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# Evaluation of Downy Mildew (Peronospora farinosa f.sp. chenopodii) Resistance among Quinoa Genotypes and Investigation of P. farinosa Growth using Scanning Electron Microscopy

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EVALUATION OF DOWNY MILDEW (PERONOSPORA FARINOSA F.SP.

CHENOPODII) RESISTANCE AMONG QUINOA GENOTYPES AND

INVESTIGATION OF P. FARINOSA GROWTH USING

SCANNING ELECTRON MICROSCOPY

by

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GRADUATE COMMITTEE APPROVAL

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This thesis has been read by each member of the following graduate committee and by majority vote has been found to be satisfactory.

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ABSTRACT

EVALUATION OF DOWNY MILDEW (*PERONOSPORA FARINOSA* F.SP.

*CHENOPODII*) RESISTANCE AMONG QUINOA GENOTYPES AND

INVESTIGATION OF *P. FARINOSA* GROWTH USING

SCANNING ELECTRON MICROSCOPY

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Quinoa (*Chenopodium quinoa* Willd.) is a pseudocereal native to the Andean region

of South America and a staple crop for subsistence farmers in the altiplano of Bolivia and

Peru.  Downy mildew is the most significant disease of quinoa caused by the pathogen

*Peronospora farinosa* f.sp. *chenopodii* Byford.  This disease greatly impacts quinoa crops

with yield losses up to 99%.  As fungicides are expensive for farmers, the development of

resistant cultivars appears to be the most efficient means for controlling downy mildew.  The

quinoa germplasm bank contains high amounts of genetic diversity, some of which exhibit

mildew resistance.  Methods for evaluating mildew severity are important for finding

resistant genotypes that are useful in breeding programs.  The main objectives of this study

were to evaluate and investigate downy mildew resistance in quinoa through several different methods. A simple inoculation method was developed for downy mildew disease assessment by placing a damp piece of cheesecloth on a leaf, pipetting a known spore solution onto the cloth, and subjecting the plants to specific humidity cycles in a growth chamber. After inoculation of five quinoa-breeding lines in a growth chamber, accession 0654 was found to be the most resistant, while genotypes NL6 and Sayana showed moderate resistance. Each of these genotypes displayed some potential for resistance breeding programs. Investigation of the growth and development of *P. farinosa* through resistant and susceptible quinoa genotypes revealed fewer sporangiophores, hyphal strands, and haustoria among leaf tissues of accession 0654 than in the susceptible Chucapaca cultivar. *Peronospora farinosa* growth was detected in leaf, petiole, and stem tissues with polymerase chain reaction (PCR) using ITSP primers designed from the internal transcribed spacer (ITS) region of the pathogen. Scanning electron microscopy (SEM) also revealed that *P. farinosa* penetrated stomata via appressoria, secreted extracellular matrices during sporangia germination, grew intercellularly in leaf and petiole tissues, and exited leaf tissue through stomata. Future research requiring knowledge of resistant quinoa genotypes, *P. farinosa* growth and development, or inoculation methods for large numbers of small quinoa plants would benefit from this report.

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**(*Chenopodium quinoa*) by in-vitro inoculation**

**Abstract**

Downy mildew caused by the pathogen *Peronospora farinosa* f.sp. *chenopoii*, is the most significant disease of quinoa (*Chenopodium quinoa*), a nutritious grain crop from the Andean altiplano, sometimes causing 99% yield loss. The inoculation of quinoa plants for resistance studies in the laboratory has proven difficult because *P. farinosa* is an obligate parasite that must have living host tissue to grow and reproduce. Therefore, a simple inoculation method was developed by placing a damp piece of cheesecloth on a leaf, pipetting a known spore solution onto the cloth, and subjecting the plants to specific humidity cycles in a growth chamber. Future research requiring inoculation of large numbers of small plants, or the simple maintenance of the pathogen on living plants will benefit from this procedure.

**Introduction**

Quinoa (*Chenopodium quinoa* Willd.) is a nutritious pseudocereal adapted to the cool temperatures and poor soil conditions of the Andean region of South America. It is also becoming a desirable crop worldwide with reports of growth in Europe, Africa, North America (3, 9), and India (4). Downy mildew is the most significant disease of quinoa caused by the pathogen *Peronospora farinosa* f.sp. *chenopodii* Byford. Yield losses are reported at 33 to 58% in resistant cultivars, and up to 99%, in some of the more susceptible cultivars (2). Disease symptoms include sporulation on the underside of the leaves, chlorosis, necrosis, and defoliation.

As an oomycete and obligate biotroph, *P. farinosa* can be difficult to manage in a laboratory as it must be maintained on living host tissue. A current method that seems to work well for maintaining *P. farinsa* isolates includes weekly transfers of infected quinoa leaves onto healthy leaves in Petri dishes with water agar (1). However, there are few reports on the inoculation of quinoa plants with *P. farinosa* (1,5). A protocol describing the procedures for inoculating quinoa would be beneficial for disease severity analyses in the laboratory, and as a simple way to maintain the pathogen. Therefore, the purpose of this paper is to describe a successful protocol for in-vitro inoculation of quinoa plants with *P. farinosa*.

**Materials and Methods**

**Plant materials**

Quinoa seeds of the cultivar Sayana were kindly provided by the PROINPA (Promotion and Investigation of Andean Products) Foundation of Bolivia. Sayana is a

susceptible cultivar commonly grown in Bolivia. Seeds were planted in 10-cm pots with commercial potting soil and thinned to three seedlings per pot after one week. Plants were grown in a greenhouse at 25-27°C. Supplemental lighting from sodium halogen lamps was used to generate a 12-h photoperiod.

**Inoculation**

*Peronospora farinosa* isolate 14B was collected from a leaf of the cultivar Sayana from a naturally infected field in Bolivia in 2005 and brought back to Brigham Young University on a Petri dish of water agar. The isolate was maintained on quinoa leaves on water agar plates in a growth chamber at 20°C with lights on and 16°C with lights off in a 12-h photoperiod (1). Sporulating leaves were transferred onto healthy quinoa leaves every seven days.

Sporangia used to make the inoculum were produced by placing ten pots of four-week-old Sayana plants in a growth chamber at 16°C with lights on and 10°C with lights off in a 12-h photoperiod. Infection was generated by placing sporulating leaves from isolate 14B onto a leaf of each Sayana plant. Humidity was increased to >95% for 24 h on day one (5,7), reduced to 60 to 70% on days two to five, increased to >95% for 24 h on day six, and reduced to 60 to 70% on days seven to 12.

Sporangia were harvested for inoculum when heavy sporulation was observed on the leaves (10-12 days after inoculation). A sporangia solution was made by placing sporulating leaves in a 50-ml conical tube filled with 40 ml of sterile deionzed water. The tube was gently shaken to remove sporangia. The solution was strained through cheesecloth and adjusted to a concentration of $4 \times 10^5$ sporangia/ml with a hemacytometer. A drop of Tween

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20 (Sigma-Aldrich, St. Louis, MO, USA) was added to the 40-ml inoculum concentration to prevent the sporangia from clustering.

Three trials were inoculated using different humidity cycles.  The first trial tested nine quinoa plants with humidity at >90% for 10 days.  The second trial tested nine plants with humidity cycles at >95% for 24 h on day one, 60 to 70% on days two to five, >95% for 24 h on day six, and 60 to 70% on days seven to 10.  The third trail tested 450 plants with the same humidity cycles as trial two, but the last humidity cycle was maintained through day 16.  Plants in all three trials were inoculated by placing a damp, sterile 1 cm$^2$ piece of cheesecloth on a single leaf of each plant.  Sporangia solution of 30 µl was pipetted onto the cheesecloth pieces.  Inoculated plants were placed in a growth chamber at 16°C with lights on and 10°C with lights off in a 12-h photoperiod.  Humidity was measured with a Hobo H8 Pro Series sensor (Onset Computer Corporation, Pocasset, MA, USA).


**Results**

Disease symptoms and sporulation occurred in plants in the second and third trials, but not in the first.  Sporangia lesions were visible on one or two leaves about 10 days after the inoculation date.  The infection progressed each day as more leaves sporulated and the sporangia density increased.  Dew was present on the plants for all 10 days in the first trial.  In trials two and three, dew dried off when humidity was decreased to 60 to 70%.  It was also observed that plants inoculated with a small sporulating leaf for inoculum production tended to sporulate earlier (on day seven) and with more severe infections than plants inoculated with the cheesecloth method (on days 10 to 12).

**Discussion**

Previous studies report that high humidity levels are important for *Peronospora* germination and sporulation (1,5,6,7,8). However, we found that low humidity levels (60 to 70%) were also important for mildew development. The fact that plants in trials two and three sporulated, and those in trial one did not, suggests that a wet period followed by a dry period is important for sporulation. The lower humidity levels on days two through five, in trials two and three, may also be important for host colonization. Dew was consistently present on the leaves in trial one, which could have limited host colonization. However, we cannot tell from this study if a dry period on days two through five is critical for *P. farinosa* development. Further research is needed to determine this.

Another successful method used in previous reports (1,5,8) of controlled *Peronospora* inoculations is to spray the plants with the sporangia solution using compressed air. This method may generate better infections because the sporangia are distributed across multiple leaves. However, it was difficult to apply in our research due to the large number plants we wanted to inoculate. Spraying plants with inoculum requires large quantities of sporangia solution and immediate placement of the plants in >95% humidity to prevent the sporangia from drying out, which was not feasible in our study.

The cheesecloth method we described was beneficial for testing large quantities of plants. We first tested a small number of plants in trials one and two. When trial two showed successful infections, the method was applied to a larger quantity of plants. The cheesecloth method required small amounts of inoculum, a limiting factor when working with an obligate parasite. It allowed the sporangia to stay moist for longer periods, and prevented the inoculum from rolling off the leaf. It was also observed that infections with

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the cheesecloth method occurred later and were not always as severe as those produced by placing a small sporulating leaf on the plant. Nonetheless having a quantifiable number of sporangia in the inoculum for disease assessment studies favors the cheesecloth method.

We report the described protocol as a successful method for growth chamber inoculations with *P. farinosa* and possibly with other *Peronospora* sp. It is an ideal method for inoculating large quantities of small quinoa plants because it requires only small amounts of sporangia solution and keeps the sporangia moist until they can be transferred to a humidity chamber. It is also useful for studies requiring inoculation of a single leaf. This protocol may aid in future resistance studies among genotypes and virulence studies among isolates.

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**Chapter 2**

**Evaluation of five quinoa-breeding lines for downy mildew**

(*Peronospora farinosa* **f.sp.** *chenopodii*) **resistance**

**Abstract**

Quinoa (*Chenopodium quinoa*) is a staple grain crop among the peoples of the Andean altiplano.  The most significant disease of quinoa is downy mildew caused by the endemic pathogen *Peronospora farinosa* f.sp. *chenopodii*.  Resistant quinoa cultivars have reported yield losses of 33 to 58% while susceptible cultivars have experienced 99% yield loss due to downy mildew disease.  Resistance is an important component of quinoa breeding, but it also needs to be improved upon in order to produce resistant cultivars.  Five quinoa-breeding lines were inoculated in a growth chamber at the four-week stage with a single mildew isolate.  Each genotype was evaluated after 16 days for the incidence and severity of sporulation among the leaves.  Quinoa genotype 0654 was found to be the most resistant, while genotypes NL6 and Sayana showed moderate resistance.  These lines display the potential for incorporation into resistance breeding programs.

**Introduction**

Quinoa (*Chenopodium quinoa* Willd.) is a pseudocereal native to the Andean region of South America. It stands as a staple crop for subsistence farmers in the altiplano, or highlands, of Bolivia and Peru (18) and is also distributed throughout regions of Ecuador, Colombia, Chile, and Argentina (2). A high nutritional content, drought tolerance, and ability to grow in saline soils have made it a desirable crop worldwide with reports of growth in Europe, Africa, North America (9,16) and India (11).

The most significant disease affecting quinoa is downy mildew caused by the pathogen *Peronospora farinosa* f.sp. *chenopodii* Byford. Downy mildew attacks the foliage of the plant causing yellowing or reddening of the leaves, depending on the genotype, and eventually defoliation. Soft, grey patches of sporangia usually emerge on the underside of the leaves acting as the primary source of inoculum, which is spread by wind and rain. The disease is endemic throughout regions of Bolivia, Chile, Colombia, Ecuador, and Peru (7), and has also been reported in Denmark (3), Canada (16), and India (11). In order to control mildew, farmers have traditionally used fungicides such as metalaxyl (6). However, they are expensive, requiring multiple applications, and may eventually be overcome by resistant isolates, as the pathogen is sexually recombinant (1,6) showing high levels of genetic diversity among mildew populations of Bolivia (15) and Ecuador (12). The development of resistant cultivars appears to be the most efficient means for controlling the disease. Large amounts of genetic diversity are prevalent in quinoa with ecotypes exhibiting varying degrees of mildew resistance. For example, valley ecotypes growing in regions where humidity is high and the disease is rampant, often display high to moderate mildew resistance, whereas southern altiplano ecotypes growing in drier regions show more susceptibility (2).

