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Effect of Ca and pH on Disease Severity of Pink Rot Phytophthora erythroseptica in Russett Norkotah Potato Solanum tuberosum

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EFFECT OF CA AND PH ON DISEASE SEVERITY OF PINK ROT *PHYTOPHTHORA ERYTHROSEPTICA* IN RUSSET NORKOTAH POTATO *SOLANUM TUBEROSUM*

by

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ABSTRACT

EFFECT OF CALCIUM AND PH ON PINK ROT (*PHYTOPHTHORA ERYTHROSEPTICA* PETHYB.) DISEASE DEVELOPMENT IN RUSSET NORKOTAH POTATOES (*SOLANUM TUBEROSUM* L.)

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Master of Science

Phytophthora erythroseptica Pethyb. is a devastating fungal pathogen of potato (*Solanum tuberosum*). The pathogen causes a disease known as pink rot. Pink rot results in necrosis and decay of tubers; and is responsible for major losses pre and post harvest. Attributes of the disease are progressing toward epidemic proportions. To help prevent such dramatic outbreaks of the disease, understanding the factors associated with incidence will provide opportunities to control the pathogen. A link between pH and disease severity has been observed. We studied the effects of pH and Ca to determine

their influence on disease development. Low pH and Ca deficiencies are often inter-related factors that can be causal of one another. The pH effect could be due to either H^+ or Ca ions. To separate their interactive effects we tested each variable individually using hydroponics and nutrient solutions. We assessed disease severity by assigning an Infection Coefficient (IC) to each root and stolon samples. The IC values were determined by quantifying DNA and then creating a ratio of host to pathogen DNA within root tissue. The DNA was measured by quantitative RT PCR. Statistical analysis showed significance in greater pathogen presence at more acidic pH and lower levels of available Ca. Significant reductions in IC values were observed when pH was elevated above pH 7. There was a notable increase in colonizing pathogen DNA at pH 5. Ca was significant, and as levels of soluble Ca increased the degree of disease severity became smaller. The effect of Ca was found not to be dependent upon pH. These results suggest immediate and cost effective applied management strategies to reduce incidence and disease severity outbreaks. Amending the soil with lime to increase soil pH and Ca content is one such potential method.

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Chapter 1.

SOLUBLE CALCIUM AND PINK ROT DEVELOPMENT IN RUSSET NORKOTAH POTATO

(Prepared for submission to Journal of Plant Disease)

Jared H. Benson, Bradley D. Geary, Jeff S. Miller, Von D. Jolley,
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Abstract

Phytophthora erythroseptica Pethyb. causes a disease known as pink rot in potatoes, which is responsible for substantial pre and post harvest tuber loss. Nutrients including Ca are associated with plant disease development and severity. The amount of available Ca in the soil affects plant health and the ability of *P. erythroseptica* to infect the host. Russet Norkotah potatoes were grown hydroponically with 3, 86, 172, and 343 mg L^{-1} Ca in association with the pathogen. The degree of disease severity was assessed by means of quantitative RT PCR. The suppression of disease by increased available Ca was statistically significant, decreasing with each increment of Ca between 3 to 343 mg L^{-1}. These data provides strong evidence that attention to Ca nutrition is a key environmental facto in disease suppression and should encourage further field evaluation.

Introduction

Pink rot is a soil borne disease caused by the fungal pathogen *Phytophthora erythroseptica* Pethyb. The pathogen has been characterized as a major pre and post harvest disease (25) of potato (*Solanum tuberosum* L.). In the United States, pink rot is a major contributor to the estimated 8-9% loss of the total potato production while in storage (1). In addition, fields have been reported with pink rot incidences as high as 70%, and consequently were not harvested (16). Such substantial losses invoke concern for control of the pink rot disease.

Preventing infection and disease outbreaks can be difficult for several reasons. First, the pathogen is soil borne and mainly affects the tubers, although severe soil infestations and root infections have been found to cause wilting of the stem (36). Few

fungicides are effective in controlling pink rot because the infections occur below the soil line, and control is dependent upon systemic delivery, for which there are few fungicides (16). Second, races of *P. erythroseptica* have developed resistance to the few effective fungicides (26). Mefenoxam is the active ingredient for the primary fungicides used to control pink rot; which acts systemically by disrupting the ribosomal RNA Polymerase of the pathogen (24). The ability for a pathogen to develop resistance to a pesticide is quickened when the mode of action is specific as in mefenoxam. Third, no potato cultivar grown in North America shows complete resistance (20); however, the degree of resistance varies between incidence and severity for cultivars. Very susceptible cultivars include: thin and red skin, Warba (8), Snowden, Norland, and Russet Norkotah (24). Moderately resistant cultivars include late season maturity types, Russet Burbank (26), Irish Cobbler, Kasota (8), and Butte (20). Finally, environmental factors such as excessive moisture (3, 21), warm temperatures (4, 10) , and acidic pH (2) have been identified as elements that have a strong influence on increasing disease incidence and development. These factors can aid in the life cycle of a pathogen and infection of hosts.

New methods to control infection need to be identified to prevent pink rot from becoming a disease of epidemic proportions. Post-harvest applications of sodium hypochlorite and various salts have been found to prevent spread of infection in storage (17), but optimal control should occur before harvest to ensure maximum yield and minimum storage loss. Phosphoric acid and fungicides containing mefenoxam are systemic and effective if applied in a timely manner. However, recently acquired resistance to mefenoxam suggests the need for development of different control methods.

The addition of calcium has been found effective in reducing incidence and severity of several diseases such as: clubroot (*Plasmodiophora brassicae*) of crucifers (*Brassica)* (19), cephalosporium strip (*Cephalosporium gramineum*) on wheat (*Triticum aestivium*) (18), root rot (*Phytophthora nicotianae*) of citrus (*Citrus*) (6), and soft rot (*Erwinia carotovora*) in potato (*Solanum tuberosum L.*) (14). Calcium is essential and considered one of the most important nutrients associated with plant defense (7). The mode of action for calcium is not always clear but could result from its role within the plant or soil. Within the plant, Ca is involved in eliciting signal transduction pathways (7) and in-membrane and cell wall integrity (23). In soil, Ca is a key cation and is integral in the cation exchange capacity (CEC) and structural composition of soil. There is potential for increased resistance to pink rot by preventing infection through better physical barriers such as the condition of the skin (27) or improved soil drainage (15). The ability of Ca to act through multiple pathways to prevent infection implies a possible role for Ca in preventing pink rot.

The effect of supplemented Ca on pink rot development was studied hydroponically by growing the plants in a low calcium solution and then transferring plants to supplemented nutrient media with varied Ca concentrations. Disease severity was calculated by similar means of Attallah et al. (1) and Benson et al. (2) with the use of quantitative Real Time PCR.

Materials and Method

Russet Norkotah potatoes were grown in an enclosed hydroponic system within a growth chamber (Percival Scientific, Perry Ia). Uniform plantlets were obtained from the University of Idaho tissue culture laboratory (Moscow, ID). Roots of four to six cm

length plantlets (20 total plants per 14 L^{-1} tank) were placed into a complete modified Steinberg solution (5) containing a minimum level (1.3 mg L^{-1})of Ca to avoid Ca loading After growth in pretreatment solution for 14 days, for relative uniformity, plants were randomly transferred into complete modified Hoagland solutions (48.5 K, 45 N, 5 Mg, 5 S, 2.1 Fe, 6.3 Cl, 3.5 mM P, 94.4 Mn, 264 B, 4.2 Mo, 24.3 Zn, 6.4 Cu, 8.3 µM Na). Calcium levels were varied using $Ca(NO_3)_2$ at 3, 86, 172, 343 mg L^{-1}. Nitrogen was balanced by providing 45 mM as KNO_3.

