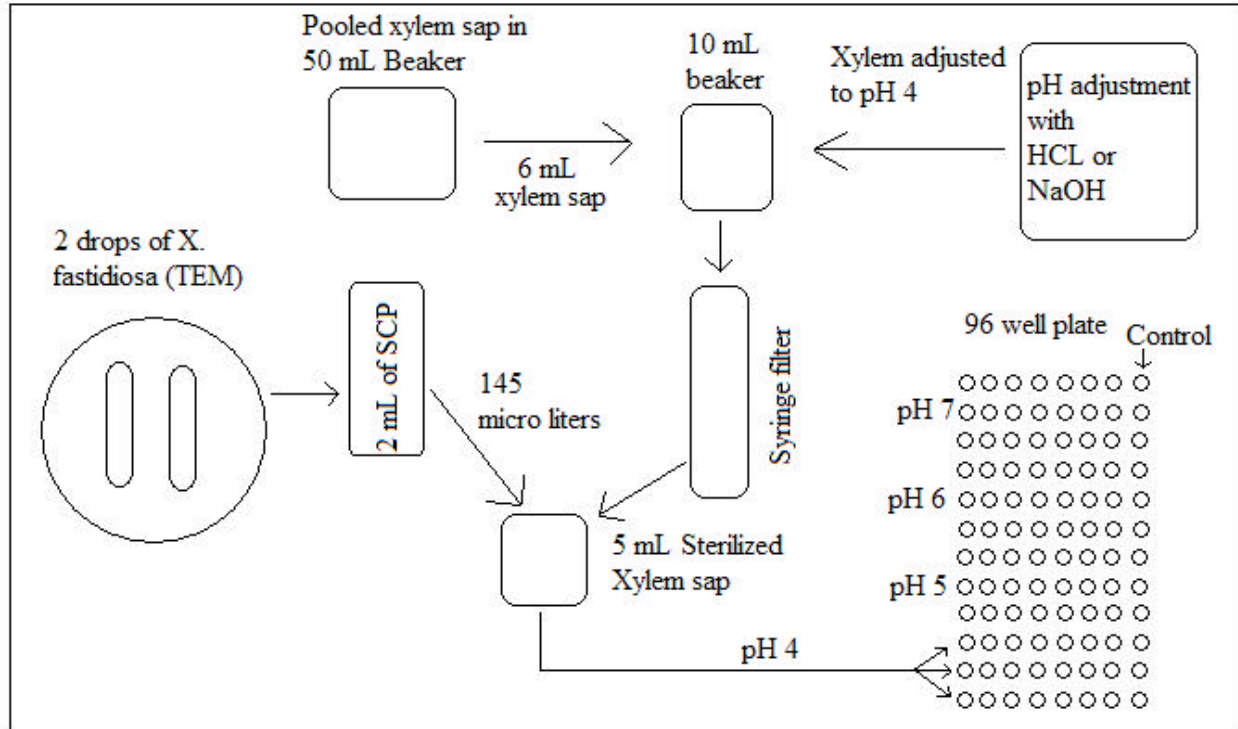| time	ph 5 vs. pH 6 vs. pH 7
0	0.85
24	0.00
48	0.00
72	0.00
96	0.01

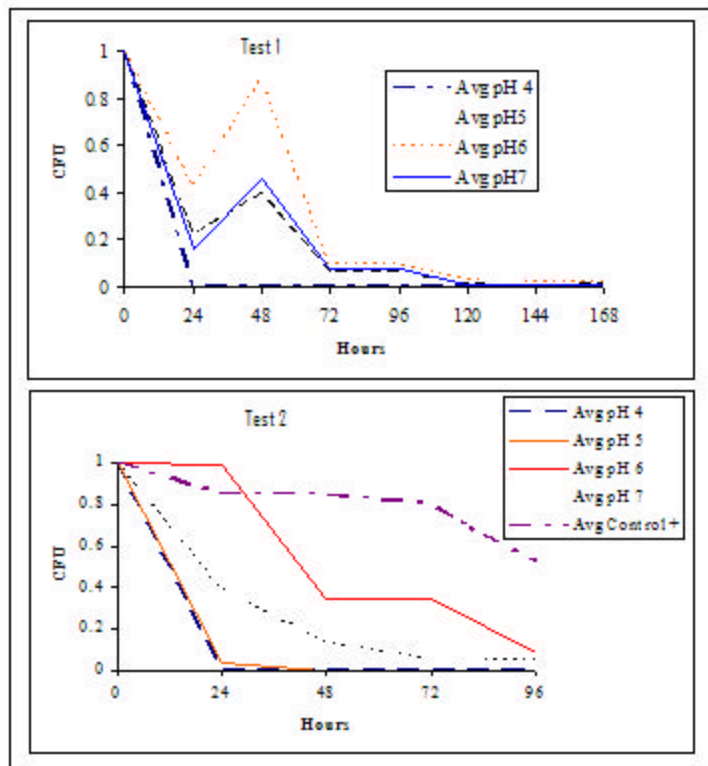Figure 5 An overview diagram of adjusting xylem sap pH and dispersing it into a 96 well plate.

Figure 6 A normalized graph of bacterial populations for test 1 and 2