Methods for evaluating mildew severity are important for finding resistant genotypes useful in breeding programs. Field evaluations can sometimes be contradictory, as mildew prevalence changes from year to year depending on the temperature and humidity levels, and virulence of mildew isolates changes among locations (4). Additionally, observed resistance in some early maturing genotypes is inconsistent. Often times, they will mature before the mildew becomes rampant, escaping infection and appearing to be resistant (4). As environmental factors have varying affects on field results, the evaluation of mildew severity under controlled laboratory conditions would be beneficial (4). Inoculation methods for laboratory evaluations have been developed for quinoa seedlings with *P. farinosa* (5,12), pea (*Pisum sativum* L.) seedlings with *P. viciae* (Berk.) Casp. f.sp. *pisi* (Sydow) (14), and Portuguese cole (*Brassica oleracea* L.) landrace seedlings with *P. parasitica* (Pers. Ex. Fr.) Fr. (13). Few studies, in general, have reported resistant genotypes in quinoa. The evaluation of small plants in the laboratory would provide results that can be compared to field trials, but without the varying affects of environmental factors.

Mildew resistance is an important characteristic that would benefit quinoa farmers if incorporated into a breeding program. The quinoa germplasm bank contains high amounts of genetic diversity, some of which have mildew resistance that may benefit quinoa breeding. The objective of this study was to evaluate five quinoa genotypes for mildew resistance under laboratory conditions.

**Materials and Methods**

**Plant materials**

Seed for five quinoa-breeding lines, Chucapaca, KU2, NL6, 0654, and Sayana, was kindly provided by the PROINPA (Promotion and Investigation of Andean Products) Foundation of Bolivia. Chucapaca is a late maturing cultivar from the Bolivian altiplano. KU2 is an early maturing breeding line from the coast of Chile. NL6, originating from Chilean lowlands, is also an early maturing breeding line. Germplasm bank accession 0654 originates from the Peruvian valley region and is late maturing. Sayana, a commonly grown Bolivian cultivar originating from the altiplano, was used as a control. Seeds for each genotype were planted in 10-cm pots with commercial potting soil, and thinned to three seedlings per pot after one week. Plants were grown in greenhouse conditions at 25-27°C. Supplemental lighting from sodium halogen lamps was used to generate a 12-h photoperiod.

**Inoculation**

*Peronospora farinosa* isolate 14B was collected from a leaf of the cultivar Sayana from a naturally infected field in Bolivia in 2005 and brought back to Brigham Young University on a Petri dish of water agar. The isolate was maintained on quinoa leaves on water agar plates in a growth chamber at 20°C with lights on and 16°C with lights off in a 12-h photoperiod (4). Sporulating leaves were transferred onto healthy quinoa leaves every seven days.

In order to generate enough sporangia for inoculation purposes, the isolate was transferred to small plants. Fifteen 10-cm pots containing four-week-old Sayana plants were placed in a growth chamber at 16°C with lights on and 10°C with lights off in a 12-h photoperiod. An infected sporulating leaf from isolate 14B was placed directly onto a leaf of

each Sayana plant.  Humidity was increased to >95% for 24 h on day one (12,13), reduced to 60 to 70% on days two to five, increased to >95% for 24 h on day six, and reduced to 60 to 70% on days seven to 12.  Humidity was measured with a Hobo H8 Pro Series sensor (Onset Computer Corporation, Pocasset, MA, USA).

The sporangia solution was prepared the same day the inoculations were made. Sporulating leaves were placed in a 50-ml conical tube filled with 40 ml of sterile deionzed water and gently shaken to remove the sporangia.  The solution was strained through cheesecloth and adjusted to a concentration of $4 \times 10^5$ sporangia/ml using a hemacytometer. A drop of Tween 20 (Sigma-Aldrich, St. Louis, MO, USA) was added to the 40 ml of solution to prevent the sporangia from clumping.

Thirty-six pots of the most uniform plants were selected from each genotype three weeks after the planting date.  Plants were randomized into a complete block design with six plants from each genotype in each of six blocks.  The plants were inoculated with mildew by placing a damp piece of cheesecloth ($1cm^2$) on a single leaf of each plant.  Sporangia solution of 30 µl was then pipetted onto the cheesecloth pieces.  The plants were inoculated in small batches and placed in a growth chamber while the cheesecloth was still moist.  Growth chamber conditions were maintained at 16°C with lights on and 10°C with lights off in a 12-h photoperiod.  Humidity was increased to >95% for 24 h on day one, reduced to 60 to 70% on days two to five, increased to >95% for 24 h on day six, and reduced to 60 to 70% the remaining time.  All plants were evaluated 16 days after the inoculation date.  The experiment was replicated five times.

**Data analysis**

All plants were evaluated for their percentage of infection based on the number of sporulating leaves per pot and the percentage of sporulation covering each leaf. Due to the uniformity of the plants, we estimated a total of 30 leaves per pot that would be susceptible to sporulation. Data was analyzed using logistic analysis with SAS version 9.1 (SAS Institute Inc., Cary, NC, USA) PROC MIXED and GLIMMIX macro to evaluate the incidence of leaf infection among each genotype. Data was recorded as a binary response where the mildew was either present or absent from each leaf. The logistic probabilities were calculated [p = exp(model) / 1 + exp(model)] using intercept and estimate values from the model. Disease severity was also evaluated using PROC MIXED with Tukey-Kramer adjustments from the differences of least squares means. These data were recorded as the percentage of sporulation covering each leaf. All five trials were analyzed together as unbalanced data, and all statistical values were considered significant at $P < 0.05$.

**Results**

There was a significant difference among genotype means ($P = <0.0001$) from an analysis of variance (ANOVA) test when calculating the incidence of infection based on the presence or absence of leaf sporulation per pot. Logistic analysis of the same data showed 0654 as having a significantly lower probability of becoming infected than Chucapaca ($P = 0.0395$), KU2 ($P = <0.0001$), and NL6 ($P = 0.0093$). The probability of infection for Sayana was not significantly different than 0654. Overall, 0654 had the lowest probability of becoming infected at 9.8% (Figure 1). Sayana, Chucapaca, and NL6 indicated a 12.4%,

13.6%, and 15.3% probability of becoming infected respectively with KU2 indicating the highest probability of becoming infected at 20.3%.

Disease severities among the genotype means were significantly different ($P =$ <0.0001) from an ANOVA test when comparing the percentage of sporulation covering each leaf (Figure 2). Chucapaca had a significantly higher disease severity than KU2, NL6, Sayana, and 0654, where $P =$ <0.0001 for each comparison. No other means were significantly different from one another. 0654 displayed the lowest level of severity (57.8%) and Chucapaca had the highest (68.9%) (Figure 2). NL6, KU2, and Sayana had severity levels of 58.3%, 59.1%, and 60% respectively.

**Discussion**

Evaluation of downy mildew severity can be difficult to assess, thus a variety of scales and methods for disease infection in quinoa have been examined for their effectiveness and accuracy (6,8). However, a strategy for rating small plants in growth chamber conditions has not been studied. We feel that the evaluation method used in this study is appropriate for growth chamber analysis because it gives an estimate of qualitative resistance by looking at the incidence of infection, and quantitative resistance by looking at severity. Incidence is important in this study because it uses logistic analysis to estimate the probability of each genotype becoming infected based on whether or not the disease was present in each leaf, thus a determination of resistance. An analysis of severity alone would not give as accurate of a representation of the degree of resistance because of the high variability of leaf severity within each genotype, and the high percentages of severity among means of all the

genotypes.  The analysis of each leaf also captures a more accurate reading than whole plant evaluations, which is less exact because the plants were so small.

Our investigation of disease severity in quinoa tended to agree with previous field studies and observations (2,17).  Breeding line KU2 displayed the highest level of incidence, but only a moderate degree of severity compared to the other genotypes.  The degree of incidence for Chucapaca was significantly less than KU2 (Figure 1); however, it showed a greater severity of infection (Figure 2).  The high susceptibility observed in Chucapaca agreed with previous field results showing 31.7% severity, which is greater than the other genotypes in the field study (17).  The percentage of infection in our study was higher than this report, at 68.9%, which may be due to ideal pathogen conditions and high inoculum loads in the growth chamber.

Accession 0654 was significantly less susceptible than the other five genotypes.  It showed the lowest probability of becoming infected, and the lowest severity of infection (Figures 1 and 2).  Valley ecotypes and late maturing genotypes, such as 0654, often display mildew resistance (2,7,17).  However, it showed a greater degree of severity in the growth chamber than was expected.  High levels of mildew resistance have been observed in 0654 in Bolivian fields over subsequent years, although the pathogen has been known to overcome the resistance when disease pressures are high (A. Bonifacio, personal communication).  The temperatures set in the growth chamber during infection (10-16°C) may have also contributed to an impact on host-pathogen interactions in 0654.  Studies on *Bremia lactucae*, downy mildew of lettuce, showed that resistance decreases at lower temperatures around 5-10°C (10).  The environmental conditions of the growth chamber were ideal for mildew growth, and resistance previously observed in 0654 may have been overcome under these

conditions. Despite the favorable conditions for *P. farinosa*, 0654 still displayed greater resistance than the other quinoa genotypes. Resistance data from the $F_2$ (15) and $F_3$ (A. Vargas, B. Geary, and M. Stevens, unpublished) progenies of a cross between 0654 and Sayana show fairly high resistance suggesting that 0654 resistance is dominant in nature.

Danielsen et al. (7) reported 33 to 58% yield reductions in the most resistant cultivars of their study, suggesting horizontal resistance in different quinoa genotypes. Sayana, used as the control cultivar in our study, is commonly grown in Bolivia and known to be susceptible to downy mildew; however, in the growth chamber it showed greater severity than 0654 (Fig. 2), but not enough to be significantly different. It was also significantly less susceptible than Chucapaca. These results may suggest some resistance in Sayana, which was also concluded from the field study performed by Swenson (15). Breeding line NL6 was similar to Sayana in that it showed some resistance in the growth chamber and in the field (17). The possible quantitative nature of 0654, Sayana, and NL6 may be beneficial to resistance breeding programs. Horizontal resistance would be difficult for the mildew to overcome, but it would also be difficult to transfer the resistance during quinoa breeding.

Further examination of downy mildew severity in 0654, Sayana, and NL6 would help in understanding the nature of the resistance in these genotypes. Additional testing of the progeny from the 0654 x Sayana cross in the field trial and growth chamber to see how the resistance reacts under high disease pressures could also be included. Future research with resistance in the genotypes of this study would benefit breeding programs and aid in finding and characterizing resistance genes using molecular markers.

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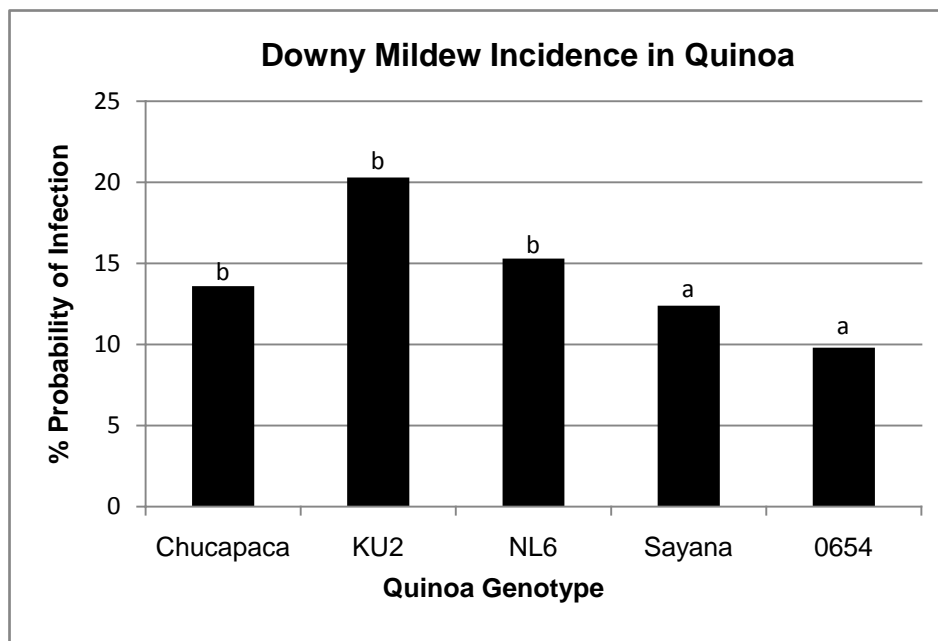
**Figure 1.** Downy mildew incidence from 30 leaves among each pot of five quinoa genotypes. Incidence was calculated as the probability of each genotype becoming infected. Means with common letters are not statistically different ($P = 0.05$).
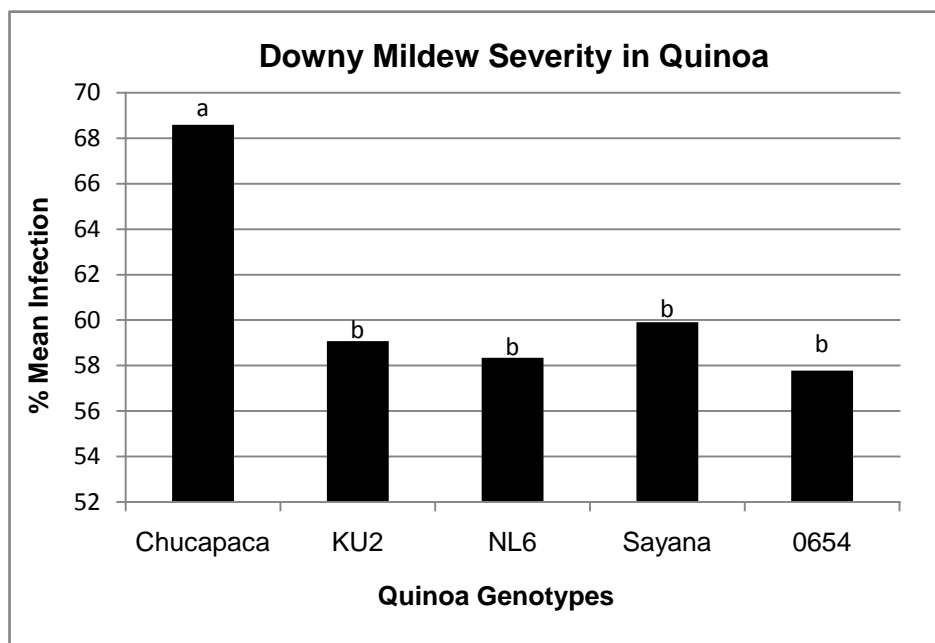
**Figure 2.** Downy mildew severity of all infected leaves from each pot of five quinoa genotypes. Severity was calculated as the percent infection of each leaf. Means with common letters are not statistically different ($P = 0.05$).