The environmental conditions cycled on a 12 hr light with high temperatures at 23^{o}C and a 12 hr night with low temperatures at 17^{o}C. The trial was repeated four times and conducted in a 4x4 Latin Square each time. Four replications of each treatment were randomly place in 16 individual 14 L buckets placed in a gray/black (outside/inside)box and covered with a white/black polyethylene lid to mimic soil conditions. Air was supplied at a constant flow to each bucket at 10 psi.

Phytophthora erythroseptica isolate 01-21 was received from the University of Idaho Research station (Aberdeen, ID). The isolate was used due to its frequent presence in the growing regions of south-eastern Idaho (16). The isolate was maintained on P_5ARPH *Phytophthora* selective medium (17 g corn meal agar, 100 mg pentachloronitrobenze (PCNB), 10 mg Rifampicin, 5 mg Primaricin, 250 mg Ampicilin). Zoospore inoculum was created according to the protocol of Salas et al. (24). Each hydroponic tank was immediately inoculated after transfer of plants to Hoagland treatment solution. Each unit received an equivalent inoculation of 20 ml of suspended zoospores at 50,000 spores ml^{-1}, which were quantified using a hemocytometer.

Plants were destructively harvested in pairs 14 days after inoculation. Root and stolon tissue were cut and removed just below the lid. Roots of both plants from each bucket were pooled together and represented a single sampling. Each sample tissue was washed in a 10% bleach solution followed by rinsing with distilled water. Root tissue was stored at -80^{o}C before lyophilizing. Using 275 mg of ground lyophilized tissue from each sample, DNA was extracted through a modification of the method described by Kidwell et al. (11). Because of smaller sample sizes, volumes, masses, and reagents used in the extraction were at 10% of the suggested values.

Infection or root was quantified and adjusted to 25 ng μl^{-1} by use of a Nanodrop 1000 spectrometer (Applied Biosystems, Foster City, CA). Disease severity was assessed by creating infection coefficients (IC) according to the methodology described by Attallah et al. (1). An IC for each sample was created based upon the ratio of host DNA to pathogen DNA (28). The use of quantitative RT PCR to amplify specific target sequences allows for sensitive reproducible results. The ribosomal polymerase B1 gene (*rpb1*) in *P. erythroseptica* and actin gene (*act*) in potato serve as the target sequences.

Absolute quantification was conducted on a ABio 7300 RT-PCR (Applied Biosystems Foster city, CA) with DNA 1 ng μl^{-1}, 200 nm each primer, and SYBR green mastermix (Applied Biosystems, Foster City, CA) in a 25 µl reaction. Each sample was run in duplicate. Positive and negative controls were fungal culture DNA and water.

Stem and above ground plant tissue was subjected to nitric - perchorlic acid digestion and Ca was quantified as described in Johnson et al. (9) and was conducted on IRIS Intrepid II XSP (Thermo Scientific Electron, Waltham, Ma).

Analysis of the infection coefficients was conducted using SAS (SAS Institute, Cary, NC). The general effects model was conducted with Ca nested within trials. Due to multiple samples for each treatment a repeated measure step was included in the analysis. A Tukey-Kramer adjustment was also incorporated to determine differences between treatments.

Results

Based on IC ratio data, augmented levels of solution Ca to potato grown in nutrient solution inoculated with pathogen reduced the pathogen load within the host tissue (Table 2). The overall effect of Ca on disease development was statistically significant ($P \leq .0025$). Eighty-six mg L^{-1} Ca can be considered the control for normal levels of available Ca. A significant increase in pathogen load (IC ratio) was observed at the 3 mg L^{-1} solution Ca level and was statistically higher than all the other solution Ca levels. The lowest pathogen load indicated by IC values was associated with the 343 mg L^{-1} Ca and thus was significantly lower than Ca treatments of 86 and 3 mg L^{-1}. Analysis of stem and leaf tissue Ca confirmed that more Ca was being taken up by the plant as solution Ca increased from 3 to 343 mg L^{-1} Ca. As expected the highest concentration of leaf and stem Ca was associated with the highest treatment level (Figure 1).

Discussion

Calcium is an essential mineral that has been shown to be important in physiological processes such as plant defense (22) and a deficiency of available soil Ca can create a more favorable condition for some pathogens to infect. However, adequate and/or excess Ca has been found to improve soil quality, increase cell wall integrity,

thicker skin netting, and ensure proper cell signaling of pathways such as calmodulin, thus reducing the incidence and severity of disease (22).

We have found that the addition of Ca reduced the IC value of *P. erythroseptica*, indicating less infection. Previous study conducted by Benson et al. (2) established pH 5.0 as the pH of greatest infection thus this test was conducted at pH 5.0 to ensure maximum infection and greatest chance that the effect of Ca could be observed. The effect of Ca on disease infection can be pH dependent (19) because one factor is often inter-related to the other. In this study, a decrease in disease severity was observed as Ca levels were elevated and pH was maintained at a constant through MES buffer at 5.0, indicating that Ca alone, and not it's influence on pH, has a direct influence on pathogen levels within potato roots.

As indicated, acidic pH proved to give the highest infection rate with pink rot. It can therefore be assumed that Ca provided at more basic pH would also impact infection, but this deserves further investigation. The decrease in infection is attributed to Ca and not N because we would have expected with an increase in solution N we would have increased plant growth and a higher susceptibility to disease. Different forms of Ca might also be more or less effective. Calcium nitrate is generally accepted as an effective and relatively inexpensive delivery method of Ca but may not be the best form to lower *P. erythroseptica* levels in potatoes. Other common sources include $CaCl_2$, $CaCO_3$, and Ca propionate, although not all have equal effects is reducing disease in potato plants (6, 14, 29). We chose $CaNO_3$ because other forms like $CaCl_2$ provides Cl, an element also associated with improved disease resistance and $CaCO_3$ which buffers the pH above 7.0

and pH above 7.0 promote less infection than more acidic pH's. We also provided N levels slightly above the adequate level to avoid needing to balance soluble N.

Since more severe outbreaks of pink rot are associated with acidic soils, supplemental Ca, in addition to raising the pH, can substantially increase protection against *P. erythroseptica* infection. An upper limit was not established for the effect of Ca in this study, although the substantial decrease in infection was still observed when Ca was applied at the highest rate of 343 mg L^{-1} was not significantly different from the 172 mg L^{-1} solution Ca level. McGuire also found in potatoes that substantial decreases in Erwinia soft rot disease were observed as Ca increased in potato tissue, and no surface decay occurred when tubers were vacuum infiltrated in solutions at 12,000 mg L^{-1} Ca (14). These results indicate that there is a possibility for pink rot infection to be almost entirely eliminated when Ca levels are raised high enough, though this may not be practical in a commercial operation.

Studies relating to Ca content and *Erwinia* soft rot of potato tubers have revealed that more susceptible varieties contain low Ca content in the tuber, but that not all resistant cultivars contain high tuber Ca content (13). Since there is a lack of disease resistance genes against pink rot and Ca levels have been implicated in resistance for soft rot, then the addition of Ca would provide a boost to the natural defense of potato to pink rot and possibly many other diseases.

The mode of action for Ca to promote disease resistance was not the focus of this study and thus still remains unknown although it is likely that Ca acts through multiple means. The effect specifically for *P.erythroseptica* could result from a reduction in zoospore and sporangia pathogenicity and increasing cell wall integrity by resistance to

degrading enzymes. Analysis of Ca in the leaf and stem tissue of the plant showed that more Ca was being taken up and used within the plant when grown in higher concentrations of extracellular Ca solutions which would likely strengthen cell walls and increase the plants defenses.

This study indicates that amending soil with excess Ca has the possibility to significantly reduce pink rot outbreaks which may reduce the amount of yield loss for pre- and post-harvest tubers. Augmenting the soil with soluble Ca also provides another tool to the limited control strategies for pink rot management.