**Chapter 3**

**Recognition of infection structures and development of *Peronospora farinosa* in quinoa**

**through scanning electron microscopy and detection with PCR of the internal**

**transcribed spacer region**

**Abstract**

*Peronospora farinosa* f.sp. *chenopodii* is an oomycete that infects quinoa (*Chenopodium quinoa*) grain crops in the Andean mountains of South America.  Growth and development of the pathogen through different quinoa tissues was studied using scanning electron microscopy (SEM) and polymerase chain reaction (PCR) with primers designed from the internal transcribed spacer (ITS) region of *P. farinosa* f.sp. *chenopodii*.  SEM revealed secretion of extracellular matrices from germinating sporangia and stomatal penetration.  Colonization included the intercellular growth of hyphae and the production of haustoria in leaf and petiole tissues.  Infection within resistant and susceptible host genotypes were compared by counting the number of infection structures in different quinoa tissues.  Resistant genotype 0654 had fewer sporangiophores, hyphal strands, and haustoria among leaf tissues than did the susceptible Chucapaca genotype.  PCR detected *P. farinosa* growing in leaf, petiole, and stem tissues.  The specific PCR primer for *P. farinosa* could also be used for seed certification to help minimize the spread of this pathogen.

**Introduction**

  Quinoa (*Chenopodium quinoa* Willd.) is an important food crop of the Andean region of South America.  It is a nutritious pseudocereal adapted to the cool temperatures and poor soil conditions of the Altiplano.  Because quinoa has a seed protein content higher than most cereal grains, ranging from 12 to17% (21), subsistence farmers in Northern Argentina, Bolivia, Northern Chile, Colombia, Ecuador, and Peru rely heavily on cultivated quinoa as part of a balanced diet (3,17).  Diseases influencing quinoa production are a major concern in these areas because subsistence farmers must rely on plant resistance to minimize damage.

  Downy mildew is the most significant disease affecting quinoa crops in South America (8) with yield losses reported from 33 to 58%, and even up to 99%, in some cultivars (11).  The causal agent of this disease is the obligate parasite *Peronospora farinosa* f.sp. *chenopodii* Byford.  It is endemic to regions of Bolivia, Chile, Colombia, Ecuador, and Peru (9, 11).  Disease symptoms include dark lesions, composed of sporangia, which develop on the underside of the leaves and cause chlorosis, necrosis, and defoliation.  Infection is spread by the movement of sporangia through wind and rain, as well as by oospores that are known to remain in quinoa seeds, old leaf tissue, and in the soil (8).  Controlling mildew can be difficult.  Fungicides, such as metalaxyl, are effective (10), but expensive for subsistence farmers (9).  Therefore, the development of resistant cultivars could be the most efficient means of control.  Mildew resistance has been observed in certain quinoa genotypes, such as the Peruvian accession 0654, although few resistant cultivars with quality grain are currently available for farmers.  An understanding of the infection processes in resistant genotypes could help in selecting resistant plants for breeding programs.

Scanning electron microscopy (SEM) has been used to investigate growth processes of different true fungi.  The pathogen *Sclerotinia sclerotiorum* of rapeseed (*Brassica napus* var. *oleifera*) directly penetrates leaf cells via a single germ tube and appressorium and exits leaf tissue through stomata (16).  *Puccinia thalspeos* Schub., which infects the weed Dyer's woad (*Isatis tinctoria* L.), also penetrates leaf cells directly through a germ tube and appressorium, and hyphae inside the leaf tissue grow intercellularly (18).  Reports on the infection processes of *P. farinosa*, and oomycetes in general, are limited.  There are currently no reports of SEM studies with downy mildew of quinoa.  Investigation with methods other than SEM has shown that *P. farinosa* grows intercellularly within quinoa tissues (9).  Sporangiophores are 167-227 μm long and 11-14.8 μm in diameter, and sporangia are oval shaped and 25.7-31.9 μm long with a 19.3-24.3 μm diameter.

Little is known about how quinoa mildew infection develops, or how it reacts with resistant genotypes.  An understanding of the infection processes is important in order to better manage the disease.  The purpose of this study was to visualize growth and development of downy mildew on quinoa, and compare infection structures within four different tissues of resistant and susceptible quinoa genotypes using SEM.  Additionally, polymerase chain reaction (PCR) primers specific to the internal transcribed spacer (ITS) region of *P. farinosa* were designed and implemented in order to verify the presence of the pathogen in selected tissue sections.  A PCR-based marker for downy mildew would be useful for seed certification laboratories worldwide because it is inexpensive, easy to generate, and highly reproducible.  It would also be beneficial for identifying latent infections in host tissues and for further molecular investigations.

## Materials and Methods

### Plant materials

Quinoa seeds for the cultivar Chucapaca, and accession 0654 were kindly provided by the PROINPA (Promotion and Investigation of Andean Products) Foundation of Bolivia. Chucapaca is a commonly grown Bolivian cultivar susceptible to downy mildew (24), and 0654 is a Peruvian accession that has shown consistent mildew resistance in the field (A. Bonifacio, personal communication). Seeds for each genotype were planted in 10-cm pots with commercial potting soil and placed in a greenhouse at 25-27ºC. Supplemental lighting from sodium halogen lamps was used to generate a 12-h photoperiod. After three weeks, several plants of each genotype were selected for inoculation.

### Inoculation

*Peronospora farinosa* isolate 14B was collected from a leaf of the cultivar Sayana from a naturally infected field in Bolivia in 2005 and brought back to Brigham Young University on a Petri dish of water agar. The isolate was maintained on quinoa leaves on water agar plates in a growth chamber at 20°C with lights on and 16°C with lights off in a 12-h photoperiod (9). Sporulating leaves were transferred onto healthy quinoa leaves every seven days.

Sporangia were generated on living plants to produce sufficient inoculum for infections by placing sporulating leaves from isolate 14B onto fully expanded leaves of fifteen small susceptible plants in a growth chamber at 16°C with lights on and 10°C with lights off in a 12-h photoperiod. Humidity was increased to >95% for 24 h on day one (19,23), reduced to 60 to 70% on days two to five, increased to >95% for 24 h on day six, and reduced to 60 to 70% the remaining days. Humidity was measured with a Hobo H8 Pro

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Series sensor (Onset Computer Corporation, Pocasset, MA, USA). Sporangia were harvested 12 days after inoculation by placing sporulating leaves in a 50-ml conical tube with 25 ml of distilled deionized water and gently shaking the tube. The sporangia solution was strained through cheesecloth and adjusted to a concentration of 4 x $10^5$ sporangia/ml using a hemacytometer. A drop of Tween 20 (Sigma-Aldrich, St. Louis, MO, USA) was added to the 25 ml of solution to prevent sporangia from clustering.

Inoculation of four-week-old Chucapaca and 0654 plants was performed by placing a moist piece of cheesecloth (1 cm$^2$) on the 4$^{th}$ leaf up from the bottom of each plant. Sporangia solution of 30 µl was pipetted onto the cheesecloth pieces and plants were placed in a growth chamber at 16°C with lights on and 10°C with lights off in a 12-h photoperiod. Humidity was increased to >95% for 24 h on day one, reduced to 60 to 70% on days two to five, increased to >95% for 24 h on day six, and reduced to 60 to 70% the remaining days.

Tissue sections were collected 12 days after inoculation. Two adjacent 5-mm$^2$ tissue sections were taken from the following regions of each plant: the central part of a sporulating leaf, the petiole of a sporulating leaf, the stem between sporulating leaves, and a small non-sporulating leaf near the apical meristem. The tissue sections from each region were subjected to PCR for amplification of *P. farinosa* DNA and SEM preparation.

**Scanning electron microscopy**

Quinoa tissue samples infected with *P. farinosa* (5 mm$^2$) were fixed in 2% glutaraldehyde in 0.06 M sodium cacodylate buffer (pH 7.3), aspirated in a vacuum with a drop of Teepol liquid detergent (Harvey Waddington, Kent, UK) for 5-15 min until pieces became submerged. Samples were removed from the aspirator and refrigerated overnight.

They were then washed three times in 0.03 M sodium cacodylate buffer (pH 7.2-7.4), and dehydrated through an ethanol series of 10, 30, 50, and 70% washes.

All samples were submerged in liquid Freon and fractured with a razorblade in liquid nitrogen according to the technique developed by O'Donnell and Hooper (20). After cryofracturing, the pieces were placed directly back into 70% ethanol and rehydrated through an ethanol series of 50, 30, 10% ethanol, and three buffer washes.

Samples were post-fixed with a 1:1 solution of 2% osmium tetroxide (Electron Microscopy Sciences, Hatfield, PA, USA) in 0.03 M sodium cacodylate buffer and refrigerated overnight. The samples were next washed six times with distilled water and again dehydrated in an ethanol series of 10, 30, 50, 70, and 95% washes. Dehydration was continued with three washes of 100% ethanol and three washes of 100% acetone. All washes during sample preparation were changed at 10-min intervals. The samples were critical-point dried with liquid $CO_2$ in a Pelco™ CPD2 drier (Ted Pella, Inc., Redding, CA, USA), mounted with double-sided carbon tape on specimen stubs, and sputter-coated for conductivity. Samples were viewed under a Philips XL30 ESEM FEG (FEI, Hillsboro, OR, USA) or JSM 840a (JEOL, Tokyo, Japan).

In addition to the samples prepared as above, some mildew infected quinoa leaves were cut into 5-mm$^2$ pieces and placed directly onto a stub in the environmental scanning electron microscopy (ESEM) chamber. ESEM is a function of the microscope that allows biological samples to be visualized in a wet environment in order to observe their natural form and requires simple preparation. Samples were viewed in a low vacuum mode with a Philips XL30 ESEM FEG.

SEM was used to observe the general growth and development of *P. farinosa*, as well as observe the intensity of infection between susceptible and resistant genotypes. The number of infection structures in susceptible Chucapaca and resistant 0654 were counted in three different plant regions under specific magnification fields (leaf surface 126x, cryofractured leaf 800x, and petiole tissue 1025x). On the leaf surface, the number of stomata with emerging sporangiophores was counted. The sporangiophores themselves were usually twisted together and difficult to count individually, thus necessitating the counting of stomata with sporangiophores. In the fractured leaf and petiole tissues, the total number of hyphal strands and haustoria were also counted.

**PCR of infected tissue sections**

The presence of *P. farinosa* in the quinoa tissue sections studied with SEM was verified using PCR with ITSP primers (Table 1) as designed below. Because the tissue sections were too small to extract and isolate a sufficient quantity of mildew DNA, a protocol modified from Yang et al. (26) was used to amplify DNA directly from the tissue pieces. Each tissue section (5 mm$^2$), weighing 5-15 mg, was ground with a pestle in 100 µl of detergent-lysis solution [20 mM Tris (pH 8.0), 5 mM EDTA (pH 8.0), 400 mM NaCl, 0.30% SDS, 0.60% Tween-20] (26), incubated at 80ºC for 2 h in a GeneAmp PCR system 9700 thermocycler (Applied Biosystems, Foster City, CA, USA), and directly cooled to 4ºC. The lysates were centrifuged at 6,000 rpm for 5 min and the supernatants were reserved for amplification.

The PCR reaction mixture contained 1x Cresol Red (Sigma-Aldrich), 1x BloodDirect™ buffer 1 (Novagen, EMD Biosciences, Gibbstown, NJ, USA), 1x BloodDirect™ buffer A, 0.2 mM dNTP, 0.25 µM of each ITSP primer, 0.05 U of

JumpstartTaq™ (Sigma-Aldrich), and a 0.50 µl aliquot of the tissue supernatant in a total volume of 20 µl. All PCR reactions were amplified in a GeneAmp PCR system 9700 thermocycler at the following temperature cycles: 1 cycle at 94ºC for 5 min; 40 cycles at 94ºC for 30 s, 52ºC for 30 s, 72ºC for 1 min; and a final extension cycle at 72ºC for 7 min (24). The final PCR product was separated and visualized in a 1% agarose (GenePure LE Agarose, ISC BioExpress, Kaysville, UT, USA) gel in 0.05x TBE [4.4 mM Tris, 4.4 mM boric acid, 1.07 mM EDTA (pH 8.0)] at 90 V for 1 h, stained with ethidium bromide (5 µl/100 ml of TBE) and visualized with UV light.