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Tables and Figures

Table 1. Primers for target genes of *Phytophthora erythroseptica* and *Solanum tuberosum*.

Organism	Gene	Primer sequence (5'→ 3')
P.erythroseptica	*rpb* 1	GAT GAA ACT AAG CGC CTT CTC CGA CAA TAG TCT TCA AGG TGG AT
S. tuberosum	*act*	TGA ACA CGG AAT TGT CAG CA GGG GTT AAG AGG GGC TTC AG

[a]Primers designed by Attallah et al. (3)

Table 2. Effects of four levels of solution Ca on *Pythophthora erythroseptica* colonization in Russet Norkotah roots.

	Mean		P value at		
Ca	IC value[a]	P value	86	172	343
3	0.9338	<.001	0.0286	0.0019	0.0005
86	0.8646	<.001		0.1204	0.0257
172	0.8191	<.001			0.3655
343	0.7938	<.001			

[a]IC value, is a ratio based upon constitutively expressed genes in the host and pathogen. These values correspond well to disease development, as colonization of the pathogen progresses, the amount of product from the pathogen increases and the ratio gets larger.

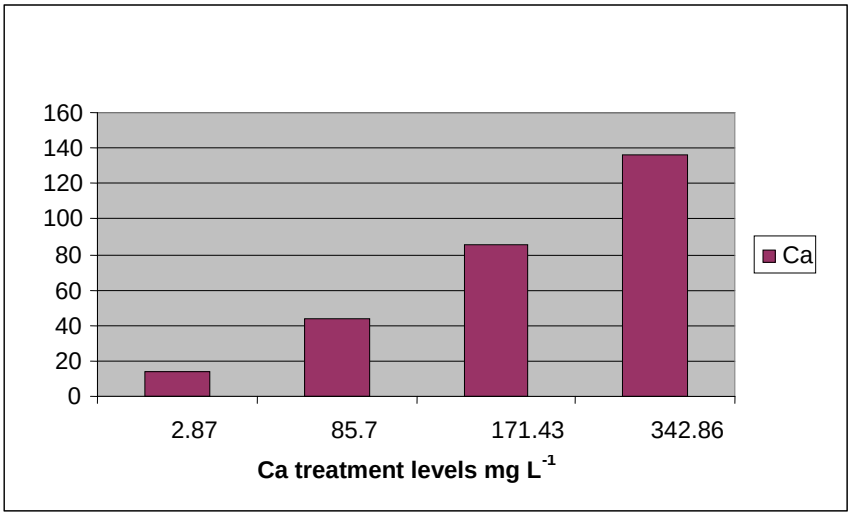

Figure 1. Calcium concentration of leaf and stem tissue of Russet Norkotah when grown in 3, 86, 172, 343 mg L^{-1} Ca in solution.

Chapter 2.

VARIABLE PH AS A FACTOR IN DEVELOPMENT OF PINK ROT IN RUSSET NORKOTAH

(Prepared for submission to Journal of Plant Disease)

Jared H. Benson, Bradley D. Geary, Jeff S. Miller, Von D. Jolley, Mikel R. Stevens

Abstract

Pink rot caused by the pathogen *Phytophthora erythroseptica* Pethyb. causes severe pre and post harvest losses in potato. Disease outbreaks are associated with excessive soil moisture, moderate temperature, late season development, and a lack of potato cultivar resistance. The severe loss of potato due to pink rot suggests the need for better information on the factors associated with disease development. The effect of pH has been regarded as a strong influence on disease development as observed by previous studies and by commercial potato growers. A hydroponic growth system was established to study the effects of pH on *P. erythroseptica* infection and colonization in potato roots. Russet Norkotah plantlets were grown in hydroponic nutrient solutions buffered at pH 5, 6, 7, and 8 using MES and evaluated for infection by means of quantitative Real Time PCR. Acidic pH solutions had a higher proportion of pathogen to host DNA in root tissue, indicating increased infection and colonization of the pathogen. Results indicate that pH has a unique and significant influence on the levels of infection from *P. erythroseptica* and that commercially grown potatoes would likely have less tuber rot if they were grown in soils with pH is above 7.

Introduction

It is estimated that 8-9% of the total potato (*Solanum tuberosum* L.) production in the United States is lost to tuber rots following harvest but during storage (1). Pink rot, caused by the fungal pathogen *Phytophthora erythroseptica* Pethyb. has been identified as a major post harvest disease (14, 25). The storage environment: high humidity, temperature, poor air circulation, close proximity (1) in combination with wounding (22)

during harvest creates optimal conditions for disease development and new infections to occur, although it is proposed that the majority of infection occurs in-field before harvest (3). Fields with infections have ranged from 15% to as high as 70% (26) and were not harvested (14). Pink rot is a soil borne disease and therefore the pathogen has the ability to maintain high populations within a field for subsequent years (6).

Phytophthora populations offer several possible sources of inoculum. Infection has been found to occur through mycelium, sporangia, zoospores, and cysts (13). Zoospores create a unique approach to infection because they are the only mobile fungal structures. Either excessive or standing water provide the optimal conditions for zoospores to find and infect hosts (4, 20). Zoospores locate their hosts by means of chemotaxis. This chemical attraction has been known to act through amino acids such as: glutamic and aspartic acid (11).

Although zoospores may attack susceptible locations on roots due to chemical signals, infection typically occurs through the eyes, lenticels, wounds, and stolon (13). In-field infection is often characterized by notable symptoms at the location of infection such as: purplish or black eyes (8), enlargement of lenticels (30), spongy/granular texture (9) and discoloration at the attachment of the stolon (13). The disease mainly infects roots, stolons, and tubers and is attributed to tuber decay. Above ground plant structures were not found to be infected by zoospores or mycelium, although heavy infection of the stem has caused wilting of the plant (6).

The ability of fungal structures to cause disease is associated with several other environmental factors besides water. Temperature was found to be highly correlated with disease. The key range for infection was between 15°C and 30°C (6). Infection was most

severe at 25 °C, and was scarce below 5°C or above 35°C (10). Other environmental factors associated with disease include larger tubers (5), early lifting (9), and thin skinned or early maturing varieties (18, 19, 21).

All potato cultivars grown in North America are susceptible to pink rot, although the degree of resistance varies between incidence and severity for cultivars (18). Very susceptible cultivars include the thin and red skinned cultivars, Warba (9), Snowden, Norland, and Russet Norkotah (21). Moderately resistant cultivars include late season maturity types, Russet Burbank (18), Irish cobbler, Kasota (9), and Butte (19).

Cultivar susceptibility and recent discovery of *P. erythroseptica* races that are resistant to Mefenoxam, the active ingredient in effective systemic fungicides (17), suggest that more studies are needed in order to prevent and manage pink rot. Understanding the factors associated with outbreaks allows for appropriate management strategies to be taken to control pink rot and avoid yield loss.

Disease outbreaks in south-eastern Idaho potato growing region have suggested a potential role of pH in infection (14). The effect of pH on disease development has been documented for several pathogen-hosts interactions such as: club root of crucifers (16), Cephalosporium stripe on wheat (*Triticum aestivum*) (15), and potato scab (2). The incidence of club root of crucifers (*Brassica*) was reduced when pH was raised above 7.2 (16), while contrastingly potato scab was significantly reduced when pH was lowered to 5.2-5.0 (2).

How pH affects conditions for *P. erythroseptica* infection is not clear, although it is known that the availability of nutrients to the host is confounded depending upon the pH of the environment. The effect of soil pH is hard to separate from a depletion or

excess of particular ions, but can be evaluated through nutrient solutions in a hydroponic growth system. The objective of this study is to determine the effect of pH on the development of pink rot in Russet Norkotah potatoes grown under hydroponic conditions.