**DNA isolation and ITS amplification for primer design**

Sporangia from *P. farinosa* isolates 5, 7, and 8 were collected in Lacaya, Bolivia in 2007. Sporulating quinoa leaves were placed in a 50-ml conical tube with 25 ml of sterile water and gently shaken to remove sporangia. After the sporangia settled into a pellet, DNA was extracted from them using Qiagen DNeasy Plant Mini Kit (Qiagen Sciences, MD, USA).

The full ITS region was amplified in a two-step, semi-nested PCR assay with three primers directed at conserved regions of the 18s and 5.8s rDNA genes (Figure 1). The first PCR step used primer DC6 (Table 1), designed specifically to amplify the ITS region of Peronosporales and Pythiales orders (2), and the universal primer ITS4 (25) (Figure 1). An aliquot of the mildew DNA was used as the template. In the second PCR step, universal primer ITS4 was paired with ITS5 (25). An aliquot of the PCR product from the first step was used as the DNA template.

The reaction mixtures of both steps in the semi-nested protocol contained 10x PCR buffer [100 mM Tris-HCl (pH 8.3), 500 mM KCl, 0.01% gelatin] (Sigma-Aldrich), 1x Cresol Red, 1.5 mM $MgCl_2$ (Sigma-Aldrich), 0.1 mM dNTPs, 0.25 µM of each primer, 0.05 U of

JumpstartTaq™ and 1 µl aliquot of either mildew DNA or PCR product from the first reaction

in a total volume of 20 µl.  All PCR reactions were amplified in a GeneAmp PCR system

9700 thermocycler at the following specifications: 1 cycle at 95ºC for 2 min; 30 cycles at

95ºC for 20 s, 55ºC for 25 s, 72ºC for 50 s; and a final extension cycle at 72ºC for 10 min (7).

The final PCR product from step two was separated and visualized in a 1% agarose gel in

TBE buffer at 90 V for 1 h, stained with ethidium bromide and visualized with UV light.

**ITS cloning, sequencing and primer design**

The final PCR product was purified using the Wizard® SV Gel and PCR Clean-up

System (Promega, Madison, WI, USA) and cloned using pGEM-T Easy Vector System

(Promega).  Recombinant cells were spread on plates of Luria-Bertani (LB) media containing

ampicilin (0.025g/l).  Forty µl of an X-Gal and IPTG mixture (Research Products

International Corp., Mt. Prospect, IL, USA) were spread on each LB media plate.  Plates

were incubated overnight at 37ºC.  White bacterial colonies were selected and re-grown by

streaking in a line on new LB media plates in order to make a sufficient amount of colony to

check for the insert with PCR and to grow in LB broth.  Selected colonies were subjected to

PCR amplification, by dipping a sterile toothpick in the colony and then swirling it in a PCR

reaction containing M13 primers.  The PCR reactions were subjected to electrophoresis.

Colonies showing strong bands were selected and grown in 3 ml of LB broth with ampicilin

(0.025g/l) and incubated overnight at 37ºC in a shaker machine.  After centrifuging the

colonies in 1.5 ml tubes at 13,000 rpm for 1 min, the pellets were cleaned using Genelute

Plasmid Mini-prep kit (Sigma-Aldrich).

The eluted colonies were prepared for sequencing using BidDye and sequenced using

a 3730xl DNA Analyzer (Applied Biosystems).  Contigs were cleaned-up and aligned using

ContigExpress program (Invitrogen, Carlsbad, CA). A BLASTN search of the consensus sequence was performed in GenBank database on the NCBI website to check for congruency with other *Peronospora* species. Primers were designed with the software program Primer3 (22) and labeled as ITSP forward and reverse (Table 1).

**Results**

    *Peronospora farinosa* was consistently detected with SEM in leaf tissues of infected quinoa plants, but inconsistently in petiole tissues. It was not detected with SEM at all in the stem or in leaf tissue near the apical meristem. Sporangia germinated and penetrated stomata of the leaf tissue by creating a single germ tube and appressorium (Figure 2, A and B). The appressoria were observed as bulging oblong structures that facilitated passage through stomata. No germ tubes were seen directly penetrating the leaf cuticle. Extracellular matrices presumably secreted by the pathogen were observed in patches around germinating sporangia and appeared to secure it to the leaf surface (Figure 2, E and F). Cryofractured leaf tissue revealed hyphae growing mostly in the spongy mesophyll tissues (Figure 2, C and D). These hyphae measured 8.5-12.8 µm in diameter and wound intercellularly through the leaf tissue. Occasionally, hyphae were observed among the palisade mesophyll. Haustoria formed in the epidermal and mesophyll cells (Figure 2, C and D). Hyphae exited leaf tissue through stomata on the abaxial surface and matured into sporangiophores bearing sporangia (Figure 3, A and B). Mature sporangia measured 24-32 µm long and 16 µm in diameter, and sporangiophores varied in size from 174-250 µm long and 8-9.6 µm in diameter. Multiple sporangiophores emerged out of a single stomate and were often seen twisted together in a mass (Figure 3, B).

In cryofractured cross sections, hyphae were seen growing from leaf tissue into the cortex of the petiole (Figure 3, C) where they also grew intercellularly. Hyphae in petioles measured 7.7-14.3 µm in diameter, which was slightly larger than hyphae in the leaf tissue. Haustoria in both leaf and petiole tissues were measured to be 1.5-3 µm in diameter, but were more abundant in petiole tissue and usually longer measuring 4.7-9.4 µm compared to 4.2-7.1 µm in leaf tissue. The petiole haustoria were seen growing into the epidermal and cortex cells in a curling-corkscrew fashion (Figure 3, C). No infection structures were observed in any portion of the stem tissue or leaf tissue near the apical meristem with SEM, and no signs of the downy mildew were seen in any vascular tissues. Control samples of healthy tissue were used for comparison with infected tissues (Figure 3, E and F).

**Infection of resistant and susceptible genotypes**

*Peronospora farinosa* was observed with SEM in both resistant 0654 and susceptible Chucapaca genotypes. More stomata with emerging sporangiophores were counted on the Chucapaca leaf surface (>35 stomata) than on the 0654 leaf surface (19 stomata) (Table 2, Figure 4, A and B). Chucapaca displayed 21 total hyphal strands and haustoria, while 0654 had 16 (Figure 4, C and D). Within the petiole tissue, 36 total hyphae and haustoria were counted in 0654 (Figure 4, E), but no signs of infection were observed in the Chucapaca petiole. No infection structures were found in the stem tissues of either gentoype.

**Detection of ITS region with PCR**

After sequencing the entire ITS region, it was found to be 866 bp long and 99% congruent with other *P. farinosa* species in GeneBank. The ITSP primers consistently amplified bands at 866 bp from positive controls during PCR with conventional buffer and direct PCR buffers (Figure 5, A and B; Figure 6).

The ITS region from infected leaf tissue was also consistently amplified during testing of the ITSP primers (Figure 5, A and B). However, it was inconsistently amplified in stem and petiole tissues. DNA extracted from petiole and stem tissue of infected quinoa plants yielded a faint band from the petiole and a strong band from the stem after PCR with conventional buffer (Figure 6). When testing direct PCR with infected quinoa tissues, a band was successfully amplified from stem tissue 8 of 33 repeated PCR reactions with tissue samples from different infected plants (gels not shown). Amplification from petiole tissues with direct PCR occurred more frequently, but was still inconsistent. No bands were amplified in leaf tissue near the apical meristem.

When analyzing tissue from susceptible and resistant genotypes, three petiole samples from both genotypes were submitted to direct PCR in order to ensure some detection since we were previously getting inconsistent results from these tissues. Petiole samples were selected from each genotype that did and did not reveal a band, and were observed with SEM to see if *P. farinosa* was indeed detected by the PCR in these samples. Two of the three 0654 petiole samples revealed bands with direct PCR (Figure 5, A), although, none of the three Chucapaca petioles, or any of the stem tissue or leaf tissue near the apical meristem in either genotype revealed bands (PCR of the stem and leaf tissue near the apical meristem for 0654 were ran on a separate gel not shown). Hyphae and haustoria were observed with SEM in the first petiole sections of both Chucapaca and 0654 even though it was not detected with PCR in either sample. The ITS region was successfully amplified from infected leaf tissues of both quinoa genotypes studied with SEM (Figure 5, A). Infection structures were observed with SEM in all of the tissue samples that yielded ITS bands from PCR. ITS bands were

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successfully amplified in three positive controls, where as neither of the negative controls yielded bands (Figure 5, A and B).

**Discussion**

Our SEM observations of the infection and development of *P. farinosa* f.sp. *chenopodii* generally agree with the findings reported in previous studies of *Peronospora* infections (5,6,9,12,14). We found that the pathogen grows through certain tissue types within the plant, severely infecting leaf mesophyll tissue of mature leaves, but not infecting younger leaves actively dividing near the apical meristem.

In our study, we noticed the formation of appressoria, but did not observe direct penetration of the cuticle as described in previous reports with *P. parasitica* (1,6). Germinating sporangia were only seen entering host tissue via stomata, and appressoria were usually associated. Achar (1) observed mostly stomatal penetration among *P. parasitica* of *Brassica oleracea* cotyledons and occasionally direct penetration.

The extracellular matrices seen on the cuticle and germinating sporangia appeared to be an adhesion mechanism to stabilize the sporangia during stomatal penetration. Carzangia et al. (5) describes the secretion and make-up of extracellular matrices among *P. parasitica* of *Brassica* spp. (containing ß-1,3 glucans and proteins) as a means for securing the sporangia during germination and for perceiving appressoria inducing signals for penetration. Most studies of extracellular matrices in oomycetes have been performed among encysting zoospores of *Phytophthora* and *Pythium* (13,15), and few cases have reported the production of extracellular matrices among *Peronospora* sp. that do not produce zoospores (5). To our knowledge, *P. farinosa* does not produce zoospores (9).

The size of the sporangia and sporangiophores were found to be very similar to the measurements reported by Danielsen and Ames (9).  However, the sporangiophores tended to be slightly longer and thinner than previously described with some measuring up to 250 µm long.  This could be due to environmental conditions of the growth chamber, or a specific characteristic of the 14B *P. farinosa* isolate.

The hyphae observed in leaf mesophyll tissues were most often seen among the spongy mesophyll cells near the abaxial surface where there are more stomata.  Hyphae were also occasionally observed in the palisade mesophyll near the adaxial surface.  The downy texture created by the masses of sporangia and sporangiophores emerging from the stomata are usually produced on the undersides of the leaves, which is presumably why we would most frequently see the hyphae in the spongy mesophyll cells.  However, during heavy infections in the field and growth chamber, sporulation has also been observed on the adaxial surface.

In infected regions of the petiole cortex, the hyphae measured slightly larger in diameter than hyphae found in the mesophyll cells.  The thicker cell walls of supportive tissues in the petiole, such as collenchyma, (4) may induce hyphae in those regions to grow thicker for structural integrity in order to pass between plant cells.  Because of their mass, it is also possible that they are a different type of infection structure associated with haustoria and nutrient acquisition.

Chucapapca had a greater amount of sporangiophores on the leaf surface (Figure 4, A) and a greater number of hyphal strands and haustoria in mesophyll tissues (Figure 4, C) than 0654 (Figure 4, D).  The fact that we saw fewer *P. farinosa* structures in the leaves of 0654 suggests that some plant-fungus interaction may be limiting the amount of infection

generated in the resistant genotype. The infection was clearly present in the resistant 0654, but it produced fewer sporangiophores and therefore, fewer sporangia for spreading the infection. Accession 0654 has been observed to exhibit resistance in the field; however, the pathogen has been known to overcome the resistance when disease pressures are high (A. Bonifacio, personal communication). The environment in the growth chamber is ideal for the mildew, and it may be that the pathogen overcame the resistance in 0654 because of the high disease pressure. Even if the resistance was overcome, our studies show that a heavily infected 0654 leaf exhibits fewer infection structures than a heavily infected susceptible leaf.

From our observations of mildew development within the plant, we noticed that 12 days after inoculation of a single leaf, and with the appropriate humidity cycles, sporulation occurred on several leaves of both resistant and susceptible plants. This suggests that the pathogen traveled from one leaf to multiple leaves above and below the inoculation point. We hypothesized that the fungus was growing through the petiole and stem tissues because sporulation would appear throughout much of the plant before there was adequate time and humidity levels for new sporangia to germinate, colonize, and sporulate. For this reason, we expected to consistently detect *P. farinosa* in petiole and stem tissues with both SEM and PCR, especially in heavily infected plants. Primers specific to the ITS region of *P. farinosa* were designed as a means for verifying the presence of the pathogen in the tissues observed under SEM. However, the PCR did not always detect the pathogen in petiole and stem samples even though the plants showed sporulation across multiple leaves. The inconsistent detection of downy mildew in the petiole and stem tissues may be due to several reasons.

One possible explanation may be that *P. farinosa* sends small hyphal strands around the exterior of the petiole and stem until it reaches stomatal openings of a leaf. If these

hyphae grew in small quantities, they may not have been detected with PCR. However, we did not observe such exterior signs of the fungus. An additional explanation for the inconsistency of PCR detection is that petiole and stem tissues may disrupt amplification during direct PCR. This would explain why *P. farinosa* was seen in the petiole with SEM, but not detected with PCR in a section taken only 1 cm away. Very few reports have been made on the use of direct PCR buffers with plants (26). Future studies should include testing the functionality of the direct PCR with petiole and stem tissues.

Another possible explanation may be that as *P. farinosa* grows away from the mesophyll tissues, it generates a different type of infection structure that is smaller and in lower quantities in order to conserve energy as it travels through the petiole and stem until it reaches more nourishing mesophyll tissues, thus evading detection of the PCR and SEM in the base portions of the petiole and much of the stem. In our study, petiole samples submitted to SEM were taken from the region closest to the leaf, while those submitted to PCR were taken from the region closest to the stem. It may be that the detection with PCR was dependant on the size and amount of mycelium structures produced in the stem and petiole tissues being tested. The bands yielded from conventional PCR with infected petiole and stem tissues, and the occasional bands from direct PCR with similar tissues, leads us to believe that the pathogen does grow through the petiole and stem, although at what levels and in which tissues we are unsure. Staining, tissue sectioning, and visualization with light microscopy of petiole and stem pieces may prove useful in verifying whether *P. farinosa* is indeed traveling within the petiole and stem tissues and how it is growing. As oomycetes are aseptate pathogens, the nuclei are free to cluster in regions of the hyphae. The *P. farinosa* nuclei of hyphae in quinoa stem and petiole tissues may cluster in various regions as it

travels, which would also explain why PCR inconsistently amplified the mildew DNA in these tissues.

Increasing our understanding of how *P. farinosa* infects quinoa is important to finding methods of control, especially when few studies have been performed with this destructive disease.  Knowledge of infection among resistant quinoa will also prove significant when selecting genotypes for breeding programs.  The use of direct PCR and specific *P. farinosa* primers would be useful in future studies limited to small tissue samples, or other molecular studies with involving this fungus.

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**Table 1.** PCR primers used to amplify the full ITS region of *P. farinosa* f.sp. *chenopodii.*

| Primer | Sense | Sequence | Detects |
|--------|-------|----------|---------|
| DC6[a] | Forward | GAGGGACTTTTGGGTAATCA | Peronosporales |
| ITS4[b] | Reverse | TCCTCCGCTTATTGATATGC | Universal |
| ITS5[b] | Forward | GGAAGTAAAAGTCGTAACAAGG | Universal |
| ITSP | Forward | GAACCTGCGGAAGGATCA | *P. farinosa* f.sp. *chenopodii* |
| ITSP | Reverse | AGTTCAGCGGGTAATCTTGC | *P. farinosa* f.sp. *chenopodii* |

[a] Bonants et al. (2)
[b] White et al. (25)

**Table 2.** The number of *P. farinosa* fungal structures observed with SEM in different tissues of susceptible and resistant quinoa genotypes.

| Tissue | Genotype | Count | Magnification |
|---|---|---|---|
| Leaf surface | Chucapaca[a] | >35[c] | 126x |
| | 0654[b] | 19[c] | 126x |
| Leaf cells | Chucapaca | 21[d] | 800x |
| | 0654 | 16[d] | 800x |
| Petiole cells | Chucapaca | 0[e] | 1025x |
| | 0654 | 36[e] | 1025x |

[a] Susceptible genotype
[b] Resistant genotype
[c] Number of stomata counted from which sporangiophore masses grew
[d] Number of total hyphae and haustoria counted among fractured leaf cells
[e] Number of total hyphae and haustoria counted among fractured petiole cells
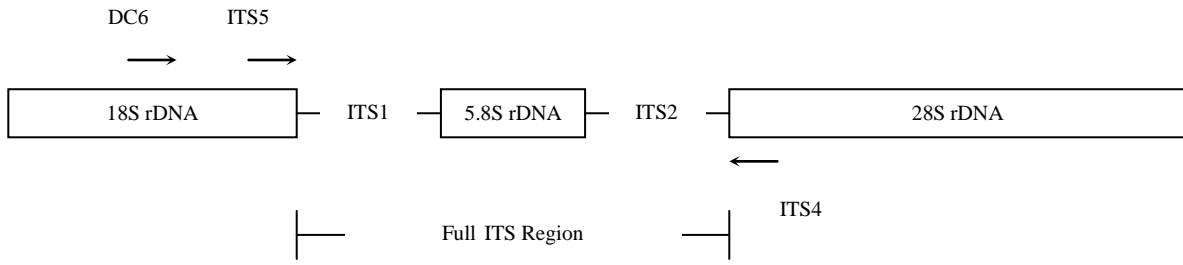
**Figure. 1** Location of ITS region, and primers within rDNA genes running 5' to 3'.
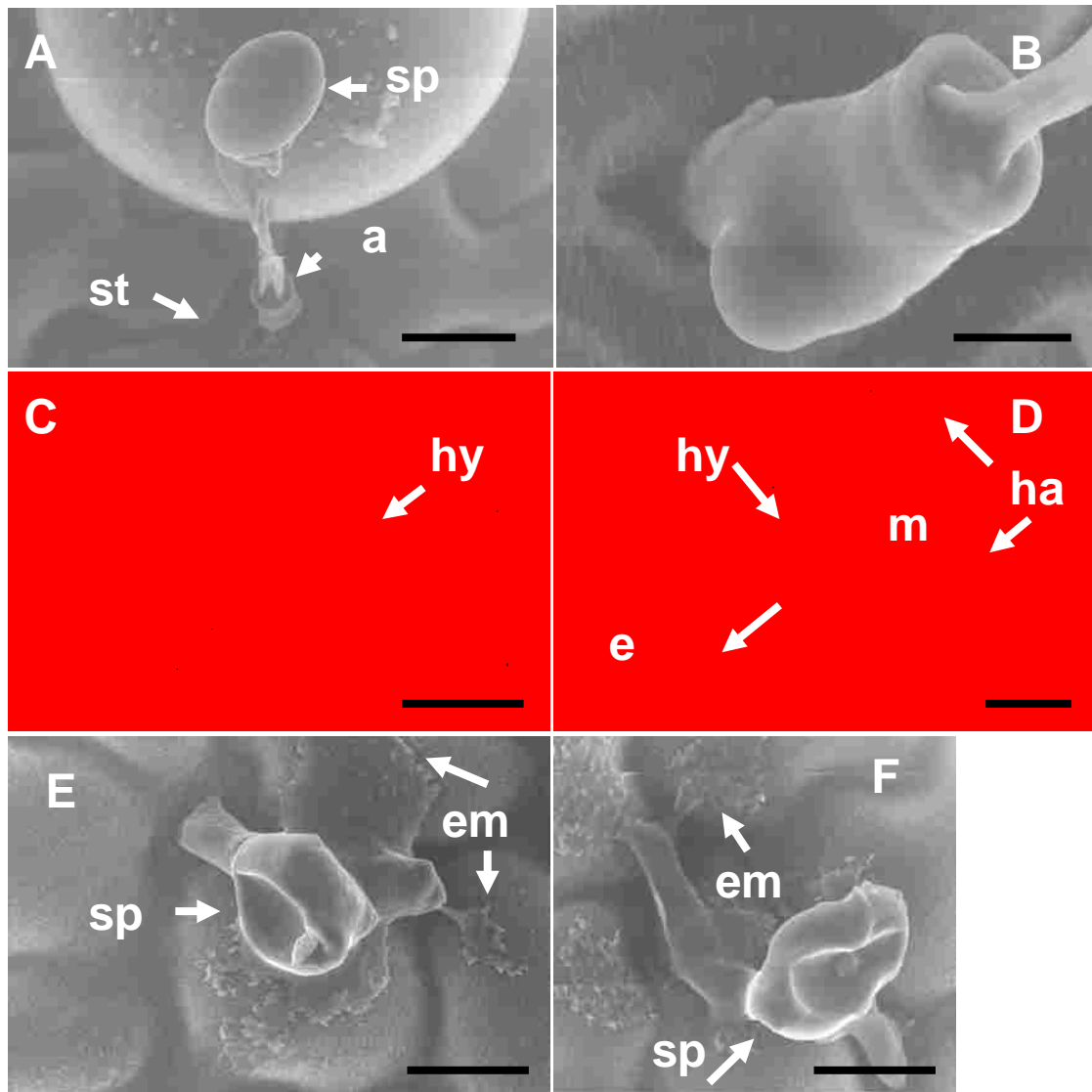
**Figure 2.** (**A**) Germinating sporangium (**sp**) with appressorium (**a**) penetrating a stomate (**st**) (Bar = 20 µm, ESEM). (**B**) Appressorium penetrating a stomate (Bar = 5 µm, ESEM). (**C**) Hyphae (**hy**) growing intercellularly in spongy mesophyll tissue (Bar = 100 µm). (**D**) Hyphae (**hy**) and haustoria (**ha**) growing into epidermal (**e**) and mesophyll (**m**) cells of a leaf (Bar = 20 µm). (**E and F**) Extracellular matrices (**em**) secreted from germinating sporangia (**sp**) (Bar = 20 µm, ESEM).
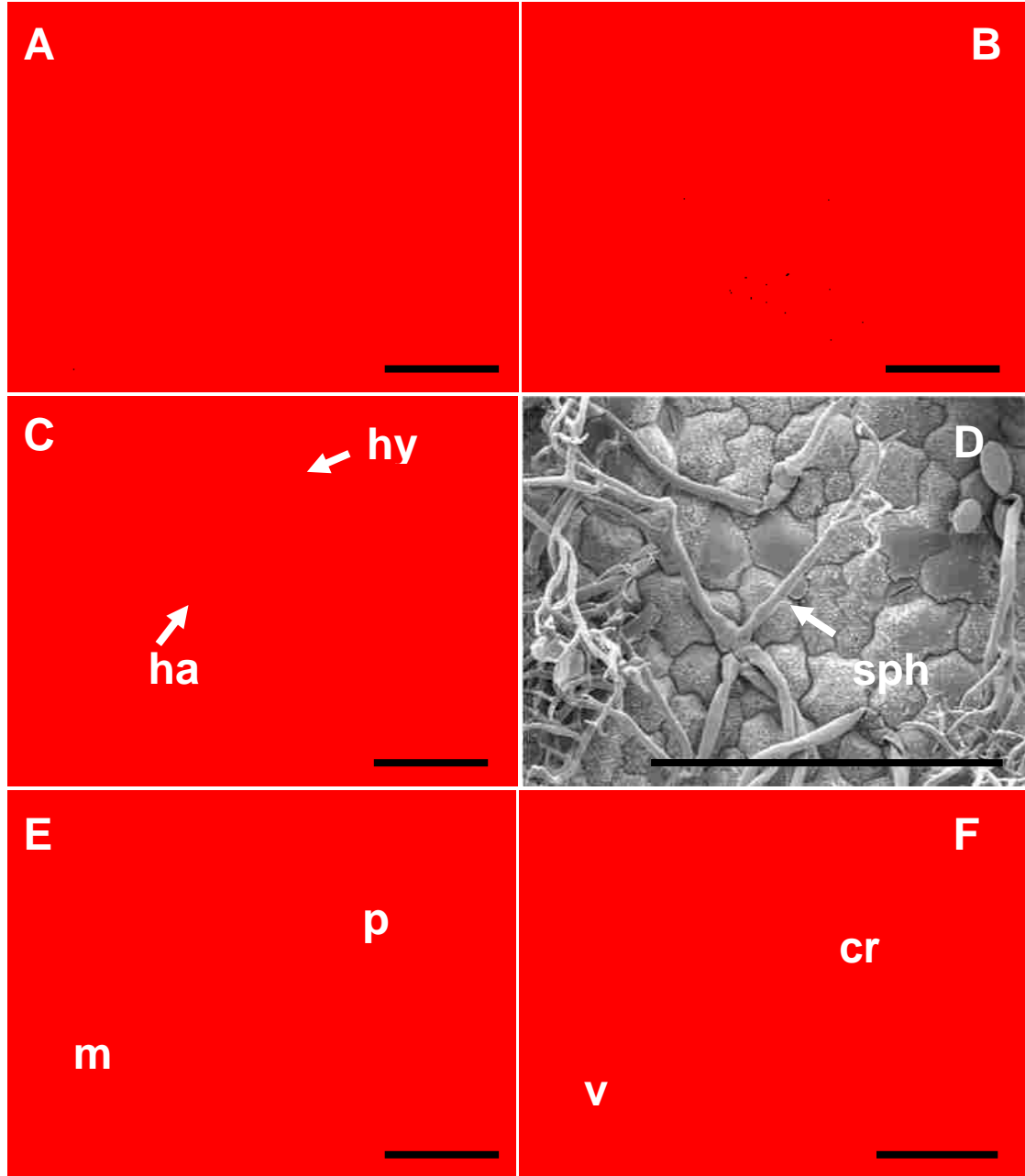
**Figure 3.** (**A**) Sporangiophores exiting stomata (Bar = 50 µm). (**B**) Mature sporangia attached to a mass of branching sporangiophores (Bar = 100 µm). (**C**) Hyphae growing intercellularly (**hy**) and haustoria (**ha**) growing into petiole cortex cells (Bar = 20 µm). (**D**) Multiple sporangiophores (**sph**) emerging from a single stomate (Bar = 200 µm). (**E**) Healthy leaf tissue with spongy mesophyll (**m**) and palisade (**p**) cells (Bar = 50 µm, JEOL 840). (**F**) Healthy stem tissue with cortex cells (**cr**) and vascular system (**v**) (Bar = 50 µm).
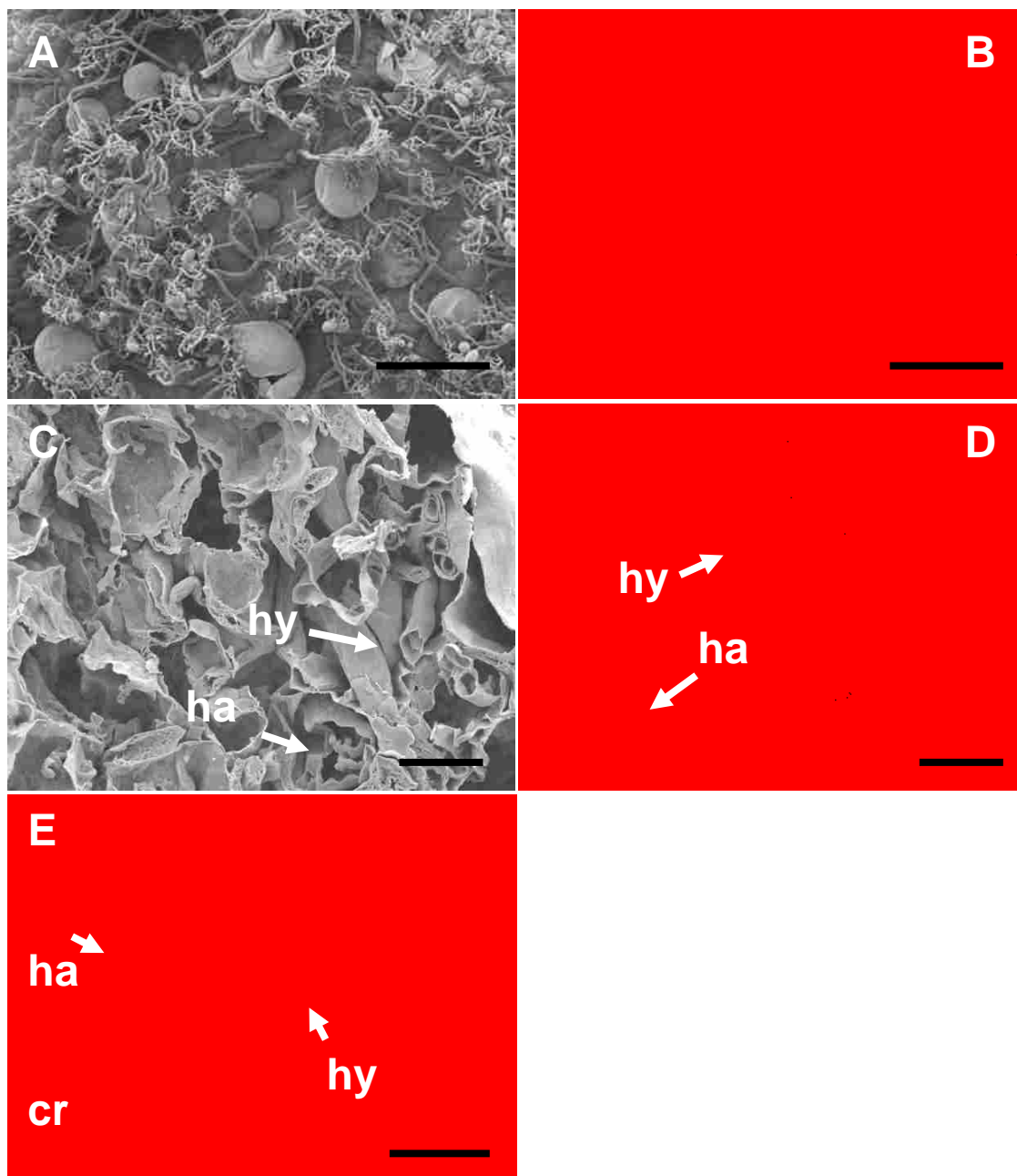
**Figure 4.** (**A**) Sporangiophores on the leaf surface of Chucapaca (Bar = 200 µm). (**B**) Sporangiophores on the leaf surface of 0654 (Bar = 200 µm). (**C**) Hyphal strands (**hy**) and haustoria (**ha**) in fractured Chucapaca leaf tissue (Bar = 20 µm). (**D**) Hyphal strands (**hy**) and haustoria (**ha**) in fractured 0654 leaf tissue (Bar = 20 µm). (**E**) Hyphal strands (**hy**) and haustoria (**ha**) in fractured cortex (**cr**) cells of 0654 petiole tissue (Bar = 20 µm).
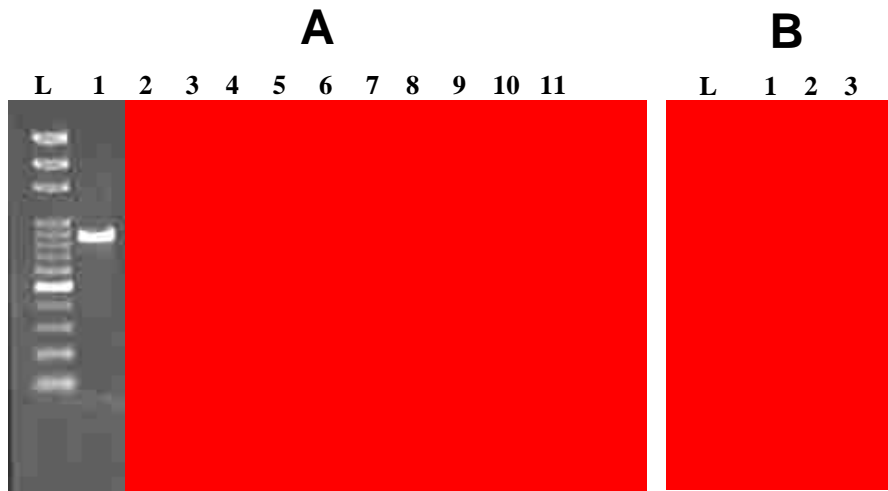
**Figure 5.** (**A**) Detection of *P. farinosa* ITS region in infected tissues of resistant and susceptible quinoa genotypes with direct PCR buffers and ITSP primers. Lane L, 100-bp DNA ladder; lane 1, leaf tissue from Chucapaca; lane 2-4, petiole tissue from Chucapaca; lane 5, stem tissue from Chucapaca; lane 6, leaf tissue near the apical meristem from Chucapaca; lane 7, leaf tissue from 0654; lane 8-10, petiole tissue from 0654; lane 11, non-sporulating leaf tissue from a infected quinoa (positive control); lane 12, leaf tissue from a healthy quinoa (negative control). (**B**) Detection of *P. farinosa* ITS region with conventional PCR buffer as controls. Lane L, 100-bp DNA ladder; lane 1, sporangia DNA; lane 2, infected leaf DNA; lane 3, healthy leaf DNA (negative control).