Materials and Methods

Russet Norkotah potatoes were grown in an enclosed hydroponic system within a growth chamber (Percival Scientific, Perry Ia). Uniform plantlets were obtained from the University of Idaho tissue culture laboratory (Moscow, ID). Roots of four to six cm length plantlets (20 total plants per 14 L^{-1} tank) were placed into a complete modified Hoagland solution (7) containing (mM), (33.5 K, 30 N, 5 Mg, 5 S, 2.1 Fe, 6.3 Cl, 3.5 mM P, 94.4 Mn, 264 B, 4.2 Mo, 24.3 Zn, 6.4 Cu, 8.3 µM Na). After growth in pretreatment solution for 14 days, for relative uniformity, plants were randomly transferred into new complete modified Hoagland solutions.. The pH treatment level was adjusted using KOH, and buffered using 2-(N-morpholino)ethanesulfonic acid (MES).

The environmental conditions cycled on a 12 hr light with high temperatures at 23^{o}C and a 12 hr night with low temperatures at 17^{o}C. The trial was repeated four times and conducted in a 4x4 Latin Square each time. Four replications of each treatment were randomly place in 16 individual 14 L buckets placed in a gray/black (outside/inside) box and covered with a white/black polyethylene lid to mimic soil conditions. Air was supplied at a constant flow to each bucket at 10 psi.

Phytophthora erythroseptica isolate 01-21 was received from the University of Idaho Research station (Aberdeen, ID). The isolate was used due to its frequent presence in the growing regions of south-eastern Idaho (16). The isolate was maintained on P_5ARPH *Phytophthora* selective medium (17 g corn meal agar, 100 mg

pentachloronitrobenzed (PCNB), 10 mg Rifampicin, 5 mg Primaricin, 250 mg Ampicilin). Zoospore inoculum was created according to the protocol of Salas et al. (24). Each hydroponic tank was immediately inoculated after transfer of plants to Hoagland treatment solution. Each unit received an equivalent inoculation of 20 ml of suspended zoospores at 50,000 spores ml^{-1}, which were quantified using a hemocytometer.

Three repeated trials were conducted in a 4x4 Latin square. The sixteen experimental units (hydroponic tanks) were identical except for K, which was used to regulate pH levels. The four pH treatments: 5.0, 6.0, 7.0, and 8.0 (+/- 0.1) were representative of soil found in the Snake River Plain of southern Idaho. The pH for each treatment was buffered using MES and was adjusted by means of adding KOH until the target pH was reached. Since K was already adequate for growth and K is often in excess in soil culture, the differential level of K caused by these additions should be minimal.

Plants were destructively sampled in pairs every three days for two weeks after inoculation for a total of 5 samples. Root and stolon tissue were cut and removed just below the lid level. Roots of both plants from each sampling time from each bucket were pooled together and represented a single sampling. Each sample tissue was washed in a 10% bleach solution followed by rinsing with distilled water. Root tissue was stored at -80^{o}C before lyophilizing. Using 275 mg of ground lyophilized tissue from each sample, DNA was extracted through a modification of the method described by Kidwell et al. (12). Because of smaller root sample sizes, volumes, and masses of samples, reagents used in the extraction were at 10% of the suggested values.

Samples were quantified and adjusted to 25 ng ul^{-1} by use of Nanodrop 1000 spectrometer (Applied Biosystems, Foster City, CA). Disease severity was assessed according to the methodology described in Attallah (1).

Samples were analyzed by quantitative RT PCR, and assigned a cycle threshold (ct) value when a minimum level of fluorescent DNA was detected. These ct values correspond to initial DNA quantities and were used to create a ratio between the amount of host DNA and colonizing pathogen DNA. The ratio is called an infection coefficient (IC) and was compared to other IC values (27). The ribosomal polymerase B1 gene (*rpb1*) in *P. erythroseptica* and actin gene (*act*) in potato serve as the constitutively expressed target sequences.

Absolute quantification was conducted on a ABio 7300 RT-PCR (Applied Biosystems Foster City, CA) with DNA 1 ng μl^{-1}, 200 nm each primer, and SYBR green mastermix (Applied Biosystems, Foster City, CA) in a 25 µl reaction. Each sample was run in duplicate. Positive and negative controls were fungal culture DNA and water, respectively.

Analysis of the infection coefficients was conducted using proc mixed in SAS (SAS Institute, Cary, NC). The general effects model was conducted with pH nested within trials. Due to multiple samples for each treatment a repeated measure step was included in the analysis. A tukey-kramer adjustment was also incorporated to determine differences between treatments.

Results

Based on IC ratio calculation, potato grown hydroponically at various pH levels developed significant differences ($P \leq 0.05$) in the amount of colonizing *P.*

erythroseptica (Table 2). Analysis of variance (ANOVA) on the overall effect of pH on *P. erythroseptica* infection was (P = .027). The IC value was inversely related to pH i.e. as pH became more acidic the IC values were larger. The lowest IC value was observed at pH 8.0 and the highest was at pH 5. The latter IC value was significantly higher associated with pH 8.0 (P = .0051). Statistical differences were also observed between pH 6.0 and 8.0 (P = .0296) and 7.0 and 8.0 (P = .055).

As expected, infection increased over time (table 3), but the interaction beween time by pH was not significant. The increase in pathogen presence as assessed by IC values over time has an approximately linear trend to increase for each treatment level (Figure 1). When comparison of the interaction of sampling times within treatment level was accounted for, statistical significance was only present in the comparison of the fifth sampling time between pH 5 to 7.0 and 8.0 (P = .0273 and .005) in addition to 6.0 to 8.0 (P = .0448). The model was fit according to AR1 and was not found to have serial correlation.

Discussion

We have developed a system in which a specific abiotic variable, pH, can be assessed for its effect on disease development. By hydroponically growing plants in buffered and specific pH levels, we were able to discern the effect of pH on *P. erythroseptica* infection is discernable. The results confirmed what has been observed in the field (Unpublished data, 2008, Jeff Miller); as the pH became more acidic the infection became more severe. Larger infection coefficient (IC) values represent higher pathogen load and subsequent greater disease severity. These IC values correspond to disease severity accurately in that they relate the amount of pathogen DNA to host DNA

as a measurement of infection (28). These IC values can be accurately quantified because the high sensitivity of quantitative RT PCR allows for discrete differences in pathogen concentrations to be distinguished.

The severity of the disease was significantly higher at pH 5 than 6, 7, or 8, but no upper or lower limits were established. *In vitro* studies revealed that the pathogen is unable to grow below pH 3.2 (6). No upper limit has yet been established for the failure of growth of the colony due to inability to maintain media above pH 10 (6). The optimum growth of the fungus was at a pH of 7.0. These *in vitro* pH growth values can be extended to field conditions, but pH below 4.5 and above 8.5 would be unlikely in field and thus indicate an extreme range for ideal potato production.

The pH in the hydroponic solution was regulated by use of MES and was adjusted by addition of KOH. This base was found much more effective in raising the pH of the acidic modified Hoagland solution than NaOH, $Ca(OH)_2$, or other bases available. Also, because the higher the pH needed, the greater amount of KOH added was larger. The use of KOH was practical because solutions at each pH already had adequate K for potato growth and additional K would not injure potato nor impact infection. Potato and other plants often are grown in soil conditions of excess K and resultantly can be a luxury consumption. Therefore, KOH was the best option for raising the pH.

Studies have revealed that pH is strongly associated with disease severity (2, 16). The effects pH can have on soil attributes, nutrient availability, and plant metabolism can create more ideal conditions for infection to occur even when pH is not necessarily damaging to the host. Low pH can sequester nutrients and promote mineral deficiencies in plants with resulting developmental problems and weakened plant defenses. Soil

quality is also influenced by pH. The structural relationship of soil particles in acidic environments can decrease the drainage capacity by deflocculation of clay particles, allowing water to accumulate and assisting zoospore movement.