**Figure 6.** Detection of *P. farinosa* ITS region in infected petiole and stem tissue with conventional PCR buffer and ITSP primers. Lane L, 100-bp DNA ladder; lane 1, DNA from petiole tissue; lane 2, DNA from stem tissue; lane 3, DNA from sporangia (positive control); lane 4, DNA from healthy quinoa tissue (negative control).

**Chapter 4**

**Literature Review**

**Introduction**

Quinoa (*Chenopodium quinoa* Willd.) is one of the most important food crops of the Andean region, not only because of its unique nutritional value, but because very few crops can withstand the harsh environment of the Altiplano (10). It is drought tolerant, salt tolerant, and thrives at high elevations. The protein content of its seeds is higher than that of most cereal grains, and its nutritional levels are close to the recommended dietary values established by the Food and Agriculture Organization of the United Nations (FAO) (34). Although quinoa was once a staple crop throughout much of western South America, its production is now restricted to a few regions. Quinoa is one of few crops grown by farmers in the altiplano. Other crops include potatoes, fava beans, and forage crops of either barley or oats (5).

Many pests affect quinoa production including insects, birds, and wild hares (5). However, quinoa's most important threat is from downy mildew caused by the pathogen, *Peronospora farinosa* f.sp. *chenopodii* Byford. This disease is a fungus-like pathogen endemic to South America. It is an obligate parasite that attacks the foliage of the plant causing chlorosis and defoliation. It spreads through sporangia found in lesions on the undersides of leaves, and through oospores. These structures can remain dormant for long periods of time in soil and quinoa seeds. *Peronospora farinosa* can greatly affect crops causing up to 99% yield loss (13). Fungicides have traditionally been used to control downy mildew, but they are expensive for the majority of quinoa farmers, which manage small to medium sized production farms (11). A practical solution for keeping mildew levels low is to develop quinoa lines that are genetically resistant to *P. farinosa*.

Some varieties of quinoa are more susceptible to downy mildew than others, while some exhibit complete resistance. Quinoa landraces contain a vast amount of genetic variation (5) including genes controlling resistant traits that can be selected for breeding.

The development of polymerase chain reaction (PCR) primers for *P. farinosa* will also benefit the management of downy mildew in many ways. PCR primers are required for the use of molecular marker assays, which can better help to understand the pathogen. They are also useful for detecting the presence of a latent pathogen in the host tissue. Quinoa's oospores often lie dormant and undetected in soil, seed, and plant tissue. When a new season begins and oospores become active, and quinoa seedlings become infected. The early detection of downy mildew in quinoa seed may help prevent crop loss, and PCR primers can help to achieve this. The use of conserved genetic sequences aids in primer design by amplifying specific DNA regions in the pathogen that can then be sequenced and tested.

**Quinoa**

*Chenopodium quinoa* Willd. is a nutritious pseudocereal with an interesting history. Its common name is quinoa and it was once a staple crop throughout the western countries of South America. As an ancient Incan crop, its cultivation has survived for thousands of years serving local inhabitants as a source for food, dyes, and religious ceremonies. It has often been called *chisiya mama*, which means "mother grain" in the Incan language of Quechua (36). After the Spanish conquest in the early 1500's, cultivation declined as quinoa and other grains with religious significance were prevented from being grown (5). Major crop production has since become isolated to the Andean region and high plateaus around Lake Titicaca (11,40).

Quinoa's origins lie in the Altiplano near Lake Titicaca in the Andean Mountains of

South America. The earliest domestication of quinoa dates back 7000 years (11,40).

Archeological remains of quinoa have been reported in Peru, Chile, and Bolivia, some dating

back to 5000 BC (40). During the reign of the Incan Empire from the 1400-1500's, quinoa

was cultivated from Columbia to Chile, and its many names signify the importance it had

throughout these different regions in South America. It has been referred to as "jupha" by

the Aymara in Bolivia, "suba" by the Chibcha in Columbia, "dahue" by peoples of the

Atacama Desert in Chile, and "kinua" in the widespread language of Quechua (5,40).

Quinoa can be used in a variety of ways. Young leaves can be cooked or eaten fresh,

and the grain is generally boiled like rice, or ground into flour for baked goods (5).

Preparation of the quinoa grain for consumption is a somewhat tedious process. The pericarp

of the seeds contains bitter soap-like compounds called saponins that foam when rinsed in

water. In order to remove the saponins, the seeds must be either threshed or soaked and

washed repeatedly. High amounts of saponins can be toxic to red blood cells and must

therefore be removed (40). Traditional uses of the grain have been studied and are being

reintroduced into local populations of Bolivia. Such uses include making quinoa into soups,

biscuits, cakes, breads, and fermented beverages, or popping it like popcorn. Quinoa is also

used as feed for animals after harvest and threshing, and Andean women wash their hair with

the saponin water leftover from soaking the seeds (5,10).

The nutrition content of quinoa seeds is extremely valuable. Its protein content

ranges from 12 to 17% depending on the variety, and is greater than that of any other grain,

but less than the protein content of legumes, which is 20 to 30% (40). The quality of

quinoa's amino acid composition is exceptional. It is more completely balanced than other

major cereals.  Lysine is a scarce amino acid among edible plants, and is found in high levels in quinoa seeds (23,40).  When eaten in combination with other nutritious foods, it can be used as a meat substitute because of its high protein and amino acid content (36).  It is also high in calcium, phosphorus, and iron minerals.  The fat content is 4.1 to 8.8%, and with a high caloric content, it is considered to be a high-energy food.  It also has more of the essential nutrients needed to sustain human life than any other plant or animal (23,34).  In general, quinoa has valuable nutritional qualities and the potential to improve many lives through a balanced diet.  However, farmers frequently cannot produce enough to sustain the needs of their families.  Crop rotation often includes potatoes, quinoa, and fava beans. Without sufficient combinations of these foods, their diets are nutritionally unbalanced (5).

After surviving thousands of years of cultural repression, this high quality grain has again become the food of preference.  Today, subsistence farmers in Northern Argentina, Bolivia, Northern Chile, Colombia, Ecuador, and Peru heavily rely on locally cultivated quinoa as part of a balanced diet (5,23,34).  Some of the most important regions of quinoa production on a larger scale include provinces of the Andes Mountains: Arequipa, Ayacucho, Cajamarca, Cusco, Junin, Puno (all of Peru), and La Paz of Bolivia (11).  It is also being cultivated on a lesser scale in North America and Europe where it is more difficult to grow (13).

**Morphology of quinoa**

Quinoa is an erect dicotyledonous herb that grows between 0.7 and 3.0 meters tall. The leaves alternate along a woody stem, which can be branching or unbranching depending on the variety.  A large inflorescence forms at the apex of the plant and often, smaller clusters from the axils.  First, small plain flowers emerge on a panicle and by the end of the

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growing season a large seedhead is formed. Quinoa flowers are very small and not easily

recognized as a flower. They are small, green, and self-fertilizing. Both female and

hermaphroditic flowers are present with the females toward the center of the group and the

hermaphrodites around the edges. The seeds are achenes and come in a variety of colors.

They are about 2-3 mm in diameter, conical or cylindrical in shape, and generally flat. The

embryo typically takes up 60% of the seed, which explains its high protein content (40).

The length of the roots is related to the plant's height, and often, they will be as long

as the plant is tall with excessive branching extending from the taproot. Quinoa has broad

leaves that are lanceolate in the upper portions and rhomboidal in the lower portions. The

leaves are generally lobed at the margins and are covered with a powdery pubescence that

easily rubs off (36,40). White or purple colored pubescences are spherically shaped vesicles

that hold crystals of calcium oxalate.

Quinoa's morphology has evolved to adapt to the extreme growing conditions of the

Altiplano. As a drought and saline tolerant plant, quinoa is one of the few crops that can

survive in the Andean Mountains. Most varieties perform best at high elevations between

2,000 and 4,000 meters and in cool, semi-arid temperatures—characteristic of the Altiplano.

Frost is also common in these mountains. Some varieties can endure temperatures as low as

-4°C (34). Quinoa is markedly known for its drought resistant qualities and tolerance of poor

soils. Droughts are frequent in the Altiplano due to the poor distribution of rains throughout

the season (40), and quinoa is one of the few crops that can withstand its effects. Its narrow,

erect habit, pubescent leaves, and branching root structure are all adaptations that have

helped it survive frequent years of drought. Certain varieties are also tolerable of saline soils

as well as acidic or alkaline soils.

**Genetic variation of quinoa**

There are over 250 species of *Chenopodium*. Some of quinoa's well-known relatives, sugar beets and spinach, are both belonging to the Chenopodiaceae family (40). The principle chromosome number of quinoa is x = 9. It is usually allotetraploid with wild species being either tetraploid or diploid. There are many other species of quinoa, some domesticated and some wild. Domesticated species, also grown in South America, include tetraploid huauzontle (*C. nuttalliae* Safford) and diploid cañihua (*C. pallidicaule* Aellen) (34). Wild species include *C. hircinum*, *C. murale*, and *C. ambrosoides* found in South America, as well as the species *C. album* found in North America and England and commonly known as lamb's quarters or fat hen (40). Quinoa's diversification also comes in a variety of colors and cultivars, seeds may vary from white to, red, yellow, purple, and black.

Classification of quinoa has defined the different varieties into five fundamental categories based on their habitat. First, valley types grow in the Andean valleys at elevations ranging from 2,000-3,600 meters and have a long growing season. Second, altiplano types grow near Lake Titicaca at higher elevations around 4,000 meters. They are more tolerant of frost and have a short growing season. Third, salar types are native to the salt flats of Bolivia's southern Altiplano. They grow at elevations around 4,000 meters, are extremely salt tolerant, adapted to soils with pH greater than 8.0, and their seeds contain large quantities of saponins. Fourth, sea level types are typically found in southern Chile, have a long photoperiod, and produce seeds with high amounts of saponins. Fifth, a subtropical type is found in the subtropical regions of Bolivia and has very green foliage, which changes to an orange once it reaches maturity. Little information is available about this type because it is a

newly discovered variety (36,40). The different classifications show how quinoa has adapted to various environmental conditions.

**Genetic conservation of quinoa**

Crop production in the Altiplano of Bolivia covers about 35,000 hectares where the central and southern regions produce the majority of Bolivia's quinoa. Subsistence farming is the primary means of quinoa's cultivation in South America. On larger commercial scale farms in specific provinces of the Altiplano in Peru and Bolivia, quinoa production has proved to be a successful crop. Some areas employ agricultural mechanization for commercial production, which provides 50 to 60% of their annual income. With an average yield of 500 kg per hectare, these regions export about 60% of their crop. Quinoa production in South America is currently limited due to problems with drought, birds, insects, and other pests like downy mildew (5). Understanding quinoa's diversity and genetic makeup will better help researchers address the problems that are limiting quinoa production.

As an allotetraploid derived from crosses between different species, quinoa's somatic chromosome number is $2n = 4x = 36$. Even though it exhibits tetraploidy, the suppression of homoeologous pairing during meiosis causes quinoa to be functionally diploid. The plants are generally inbred and self-fertile, conserving alleles within each variety. As genetic variability is a limiting factor in plant breeding, the phenotypic diversity among cultivated quinoa accession lines, as well as related wild species, is beneficial to the development of new varieties (34) because it is easier to select and breed for traits when a variety of genes are present in a species. Genetic conservation of quinoa species is therefore an important aspect of quality improvement and food security (5). An important factor that helped the Incas achieve high yields was the use of multiple quinoa varieties adapted to different regions

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across the land depending on their environmental niches. The existence of so many quinoa species today is evidence of the Inca's extensive cultivation and understanding of genetic diversity.

During the Green Revolution the ideal of modernized agriculture spread throughout many developing countries. The practice of abandoning traditional crops was adopted in the Altiplano, which suppressed genetic conservation of quinoa species. Focusing current research on the yield improvement of multiple varieties based on their adaptation to specific habitats has greater advantages than developing a single high yielding variety in place of traditional varieties (10,34). The conservation of quinoa cultivars will help maintain genetic resources for usage in finding new varieties and desirable characteristics such as resistance to downy mildew and other problematic pathogens.

**Effects of downy mildew on quinoa**

Downy mildew is the most significant disease of quinoa crops in South America (11). It is caused by *Peronospora farinosa* f.sp. *chenopodii*, a parasitic pathogen that attacks the foliage of the plant. Downy mildew looks and acts like a fungus, although it is not a true fungus. It has distinct characteristics that separate it from the Fungi kingdom. It is endemic to regions of Bolivia, Chile, Colombia, Ecuador, and Peru. The first sign of infection includes necrotic spots on the tops of the leaves followed by gray lesions of mildew spores covering the underside of the leaves. Spore production leads to severe chlorosis and eventually defoliation (12). Reduced photosynthetic ability weakens the plant and halts seed production. Defoliation is the major cause of yield loss, ranging from 33 to 58% reduction, and in some cultivars 99% loss (13). Some quinoa cultivars are more resistant to downy

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mildew and react with a hypersensitive response.  In such cases, although the disease is present, only mild symptoms will occur (12).

Few studies have been made regarding the effects of downy mildew on quinoa.  Some studies have used the area under disease progress curve (AUDPC) as a model to evaluate the relationship between yield loss and quantitative resistance in the plants.  Disease evaluation methods and infection ratings have also been developed.  The three-leaf method uses the average percentage of infected leaf area from three leaves randomly selected from the upper, middle, and lower regions of the plant to access infection.  A five-scale infection ranking method is also used to determine the severity of infection with zero showing no infection and five showing severe infection (14).  These methods are useful in assessing yield loss and looking for resistance in select cultivars.

**Downy mildew of quinoa: *Peronospora farinosa* f.sp. *chenopodii***

The downy mildews are host specific fungal-like pathogens that belong to the phylum Oomycota meaning "egg fungus."  The oomycetes are a unique group of protists that create oospores, or eggs, during their sexual reproductive cycle and include various white rusts, water molds, and downy mildews (33).  Oomycetes are very similar to fungi, but certain genetic, molecular, and reproductive characteristics separate them from the Fungi kingdom and categorize them in the Chromista or Straminipila kingdom.  Although their growth habits and nutrient absorption are very similar, oomycetes are more closely related to golden-brown algae than to fungi (25).  The cell walls of true fungi consist mostly of chitin, and they contain septa that separate the cells.  In contrast, the cell walls of oomycetes are mostly made of cellulous with traces of chitin, and they do not have septa, their hyphae are coenocytic

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(7,8,25). Oomycetes also exhibit a haplomitotic B ploidy cycle in which meiosis occurs only during the sexual cycle resulting in a dominant diploid phase where asexual structures are all diploid (15,16). Many oomycetes produce zoospores, which propagate the asexual reproductive cycle. Zoospores are biflagellate cells that are generated inside asexual spores through protoplasmic cleavage. As motile, uninucleate cells, they disperse through water and then germinate on the host surface (15,29). The oomycota lifecycle is also different in that it produces diploid oospores that form during the sexual part of its reproductive cycle. The heterothallic nature of certain oomycetes, and particularly the downy mildews, allows for constant genetic variation within species.

Hundreds of different oomycetes exist, each infecting their own host species. Some well-known species have caused historical crop epidemics and continue to bother crop production around the world. *Phytophthora infestans* causes late blight of potato and lead to the Irish potato famine of 1846 (44). *Plasmopara viticola*, which infects grapevine, was first reported in Europe in 1878 and currently affects vineyards around the world (26). The species *Pythium insidiosum* is infectious among different mammals including humans. It begins infection in the skin and can invade the blood and bone tissue (25). Additional plant host species infected by oomycetes include members of the Cucurbitaceae (cucurbit), Asteraceae (lettuce, sunflower), Brassicaceae (broccoli), Rosaceae (rose), Solanaceae (tobacco), Chenopodiaceae (spinach, quinoa) and hundreds more dicotyledonous families.

From the fungal order Peronosporales and the family Peronosporaceae, *Peronospora* is the genera of many different downy mildews including *P. farinosa* f.sp. *chenopodii*. The classification of *Peronospora*, or oomycota in general, is under some debate. It has not been agreed upon as to whether oomycota should be classified as Chromista, which suggests

descending from an ancestry of photosynthetic endosymbionts, or Straminipila having

ancestry from heterotrophs (16). However, strong evidence suggests categorizing them in the

kingdom Straminipila based on the morphology of the zoospores, nucleotide sequencing of

ribosomal RNA, and other molecular data (16,33). Evolutionary ideas about the origin and

development of downy mildews present hypotheses of co-evolution. Rather than evolving

linearly from common ancestors, co-evolution suggests that downy mildews would have

evolved as their host species evolved. More specifically, the evolution of secondary

metabolites within the dicotyledonous plant would have affected the development of the

downy mildew. As the two organisms changed, the downy mildew species would have

developed independent of each other and at different times and locations (16,19). Clues of

origin and evolution also include the idea of horizontal gene transfer across kingdoms.

Evidence of a necrosis-inducing peptide gene that stimulates a defense mechanism in the host

plant has been identified in oomycetes, fungi, and bacteria (29,44 ). Although these phyla

are not related through phylogenetics, the described evidence links their origins.

      The downy mildews have proven to be a significant group of pathogens among

important crop species. Only moderate amounts of research have been conducted with

*Peronospora*, however, even less work has been done on the specific species of *P. farinosa*

f.sp. *chenopodii*. Therefore, information about this species is limited. The *Peronospora*

*farinosa* species are specific to three host plant genera within the family Chenopodiaceae:

*Beta*, *Spinacea*, and *Chenopodium* (11). As an obligate parasite, or biotroph, *P. farinosa* f.sp.

*chenopodii* can only survive on quinoa species. Downy mildew of quinoa requires specific

conditions for germination and infection. It proliferates in humidity above 80% and in cool

temperatures between 15 and 20°C (12). During dry and warm conditions, the infection lies

dormant as oospores in quinoa seeds and surrounding soil.  It can also survive in host tissue

until environmental conditions are favorable for continued growth and spore production.

**Morphology and life cycle of downy mildew**

The growth habits of *P. farinosa* are very similar to true fungi.  Asexual structures of

the pathogen include hyphae, sporangiophores, and sporangia.  After landing on a leaf, the

sporangia send out a germ tube, which grows into the leaf generating vegetative hyphae.  The

hyphae develop and spread in between the leaf cells forming a mass of filamentous

mycelium.  Haustoria are extended from the hyphae to penetrate the cells and absorb

nutrients from it using digestive enzymes.  Once the hyphae are established, the pathogen

emerges from inside the leaf and forms sporangiophores that extend from the leaf surface.

Sporangiophores are branching structures that grow to be 167-227 μm long and produce

sporangia at the tips when a specific length is reached, thus sporangia mature at the same

time.  The sporangia are spores that cause secondary infection among quinoa plants.  They

drop from the sporangiophores at maturity, germinate on the leaf surface, and continue the

infection cycle.  The sporangia of *P. farinosa* have a very distinct oval shape at about 25-32

μm long and 19-24 μm wide.  A small projection, called a papilla, can usually be found at

one end.  They are light brown in color and are quite transparent when viewed under a light

transmission microscope (12,15).  Many of the oomycete species are recognized for their

production of zoospores.  However, not all *Peronospora* species produce zoospores (8,19).

*P. farinosa* f.sp. *chenopodii* germinates directly from the sporangia, and does not produce

zoospores (12).

Sexual structures of the pathogen include antheridia, oogonia, and oospores.

Antheridia and oogonia are sexual gametes developed from thalli that fuse together as paired

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gametangia to create diploid oospores.  The antheridia are male gametes, transparent, lobed, and oval in shape.  The oogonia are female gametes that are rounded and globular in shape (12).  In many oomycetes, the antheridia are hormonally attracted to the oogonia and become attached to it.  A fertilization tube from the antheridium carries a male haploid nucleus into the oogonium and fertilization occurs (15).  The single aplerotic oospore fills only the central part of the oogonium.  It is released with a thick, translucent, outer wall that darkens upon maturity and is 39-50 μm in diameter (12).  Oospores are the pathogen's primary source of inoculum.  They are durable structures that overwinter in the soil or quinoa seeds.