The lack of statistical significance for time could be attributed to a short time in association with the pathogen and stage of growth for the potato plants. Since the disease is usually associated with warm

Potato can adapt to acidic conditions (30). For example, potato grown in low pH environments, matured earlier, and were also found to have a higher tuber to top ratio (23). Thus, although not developmentally hindered in acid soils, potato may manifest an increase in early tuber material and increase the chance of infection to occur. The correlation between early maturing varieties and low pH facilitating earlier maturation and higher susceptibility is indicative of a physical condition which is more optimal for the pathogen than the host. Although there time was not statistically significant as a treatment nor between pull times we did notice increase in IC values. The disease is associated more with mature plants and tubers than it is with young plants. Initiation of tuber development while being grown hydroponically is not common and requires changes in pH (29), therefore we decided to use younger plants. Since a difference between the pHs was observed at such an early stage, we can assume that disease progression would continue.

Understanding that acidic conditions are associated with higher incidences and disease severity of pink rot suggests a cultural method for management. The addition of lime is frequently used to raise the soil pH of fields and should reduce *P. erythroseptica* infection. Lime can be applied on a precision farming basis to fields in which pH is not

uniform to reduce hot spots of pink rot incidence. This study has helped define a possible cultural method as an additional option to fungicides in managing outbreaks of pink rot . This could be increasingly important as resistance to the few effective fungicides is already apparent in *P. erythroseptica* isolates (24).

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Tables and Figures

Table 1. Primers for target genes *Phytophthora erythroseptica* and *Solanum tuberosum*. [a].

Organism	Gene	Primer sequence (5'→ 3')
P.erythroseptica	*rpbI* 1	GAT GAA ACT AAG CGC CTT CTC
		CGA CAA TAG TCT TCA AGG TGG AT
S. tuberosum	*act*	TGA ACA CGG AAT TGT CAG CA
		GGG GTT AAG AGG GGC TTC AG

[a]Primers designed by Attallah et al. (3)

Table 2. Effect of sample time on *P. erythroseptica* infection levels in Russet Norkotah potato roots[a].

	Mean		P value at sampling time			
Time[b]	IC value		2	3	4	5
1	0.7381	<.001	0.0069	0.0005	<.0001	<.0001
2	0.7681	<.001		0.1822	0.0077	<.0001
3	0.7874	<.001			0.0997	0.007
4	0.8127	<.001				0.0184
5	0.8658	<.001				

[a]Sampling times were every 3 days after inoculation. Studies were conducted at pH 6.
[b] Sample time 1 is 3 days after inoculation (DAI), 2 = 6 DAI, 3 = 9 DAI, 4 = 12 DAI, and 5 = 15 DAI.

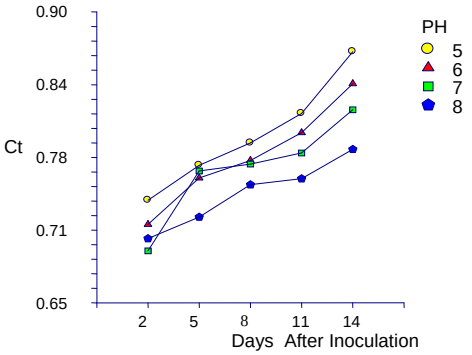

Figure 1. Interaction of pH on *P. erythroseptica* infection in Russet Norkotah potato roots over a 14 day time period. Statistical significance was not found for time or the interaction pH and time.

Appendix A

LITERATURE REVIEW

Introduction

Prevention and treatment of disease in agronomic crops is of paramount concern for several reasons. First, there is a continual decrease in existing land that is capable of cultivation and irrigation. Second, a growing population requires an increased output. Finally, as diseases develop resistance to natural, chemical, and physical barriers, new control methods must be developed and integrated. Plant pathology addresses these concerns to preserve yield and increase production efficiency; particularly in important food crops like potato which is an essential source of nutrition to many people throughout the world.

As an agricultural crop the potato is one of the most important dicot. It is the fourth largest crop in tonnage produced behind wheat, rice, and maize. As a food source it is a rich supply of carbohydrates, potassium, phosphorus, protein, niacin, and other essential minerals (9).

Potato is in the family *Solanacea,* and is one of a few members that produce tubers. The genus *Solanum* contains over 2,000 members, many of which are poisonous and/or prominent horticulture and agricultural crops. Members of *Solanum* include: tomato (*Solanum lycopersicum*), eggplant (*S. melongnea*), tobacco (*S. nicotiana*), peppers (*S. capsicum*), and petunia (*S. petunia*).

Annuals and perennials are both found in the family; potato is annual in nature, but have been called perennials because it has the ability to reproduce vegetatively (55). The tuber which is edible, is an enlarged stolon that originates from the apical cell. The height of the plant usually ranges from two to five feet. The leaves are alternate and spiral in a counterclockwise direction. Flowers can be determinate or indeterminate

depending on the cultivar, with five lobes on the corolla. The petals can vary in color from white to yellow, purple, blue, or striped.

The center of origin for genetic diversity and domestication of potato is in South America. It has been estimated that the potato has been in cultivation since 200 A.D. (9). The ability of potato tubers to be grown in difficult environments such as high elevation and colder temperature, made the crop ideal for civilizations living in the Andean ranges. Salaman (52) stated that "discovery of the potato and frost resistant types and methods of preserving tubers, man could live at higher altitudes and gain mastery of South America." The plant is ideal for cultivation because a high yield can be obtained in limited space and each tuber contains a rich supply of amino acids and nutrients. A concern for production is that the potato is susceptible to many diseases which must be controlled to obtain maximum production.

Each year approximately 20% of the potatoes grown in the United States are lost to diseases (27). A substantial portion (8 to 9%) of the total yield is lost post harvest; (1.3-1.9 million tons, corresponding to $200 million) in storage (3). Diseases of potatoes can come from multiple sources, but the most significant are associated with fungal origins (39). An escalating fungal disease of potato is pink rot caused by *Phytophthora erythroseptica* Pethyb. Fields infected with pink rot have ranged from 10 - 75% infection and often times were not harvested resulting in total yield loss (35). The disease has also devastated germplasm collections in Peru and many diploid relatives (63).

Pink rot was first observed in 1909 near Clifden, Ireland (43). Pethybridge ascribed the name pink rot to *P. erythroseptica* due to the salmon color the inner tissue becomes after being exposed to air. The disease has since spread internationally via

infected seed and has been identified in major potato producing countries (66). Pink rot has become a major pre and post harvest disease (36, 42, 59) and is responsible for considerable yield loss. Therefore, an understanding of the nature of the *P. erythroseptica* pathogen is critical for the epidemiology and control of pink rot in order to prevent the disease from reaching epidemic proportions.

Escalating circumstances involving pink rot resistance to the systemic fungicide mefenoxam draws attention for the need to identify new methods to prevent and control the disease. Understanding the relationship of specific factors such as Ca and pH to the host and pathogen can allow for better prevention of the disease. This research regarding the effect of pH and Ca on pink rot was conducted with the intent to further understand environmental factors associated with disease development and possible implications to disease management.

Pink Rot

The origin of pink rot was in Ireland. The disease subsists in the soil and favors water (68). Free water facilitates the movement of mobile fungal structures called zoospores. Infected tubers used for seed are responsible for the widespread outbreaks. Pink rot has been recorded in Holland (44), Bulgaria (2), and Peru (63). Early reports of pink rot in the United States include: Oklahoma 1923 (20), Maine 1938 (6), and Massachussets 1939 (7). The disease has often been misdiagnosed as waterslain and water rot (12) because it is frequently found in areas with excess water, although further examination of symptoms help with correct disease diagnosis.

Symptoms

The most prominent symptom of pink rot is the pink discoloration that occurs within the inner tissue after being exposed to air (43). The change in color usually occurs after 15 to 20 minutes of exposure. The tissue will continue to change color until it is black. White (40) stated that the pink discoloration is the result of an oxidative reaction of metabolites from tyrosinase. A black band will often separate healthy from infected tissue (22) and will not change colors when exposed to air.