Homothallism and heterothallism is a common means of sexual reproduction within species of the Peronosporaceae family.  When gametangia fuse from the same thallus, homothallism exists.  When gametangia from two genetically different thalli fuse, heterothallism exists.  Each species behaves differently either in a homothallic or heterothallic manner, or both within some species (11,35).  Isolates of *P. farinosa* have been tested and reported to be heterothallic in nature with genetically diverse populations (11).

The downy mildew's sexual and asexual lifecycles are important in understanding its different degrees of infection. The asexual lifecycle begins as downy mildew sporangium lands on a quinoa leaf.  With adequate conditions, the little spore germinates and grows into the leaf.  Hyphae become established inside the leaf and seven days later sporangiophores emerge from the leaf's surface.  After seven to ten days, when the sporangiophores have reached their sufficient length, sporangia are produced and disseminate to other leaves by wind and water.  A secondary infection of the downy mildew proliferates with the asexual cycle as conditions of high humidity and cool temperatures ensure its progress.  The cycle can be repeated multiple times throughout the growing season.  The sexual lifecycle is

initiated as sexually compatible gametes come together to form oospores.  It occurs only once during the end of the growing season.  When conditions become favorable again, often the following year depending on rainfall and humidity, the oospores will germinate to create a new infection.  Oospores sustained in the soil and seeds usually affect young plants at the beginning of the next growing season.

**Biological effects of downy mildew on quinoa**

The molecular pathways of *P. farinosa* f.sp. *chenopodii* have not been thoroughly studied; however, certain pathways among related *Peronospora* have been examined and may be similar to those in downy mildew of quinoa.  Recent studies regarding the production of extracellular matrices have been performed with *Peronospora parasitica*.  Carzaniga et al. (8) found that the pathogen secreted two types of extracellular matrices from its germ tubes and appressoria.  It is believed that germinating sporangia use these to adhere to plant surfaces for stabilization during germination.  One substance specifically contained sugars β-1,3-glucans, mannose, N-acetylglucosamine, and N-acetlygalactosamine.  The other substance contained mostly proteins.  The germ tube penetrated the leaf tissue by first producing an appressorium which facilitates penetration by either directly piercing the cuticle or entering through a stomate.  The appressorium is created as the tip of the germ tube swells into a round or oblong structure (8,29).  Once penetration occurs, the filamentous hyphae begin to grow within the leaf.

The cell wall of the hyphae has been studied in different oomycete species and it has recently been discovered that traces of chitin exist in the cell wall when it was previously thought that they were non-existent.  Although chitin is a small component, it is believed to have some importance (8,25,29).  When the mycelium becomes established in the leaf,

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sporangiophores exit the inner portion of the leaf and grow out from its surface.

*Peronospora parasitica* emerges from its host through the stomata (8). Stomata have an interesting role in the entrance and emergence of downy mildews. The zoospores of *Plasmopara viticola* germinate through the stomata of the host leaf, which they are drawn to through the emission of specific molecules from open stomata (26). The internal processes of how downy mildews infect their hosts are not fully understood, but new strides in research are continually being made.

Scanning electron microscopy (SEM) is a useful technology for studying how oomycetes move through host plant tissue. SEM uses a fine beam of focused electrons to scan over a specimen and create an image on a TV tube as electrons reflect off the specimen. Voltages between 5 and 30 kV are used to view specimens as small as 1.5 nm (45). Different techniques of sample preparation provide a variety of information about how the pathogen attacks its host. Cryofracturing is an effective method of sample preparation that involves freeze-fracturing with liquid nitrogen and then critical point drying of the specimen. Critical point drying preserves the structure of the specimen by transiting from liquid to vapor at the critical point of the liquid—often liquid $CO_2$ is used because it has a low critical point (45). Air drying condenses the structure of the specimen through evaporation, where as critical point drying by passes the evaporation phase at the critical temperature and pressure of $CO_2$ which is 31.0°C and 74 bar (1080 psi) (45). Cryofracturing and critical point drying produce specimen images that are clearer and more detailed (38), and fractured edges of tissue can reveal mycelium and haustoria growth among plant cells. It is also easily performed and an inexpensive method of preservation (38). Environmental SEM is another method of viewing specimens. It utilizes the control of water-vapor pressure and low temperatures to view fresh

specimens without having to undergo sample preparation. It also provides information that

sample preparation would inhibit such as spore germination, tissue penetration, and other

mechanisms that occur on the surface of the tissue.

Knowledge of how *P. farinosa* f.sp. *chenopodii* moves through quinoa tissue will

help us better understand how the pathogen attacks. SEM can show how downy mildew

enters and exits host tissue, whether it passes through stomata or directly penetrates the

tissue, and also whether it grows intracellularly or intercellularly (28). In general, SEM can

provide a lot of useful information about the mechanisms of downy mildew infection.


**Genetic resistance against downy mildew**

Downy mildew is an obligate parasite that depends specifically on quinoa to sustain

its life. Quinoa provides some factor that downy mildew cannot obtain from anywhere else.

It may be specific metabolites, certain levels and rates of nutrients, or some other variable

only found in the plant (16). Whichever factor or combination of factors it is, a resistant

variety of quinoa poses some metabolic pathway, or physical interaction (2) that disrupts the

pathogen's ability to survive in the host. Resistance genes within the host protect the plant

from infection by producing metabolites that interact with molecules emitted by the

pathogen. The conservation of genetic variation in quinoa cultivars has provided for the

development of resistance genes against downy mildew. A range of resistance levels exists

among quinoa, some highly susceptible, some moderately susceptible, and others resistant to

downy mildew. Varieties that are resistant show minimal signs of necrotic spots, and no

signs of spore lesions (12). It is evident that certain varieties of quinoa are not affected by

downy mildew, but the type and nature of resistance in quinoa have not been well studied

(37) and the mechanisms of resistance genes in general are not thoroughly understood. However, mechanistic models describing interaction between the pathogen and a resistant host have been studied, as well as different compounds emitted by the pathogens.

Plant pathogens begin infection by transferring protein compounds, called effectors, or avirulence factors, from avirulence genes into their host. Avirulence genes are named for the hypersensitive response they induce within plants that carry resistance genes (32,43). Two different types of effectors are involved in the infection process. Pathogenicity factors are effectors used to ensure the initiation of infection and colonization of the pathogen, and aggressiveness factors contribute to the effectiveness of the infection process, which determines the pathogen's vigor (22,29). Effectors are generated by a set of avirulence genes that encode for specific proteins, which plants have evolved to recognize, hence their ability for resistance. As a pathogen transmits effectors to a resistant host, the plant recognizes them through proteins generated by resistance genes. A defense reaction is then triggered and the plant responds with hypersensitive cell death localized to the area of attack (17,44). The protein products encoded by plant resistance genes have very similar structural motifs between species many consisting of leucine-rich repeats (4,17,39). The evidence of similar protein motifs suggests that plants use comparable signaling mechanisms (4). Although resistance mechanisms are not fully understood, resistance genes against oomycetes have been identified in different plant species including *Arabidopsis* (4), grapevine (18), and sunflower (34).

Qualitative resistance genes are often clustered in the plant genome (39) and can exhibit partial to full resistance against differing pathogen races. Quantitative resistance is a broader type of resistance that is governed by multiple genes. It is often referred to as field

resistance or partial resistance because it is more durable and does not bestow complete resistance to the host plant.  The symptoms are greatly reduced to a manageable level, although the pathogen is present, the plant is tolerant of it and insignificant damage occurs. Degrees of quantitative resistance are also dependent on outside factors such as the age of the plant, abiotic influences, and variability within the pathogen population. Temperature has been studied to affect resistance mechanisms in plant host species.  Often, if the temperature is moderately too low or too high, a known resistant cultivar may become susceptible to the pathogen (24).

A multiplicity of factors involved in quantitative resistance allows it to better withstand pathogen pressures.  For this reason, quantitative resistance generally displays resistance against most races.  Although it is a hardier and more durable type of resistance, it can be quite difficult for breeders and geneticists to manipulate.  It is therefore less often selected for breeding than qualitative resistance (31).  However, the durability of quantitative resistance may be the best choice when selecting for resistance in quinoa because of the genetic variation of resistance in the plant (12) and the high variability of virulence among the pathogen.

Resistance also depends on the virulence level of the pathogen within a race or isolate.  Different isolates may have different infection rates in the same cultivar.  Some isolates are able to overcome specific resistance genes in the host, while other isolates cannot (21,37).  *Peronospora farinosa* f.sp. *chenopodii* is a heterothallic pathogen believed to be constantly changing.  With a variable pathogenic population, gene-for-gene resistance may be less likely because the *Peronospora* has the ability to create many new virulence genes through sexual reproduction and genetic recombination (21), as well as adapt to new

resistance factors (37).  It is likely that a durable, quantitative type of resistance would have evolved among quinoa varieties because of the variability within populations of *P. farinosa* f.sp. *chenopodii* (11).

Testing for virulence is an important step in distinguishing between resistant and susceptible varieties of quinoa.  Severity level is a measurement of the intensity of infection and is useful because it best explains disease development.  It is determined by measuring the percentage of total infected area on the plant.  Several methods of determining severity have been used in different studies; however, a standardized method has not been established (12).  Ochoa et al. (37) developed an evaluation scale based on percentage of infection on quinoa seedling and ranked them on a scale from 0-5 with 0-2 being resistant and 3-5 being susceptible.  Another scale measures percentage of infection ranging from 0-10 (12).  Danielsen et al. (13) found the three-leaf method and AUDPC calculations to be useful when evaluating for resistance in the field.

**Detection of downy mildew using PCR primers**

The PCR is an efficient method for amplifying small amounts of DNA for use in molecular marker assays and other PCR based techniques.  It requires all of the necessary components that are present during DNA replication including primers.  DNA primers are short nucleotide sequences, usually 14-30 nucleotides long that anneal to the template DNA, acting as a starting point for extension during replication.  They typically have a high $G + C$ content of about 50% to ensure annealing to the template DNA (20).  In order to amplify desired regions of DNA, it is important to know something about the genetic makeup of the organism being tested.  A primer's specific sequence allows it to align exactly with the complementary sequences of the desired regions of DNA.  For this purpose, it is important to

select primer sequences that are specific to the organism's DNA. PCR is a practical tool used to assist in many different protocols including the detection of pathogen DNA in plant tissue.

Oftentimes a plant pathogen can go undetected in host tissue. Many pathogens experience a dormant stage during their lifecycle where they remain latent in host tissue until conditions become favorable. At this stage, their presence is nearly undetected by the naked eye. Different techniques have been developed to detect the pathogen within plant tissue, such as microscopic examination. However, PCR based methods tend to be the most efficient especially for the detection of obligate parasites (1). PCR is quick, accurate, and able to detect very small amounts of pathogen DNA (27). It is used immensely for agronomic and horticultural purposes such as testing for sources of inoculum in all kinds of plant materials including rootstock (1), weed species (27), and seed (42). It is also used to test for pathogen presence in packaged produce being shipped to the consumer (9), disease-free plant products when shipping between countries (41), and to quantify disease progression for evaluation of susceptible varieties (6,20).

The genomes of many pathogen species are unknown, which poses a problem when the use of PCR is needed. If unique primers are unavailable to aid in identification, then they can be designed from known conserved regions in the pathogen's DNA. The development of *P. farinosa* primers would make it possible to use molecular marker assays to identify latent oospores in seed and plant tissue. The sequencing of non-coding internal transcribed spacer (ITS) regions is ideal for *Peronospora* primer design (1,3,9,27,42). Ribosomal genes are highly conserved regions of DNA. The ITS regions between the 18S and 28S subunit rRNA genes are known as ITS1 and ITS2 where ITS1 lies between the 18S and 5.8S genes, and

ITS2 lies between the 5.8S and 28S genes. ITS regions are also conserved sequences of DNA; however, they exhibit interspecific variation in their sequences because they evolve faster than the rRNA genes and may alter between species of the same genus or within a population (30,46). By amplifying and sequencing the ITS regions, using universal forward and reverse primers of the conserved rDNA, a primer sequence unique to the pathogen can be generated. There are several steps involved in designing primers for ITS regions. First, forward and reverse universal primers of the 18S, 5.8S, and 28S genes are used to amplify ITS1 and ITS2 regions with PCR. Second, the amplified DNA is separated on agarose gel and bands containing the ITS regions are cut out and purified with an extraction kit. Third, the purified DNA is directly sequenced using an automated sequencer. Once sequencing is complete, the results are evaluated by performing a BLASTN search in the GenBank database (9). The newly developed primer sequences are tested for accuracy with PCR and agarose gel on multiple *P. farinosa* isolates.

**Conclusion**

Quinoa has proven to be an exceptionally important crop to the people of western South America. Its durability and nutritional value are advantageous qualities worth improving in order to provide adequate nutrition for the local people, as well as create opportunities for economic improvement through production of quinoa as a cash crop. Genetic variation within quinoa species allows for many different characteristics and adaptations to be selected for breeding. The identification and implementation of resistant genotypes will help to reduce yield loss and improve quality in quinoa crops.

Recent interest in quinoa's outstanding nutritional quality and high tolerance for extreme environmental conditions has encouraged local authorities to reevaluate its importance as a desired crop.  Bolivian government, research organizations and specific foundations are helping to ensure the progress of genetic research, conservation, and cultivar improvement.  The enhancement of quinoa varieties will help to increase food security within countries of western South America and increase the potential for competitive production by becoming a cash crop for international markets (5).

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