Cavities do not develop (19) in the diseased tissue but become soft, spongy, rubbery, and granular (22). The eventual result of infected tissue is necrosis and disintegration. An early sign of pink rot is a discoloration in the area of the stolon attachment to the tuber (31). Other external symptoms of infection can include: purplish or black discoloration of eyes (20), enlargement of lenticels (70), or a weak formaldehyde scent (22). There is not an appreciable decrease in the size of an infected tuber (7) because the disease mainly infects the roots, stolons, and tubers during the growing season without much plant decay. If there is heavy infection, particularly in the base of the stem, the above ground plant tissue will wilt (13).

The distinct color change of inner tissue was the manifest characteristic to identify pink rot, but several less detrimental *Phytophthora* species have been found to also produce a pink color of inner tissue (25). These false sub-species of pink rot include: *P. megasperma* (12, 45), *P. parasitica* (25, 45), and *P. cryptogea* (45, 51).

Alternate hosts and sources of pink rot

Phytophthora erythroseptica infects other plant genus and species and may maintain high populations in the soil. Other hosts include asparagus, pea, tomato, arrow leaf clover (45) raspberry (16), tulip (8), sugarcane, and cineraria (57). Fungal cultures from other hosts that are thought to be new sub-species of *P.erythroseptica*, have similar effects to pink rot, and researchers have attempted to label them as new sub-species from alternate hosts. The acceptance of these multiple alternate sources of pink rot is still debated (26) because of different culture morphology. Distinction between *Phytopthora* species is often difficult because there is uncertainty with the sexual reproductive nature (56), which is key to identification. The only accepted alternate source of *P.erythroseptica* is *pisi*, which was presented by Bywater and Hickman (11). Strict morphological studies have been performed to ensure proper characterization of pathogens.

Morphology

The new sub-specie *pisi*, was based on observed differences among hyphae, oogonium, and aerial mycelium. *Pisi* exhibited hyphae with very uneven diameters. The oogonium and oospore had thicker walls than Pethyb. No aerial hyphae were present when *pisi* was cultured out on multiple media types. The *pisi* name comes from being isolated from peas.

P. megasperma was thought to be a new form of pink rot (26) but rejected because of differences in the sexual structures. The key difference between *P. megasperma* and *P. erythroseptica* is the relationship of the antheridium and oogonium.

The side by side growth of the antheridia to the oogonium in *P. megasperma* is called paragynous whereas only amphigynous antheridia are observed for *P. erythroseptica* (26). Amphigynous antheridia are distinct because the antheridia will grow through the oogonium. The homothallic nature, ability to combine with identical mating types, of *P. erythroseptica* is associated with amphigynous antheridia (56). The oogonia can have two, three, or four antheridia attached to it that are uniform and spheroidal (16).

Sporangia are non-papillate (56) and can be ellipsoid, ovoid, obyriform, or distorted and are 43-69 µm long by 26-47 µm wide (21). Sporangiophores widen just beneath the sporangium (69). The hyphae are uniform and thin. When the pathogen is cultured out on media, the colony can be stellate or rosette with moderate to no aerial hyphae (57). Zoospores can be stimulated to release from sporangia by chilling (67). The taxonomic implications of zoospores are unique because they are the only motile fungal structures.

Taxonomy

Phytophthora is within the *Oomycota* family. Many devastating potato pathogens are oomycetes, such as *Pythium* and *P. infestans.* The oomycetes form an interesting group that is not considered as true, but lower fungi. It has been proposed that oomycetes be reclassified into a new kingdom as *Chromista* or *Stramenopila* (1). There are several unique characteristics of oomycetes that justify a reclassification. The attributes include: cellulose and chitin in the cell wall of the mycelium, a life cycle that is mostly spent as diploid, asexual sporangia (21), and zoospores. The zoospores contain two flagella, which generate movement. A reniform or kidney shape is distinctive of zoospores. The

nature of zoospores favors free water for movement. The flagellum can be lost and the spores can become cysts, and carry on a different path of the life cycle.

One of the most widely known *Oomycota* potato diseases is late blight of potato caused by *Phytophthora infestans.* Late blight was the scourge that caused the Irish potato famine in the1840's. The genus *Phytophthora* contains multiple pathogens detrimental to potato. *Phytophthora* is of Greek origin meaning, *Phyto*-plant *phthora*-destroyer. *Phytophthora* pathogens are non-obligate parasites, and frequently infect hosts that are higher plants (21), namely many agricultural and ornamental plants (49). Plants that are affected by *Phytophthora* pathogens will typically show symptoms of drought and starvation. Rots will symptomatically occur in the roots and lower stems (1). The ability to infect sub soil portions of the plant is exacerbated by the motile nature of zoospores.

Modes of infection

Zoospores will move toward chemical signals given off by the plant, this attraction is called chemotaxis. The stem base, stolon, growing tuber, and root can be infected by means of mycelium or zoospores: whereas the leaf and above ground stem are not infected by mycelium or zoospores (31). All of the tissue on the tuber including: lenticels, eyes, and periderm (31) could be infected by either a zoospore or mycelium inoculum source.

The defense response that occurs in reaction to the pathogen is different depending on the type and age of host tissue (40), hence the resistance of infection by above ground tissue to external application of inoculum. Above ground tissue is abundant in glycoalkaloids which have anti-fungal properties, whereas roots and tubers

have very low concentrations. The defense can also be influenced by the type of inoculum; it was found that zoospores infected faster than mycelial suspension (66). In addition to the sporangia and oospores, which can subsist in the soil for several years (68), infection can be spread by mycelium from tuber to tuber or plant organ to plant organ, infecting daughter tubers (31).

It was found that if a mother tuber was infected with pink rot, the disease has the capability to travel to the stem base, then to other roots and down stolons (31). Field infections were commonly found to initiate at the attachment of the stolon, whereas in storage the starting point originated from wounds, eyes, and lenticels (3).

How *P. erythroseptica* infects potato tissue is not exactly clear. Many fungal pathogens have polygalacturonase (15), which is an enzyme specific for degradation of cell wall and membranes. The maceration of tissue facilitates infection past physical barriers and absorption of nutrients. The mode and ability to infect the host is heavily connected to environmental factors such as moisture, temperature, plant nutrition, and inoculum density.

Factors of infection

Infection is based upon four key factors: environment, host, pathogen, and time. The relationship and ratio of these factors will determine the frequency and severity of disease. The pathogen may vary in concentration and pathogenicity, while the host may be in a dense monoculture or mixed with susceptible and resistant varieties. Environmental conditions are perhaps the most variable across time and difficult to control. Key environmental factors can include: weather, field conditions, and cultural practices.

Environmental factors can alter the state of a plant and allow more optimal settings for a pathogen to infect a host. The optimum temperature for tuber rot to take place is 25ºC (77ºF) (28). Tubers were rarely infected when the temperature was below 10ºC (50ºF) or above 30ºC (86ºF) (13). Soils that were warm and wet late in the growing season were highly associated with heavy infestations of the disease.

Moisture is critical for planting, harvest, and post harvest considerations. Areas of fields where soil is over watered, poorly drained, or has a high water holding capacity are prime locations for heavy infection. A considerable increase in the proportion and severity of infection occurred as soil moisture was raised (22). The moisture facilitates the development and movement of inoculum towards the host via zoospores. Moisture is also a key factor in storage loss due to high humidity.

In storage facilities, the humidity is kept high to avoid water loss in tubers (3), but it creates a favorable environment for the development of sporangia and zoospores. Other storage conditions like circulated air and stacked piles of tubers escalate the amount of infection by keeping inoculum densities high and more easily allowing for the pathogen to spread. It has been proposed that infection and loss during storage is aggravated by pre-harvest conditions (5).

Pieces of infected tubers that remain in storage facilities can stick to healthy tubers and act as an inoculum source. The harvesting process can also wound potatoes making it easier for infection to occur (54). This process was replicated in the lab by wounding potatoes with abrasions or cuts. Different types of inoculum such as plugs of culture, infected potatoes, and mycelial suspension were also evaluated. It was found that regardless of the type of inoculum, the occurrence of infection dramatically increased

under any form of wounding. Further trials dealing with both wounding and temperature, indicated that when temperatures were above 15ºC, infection was significantly higher (54). Potatoes are usually harvested when temperatures are between 15-20ºC. If harvested when temperatures are below 15ºC or if tubers could be immediately chilled, the occurrence of further infection and crop loss would be reduced.

Wounding and condition of the skin can also be impacted by fertilizer application. When fertilizer was applied by means of the drill method, there was no increase in the number of infected tubers per plant, but more plants had infected tubers (30). The theory could be that chemical damage from the drill to the roots and tubers facilitates pathogen infection. Additional trends include tuber size, age of plant and timing of harvesting.

Boyd (7) noticed that large tubers were more likely to be infected, which is a reverse trend for tubers infected with *P. infestans* (late blight). Older plants became infected more quickly when introduced to the pathogen than younger plants (66). The increase in root mass and volume could be the reason why older and early maturing varieties were more easily infected. Storage losses appeared to be more drastic when the potatoes were lifted earlier in the growing season (22). The problem with early lifting may be due to unnoticed symptoms of infection, which allow the disease to develop and spread in storage. Late season maturity was also associated with less disease development compared to mid and early season maturing varieties (40). Understanding these factors aids in understanding how the pathogen is overcoming defense mechanisms and how to best apply appropriate control methods.

Disease control

Methods used to prevent and control pink rot are of paramount concern because no varieties grown in United States or Canada have complete resistance to the disease. The degree of susceptibility to infection and decay vary dramatically among cultivars (41). Potatoes with thin skins such as red potatoes, were found to be the most susceptible, but thicker skinned varieties such as Norland, Shepody (41), Russet Norkotah, Snowden (53), and Warba (22) are highly susceptible.

The most resistant cultivars include: Butte (41), Irish Cobbler, Kasoata (22), and Russet Burbank (40). Since cultivars have not been established with complete resistance, other methods and management strategies are taken in order to prevent and minimize disease outbreak.

Chemical control is often a primary method of disease prevention. A difficulty with chemical schemes and pink rot, is that infection occurs below soil level in the roots, stolons, and tubers. In-furrow or systemic fungicide applications are therefore the main delivery systems. The situation is further complicated due to limited systemic fungicides which are effective at curtailing *Phytophthora* (36). Mefenoxam is the active ingredient in the primary systemic fungicides (Ridomil gold EC and Ul traflourish EC) which are able to control pink rot (60). The action for mefenoxam is through disrupting the fungal ribosomal RNA polymerase (60) which is a single location of control and tends to be easily overcome or resistant.

A second path to control pink rot is through resistance genes. Disease resistance can often be found in land races, ancestral plants, or related plants. Candidate genes from

such sources can be screened and identified through techniques such as breeding or microarrays and introgressed or transformed into commercial lines. Modifying crops genetically with new cationic peptides such as Temporin A, are being researched as alternative methods (39).

A third approach is land and crop management. Significant reductions in the occurrence of outbreaks have been noted when at least a single crop rotation was used (42). Crop rotation is dependent upon the ability of the host not to provide a green bridge to the pathogen. Non-*Solanacea* plants that are common in potato growing regions include alfalfa, sugar beets, corn, beans and wheat (41). Proper land management is critical to avoid spread of propagules which can remain from residual plant material and in the soil.

Special attention also must be given to watering methods. Often excess water can occur near pivots, low lying spots, and from improper watering. Avoiding standing water can prevent ideal environments for infection. An abundance of water fosters the creation of inoculum and also leaching of key cations such as calcium.

Calcium

Cations such as Ca, Mg, and K have been shown to be necessary for normal physiological functions such as: growth, reproduction, fruiting, and plant defense. Calcium is likely to be the most important element behind nitrogen for regulating plant defense (18). Ca is one of the most abundant elements in the earth and is critical in cell membrane and wall composition and as a free ion in signaling. In horse beans and yellow lupines up to 60% of the total Ca was found in association with the cell wall and 7% with

membranes (50). A deficiency in Ca can cause a loss of structural rigidity and allow polygalacturonase to digest cell constituents.

The concentration of calcium is highest in the leaves and other transpiring tissue. The movement of calcium is heavily regulated by movement of water. Once deposited in plant tissue, there is minimal relocation of the ion. Calcium that is delivered to the tuber is mostly delivered via small roots on the stolon and tuber (29). It is key that sufficient levels are made available to the tuber.

The ability of calcium to be delivered to the plant is dependent upon several factors. Soils which are sandy and have low cation exchange capacity (CEC) but adequate amounts of Ca, often produce plants deficient in Ca because the cation is in an immobilized form (32). Soil pH can also restrict nutrient availability. Amendments such as lime, gypsum, $CaCl_2$, $CaNO_3$, and Ca_2SO_4 can add more calcium and have been proven effective in reducing incidence and severity of diseases such as: Clubroot of crucifers with lime (38), *Phytophthora cinnamomi* with gypsum (33), and Cephalosporium stripe of wheat by liming (37).

The mode of action for calcium preventing disease has several possible mechanisms and is dependent upon the pathogen and host relationship. Methods for calcium preventing infection include: increased water movement through soil, strengthened physical barriers such as the periderm and cell walls, and inhibition of pathogen function.

With some soils such as clay, addition of calcium can help flocculate the particles and generate pores so that water can move through the soil better (34). Stagnant water has been associated as a factor favoring zoospore development and motility. The pores

decrease standing water and make the movement of zoospores towards chemical signals from hosts more difficult. The chemical signal to which zoospores respond is an accumulation of compounds such as aspartic acid and glutamic acid (24).

The attraction of zoospores to chemical signals is chemotaxis. This process can be thwarted by altering the function and behavior of fungal structures through high levels of extracellular Ca (33). The normal spiral motion of pythium zoospores is changed to swimming in circles and cannot react to chemotaxis when in the presence of an ionic Ca solution (19). It has been demonstrated that Ca can alter and even stop zoospore motility (10). *Phytophthora cinnamomi* zoospores encyst quickly when introduced to high Ca solutions (10). The production and release of zoospore in *Phytophthora* root rot of citrus was reduced when Ca-oxide and Ca-propionate were introduced (14). Growth of *Botrytis cinerea* was significantly slowed when grown in high concentrations of Ca (15). A reduction in the amount and effectiveness of inoculum can lower incidence. If the environment or pathogen cannot be altered by excess Ca, the host can be strengthened.

Cell walls and membranes form physical barriers, thus increasing calcium constituents in cell walls aid in defense because of the ability to hinder the activity of polygalaturonase (PG), which degrades wall and membrane components. It was found that addition of $CaCl_2$ reduced the effectiveness of PG activity on Ca pectate in cell walls (47). A higher amount of Ca allows a host to allocate more to the middle lamella and causing the wall to be more resistant to degradation. The ability of Ca to be effective against PG is due to its divalent cation characteristics. Calcium can bind to two galacturoneates (15). The chains of polygalacturonic residues have niches because of

rhamnose, and allow for cations to bind (46). The binding of cations prevents enzymes from the pathogen to soften or decay the cell wall.

Ca can also increase physical barriers by making the skin thicker and more resistant to bruising. Ca has been found to help with an increase in netting, which adds more layers to the periderm (64). A proper skin set will help prevent bruising and wounding of the tuber impeding spread of the disease during and post harvest.

The ability of Ca to invoke changes through various mechanisms makes it an ideal candidate to control disease. A difficulty with Ca is the form in which it is applied. Depending upon the disease, the host and soil type, the form applied can have affects on the ability to be taken up. Calcium Nitrate is a frequent amendment in addition with lime which also increases soil pH.

pH

The effects of Ca and soil pH are often hard to separate due to low pH often being attributed to a depletion of the Ca cation (23). The sole effect of pH can be difficult for a few reasons. The availability of elements for uptake is influenced by the pH of a soil and both host and pathogen have optimal pH ranges for growth. Compounding these two factors, pH becomes a significant factor affecting disease. The role of pH in infection is dependent upon the host/pathogen relationship. The frequency of potato scab is significantly reduced when soil pH is lowered to 5.2-5.0 (4). Contrastingly, clubroot of crucifers was reduced when the pH was raised above 7.2 (38). Hydroponically controlled nutrient environments have allowed for pH and Ca to be managed individually. The effects of specific variables can be analyzed through new genetic techniques based upon detection of the pathogen.

Detection

Early detection of pathogens is critical in order to prevent disease spread and economic crop losses. Symptoms of an unhealthy plant can be evidence of a disease, but not indicative of the pathogen. Many symptoms overlap between various pathogens. Several *Phytophthora* species can produce a pink color of inner tissue (25). Specific treatments are often required to manage particular disease. In addition to similar traits, visual detection of symptoms is complicated by their mildness or lack of time to expression.

Morphology and growth characteristics of the pathogen *in vitro* is often used to confirm the specific identity of diseases, but this method is time consuming (49), complicated by contamination, and isolation of multiple pathogens. Culturing out multiple pathogens has led to improper identification of pink rot (26). Visual identification of diseases can be subtle and difficult (62) therefore a more reliable approach is needed.

Reproducible, quick and sensitive methods would allow for accurate detection and evaluation of infection and disease severity. Identifying the pathogen load would help dictate the timing of planting and harvesting and use of infected tubers. The ability to detect a pathogen is critical to avoid spread and contamination.

Polymerase Chain Reaction (PCR), has become a principal method over traditional plating to detect the presence of pathogens (62). PCR has the ability to detect the presence of a pathogen in lower concentrations than via culturing on media. PCR reduces time constraints from weeks to hours. The ability to detect the pathogen through PCR is based upon amplifying a specific DNA sequence by means of specific designed

primers. The DNA is subjected to repeat cycles of varying temperatures in which the strands are denatured, primers anneal, replication of target sequence, and re-annealing of homologous strands. The amplified sequence can be confirmed through sequencing or gel electrophoresis. Newer methods of PCR have refined the amplification and detection process.

Through quantitative Real-Time PCR (q RT-PCR), quantification of the pathogen, and an increase in sensitivity is possible (61). A single copy gene serves as the target and specific primer source. A single copy gene alleviates the concern of age, stage of growth, or variation between isolates, which is often the case with rDNA genes (49). The single gene must be constitutively expressed. The continuous production of a product makes quantification possible because the starting amount can be deduced from the final product level.

This technology can be applied to plant pathology and assess disease severity. By creating a ratio between the amount of host DNA to pathogen DNA an infection coefficient (65) can be created which normalizes each sample and allows for comparison between other samples. The infection coefficient is created by using ct values. Each sample has a ct value assigned, the moment DNA passes a minimum threshold. More initial DNA would relate to a lower ct value because less time would be needed to reach the threshold. The method described by Valsesia et al. (65) has been applied to *Phytophthora* species by Attallah et al. (3) and found to be very effective in detecting pathogens.

Objective

Limited studies have been conducted regarding the role of environmental factors on pink rot infection in potatoes, and an emphasis on control methods is critical since strains of *P. erythroseptica* have been identified with resistance to mefenoxam. Resistant strains were identified in Idaho, and there has been an increase in the number of resistant strains in other states (60). Observations from growers and field consultants indicate that pH was associated with localized disease outbreaks (35).

Therefore, the purpose of this study is to test the effect of pH and Ca influence on *P. erythroseptica* infection in potato roots. Since pH problems are often correlated with Ca deficiencies it would be good to determine if pink rot development is at all pH related or if Ca alone has some influence on disease infection levels. Ca was also tested as a possible method to curtail infection by means of an amendment. Each variable can be analyzed separately through nutrient solution and hydroponics. Treatment levels of pH and Ca were 5, 6, 7, and 8, and 3, 86, 172, and 343 mg L^{-1} Ca, respectively.

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Appendix B

HYDROPONIC NUTRIENT MEDIUM SOLUTIONS

Table 1. Adjusted Steinbergs pre-treatment solution for initiation and growth of Russet Norkotah cuttings with reduced Ca content.

Steinbergs pretreatment			
Chemical	M.W.	gm L^{-1}	mMol
$FeCl_3$	162.21	162.21	0.65
KNO_3	101.10	101.10	4.50
$MgSO_4 \cdot 7H_2O$	246.50	246.50	1.25
Solution A			1.50
$Ca(NO_3)_2 \cdot 4H_2O$	236.15	300.00	
Mg(NO3)2·6H2O	256.41	70.00	
NH_4NO_3	80.04	18.75	
Solution B			1.20
KH_2PO_4	174.18	75.00	
$(NH_4)_2SO_4$	132.14	17.50	
Solution C			1.60
MnCl2·4H2O	197.91	2.34	
H3BO3	61.83	2.04	
ZnSO4·7H2O	287.54	0.88	
CuSO4·5H2O	249.54	0.20	
Na2MoO4·2H2O	241.95	0.13	
Solution K			4.90
KNO_3	101.11	24.60	
KCl	74.56	18.60	
K_2SO_4	174.27	44	
HEDTA			0.46

[a]Volume is based upon a 14 L hydroponic tank.

Table 2. Hoagland treatment solution with varied concentrations of Ca.

Hoagland			
Chemical	M.W.	gm L^{-1}	ml added[a]
$Ca(NO_3)_2 \cdot 4H_2O$	236.20	236.20	1[b]
KNO_3	101.10	101.10	45.0
$FeCl_3$	162.21	162.21	2.1
KH_2PO_4	136.10	136.10	3.5
$MgSO_4 \cdot 7H_2O$	246.50	246.50	5.0
Micronutrient Sol			8.0
$MnCl_2 \cdot 4H_2O$	197.91	2.34	
H_3BO_3	61.83	2.04	
$ZnSO_4 \cdot 7H_2O$	287.54	0.88	
$CuSO_4 \cdot 5H_2O$	249.54	0.20	
$Na_2MoO_4 \cdot 2H_2O$	241.95	0.13	
MES	195.24	195.24	36.4
HEDTA	344.21	344.21	2.1

[a]Volume is based upon a 14 L hydroponic tank.
[b]Volume used to obtain 2.86 mg L. The other treatment levels 86, 172, 343 mg L^{-1} were 30, 60, and 120 ml of $Ca(NO_3)_2 \cdot 4H_2O$ respectively.

Table 3. Complete hydroponic nutrient growth solution for Russet Norkotah potatoes.

Hoagland			
	M.W.	gm L^{-1}	ml added[a]
$Ca(NO_3)_2 \cdot 4H_2O$	236.20	236.20	30.0
KNO_3	101.10	101.10	30.0
$FeCl_3$	162.21	162.21	2.1
KH_2PO_4	136.10	136.10	3.5
$MgSO_4 \cdot 7H_2O$	246.50	246.50	5.0
Micronutrient Sol			8.0
MnCl2·4H2O	197.91	2.34	
H3BO3	61.83	2.04	
ZnSO4·7H2O	287.54	0.88	
CuSO4·5H2O	249.54	0.20	
Na2MoO4·2H2O	241.95	0.13	
MES			36.4
HEDTA			2.1

[a]Volume based upon a 14L hydroponic tank.